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Effects of Monocrotaline Pyrrole on Cultured Endothelial Cell Function

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EFFECTS OF MONOCROTALINE PYRROLE ON CULTURED ENDOTHELIAL CELL FUNCTION

By

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ABSTRACT

EFFECTS OF MONOCROTALINE PYRROLE ON CULTURED ENDOTHELIAL CELL FUNCTION

By

Cindy Marie Hoorn

Monocrotaline pyrrole (MCTP) is a putative, toxic metabolite of the pyrrolizidine alkaloid, monocrotaline. The pneumotoxicity produced by administration of MCTP *in vivo* is delayed in onset and progressive in nature and is characterized by chronic pulmonary vascular alterations. Although the mechanism by which MCTP produces lung vascular injury is unknown, there is evidence that damage to the pulmonary endothelium is involved.

A primary goal of this work was to identify alterations in cultured endothelial cell (EC) function in response to MCTP treatment *in vitro* consistent with the ability of this compound to cause lung vascular injury *in vivo*. Changes in activity of angiotensin-converting enzyme (ACE) have been associated with a number of conditions that result in lung vascular injury. A decline in the activity of this enzyme was observed that corresponded to the degree of overt MCTP-induced cytotoxicity, suggesting that altered EC ACE activity is unlikely to be a direct effect of MCTP on the enzyme but may occur in response to the delayed cell injury caused by this compound.

ECs treated with 150 μM MCTP were unable to proliferate but continued to synthesize DNA, RNA and protein, ultimately at higher levels than controls. Thus, basic cellular activity was maintained but was altered substantially by exposure to

MCTP. Altered EC production and/or release of mesenchymal cell growth factors in response to MCTP treatment could contribute to the vascular remodeling seen *in vivo*. However, untreated ECs in culture constitutively released high levels of growth-stimulatory activity, and MCTP treatment did not augment this release.

ECs from the rat, a species sensitive to the pneumotoxic effects of MCTP, exhibited cytolytic and cytostatic effects in response to MCTP treatment *in vitro* that might contribute to the pulmonary vascular changes seen *in vivo*. Persistent DNA crosslinking, which occurred in ECs treated with MCTP *in vitro*, may be responsible for the cytostasis and limited repair capacity evident in cultured ECs treated with MCTP. Treatment of cultured ECs with mitomycin C (MMC), another bifunctional alkylating agent that causes pulmonary vascular injury *in vivo*, resulted in morphologic, cytolytic and cytostatic changes similar to those caused by treatment with MCTP. As with MCTP, DNA crosslinking was observed at concentrations of MMC that were cytostatic but which caused limited cytolysis, suggesting that the abilities to crosslink DNA and inhibit proliferation are associated and may be important in the delayed and progressive injury caused by these compounds.

In summary, cultured ECs are inhibited in their ability to proliferate and demonstrate altered synthetic activity in response to treatment with MCTP. The cytostatic effects of MCTP, which impede the endothelial repair process, appear to be related to the ability of this compound to crosslink DNA. The EC morphological and functional alterations caused by MCTP are determined in part by the extent of the cytolytic changes that occur in the monolayer which create the need for repair. DNA crosslinking may be responsible for the delayed and progressive endothelial response to MCTP treatment.

"Two roads diverged in a wood, and I--
I took the one less traveled by,
And that has made all the difference."

-Robert Frost
"The Road Not Taken"

To my family,
for believing in me and supporting me
in my seemingly endless quest for knowledge.

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LIST OF ABBREVIATIONS

Ab/Am	Antibiotic/Antimycotic Solution
ACE	Angiotensin-Converting Enzyme
ACH	Acetyl Choline
ADP	Adenosine Diphosphate
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ANTU	α-Napthylthiourea
ARDS	Adult Respiratory Distress Syndrome
BALF	Bronchoalveolar Lavage Fluid
BEC	Bovine Pulmonary Artery Endothelial Cell
BK	Bradykinin
BSA	Bovine Serum Albumin
CI	Cardiac Index
CM	Conditioned Medium
CMF-HBSS	Calcium- and Magnesium-Free Hanks' Balanced Salt Solution
CO	Cardiac Output
DFMO	α-Difluoromethylornithine
DHR	Dehydroretronecine
Di-I-Ac-LDL	Acetylated Low Density Lipoprotein
DMEM	Dulbecco's Modified Eagle's Medium
EC	Endothelial Cell
ECGF	Endothelial Cell Growth Factor
EDCF	Endothelium-Derived Contracting Factor
EDGF	Endothelium-Derived Growth Factor
EDRF	Endothelium-Derived Relaxing Factor
EGF	Epidermal Growth Factor
EGIP	Endothelial Growth Inhibitory Protein
ELAM	Endothelial Leukocyte Adhesion Molecule
ET	Endothelin
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FH	Fawn-Hooded (Rats)
G-CSF	Granulocyte Colony Stimulating Factor

List of Abbreviations (cont.)

GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GMP	Granule-Membrane Protein
GSH	Glutathione
GSH-DHP	Glutathionyldihydropyrrolizine
GSH-DHR	7-Glutathionyldehydroretronecine
HBSS	Hanks' Balanced Salt Solution
HDL	High-density Lipoprotein
HETE	Hydroxyeicosatetraenoic Acid
HODE	Hydroxyoctadecadienoic Acid
5HT	5-Hydroxytryptamine (Serotonin)
ICAM	Intercellular Adhesion Molecule
IGF	Insulin-like Growth Factor
IL	Interleukin
LAS	Antiserum Directed Against Lymphocytes
LDH	Lactate Dehydrogenase
LDL	Low-density Lipoprotein
LT	Leukotriene
MAO	Monoamine Oxidase
MFO	Mixed Function Oxidase
MCT	Monocrotaline
MCTP	Monocrotaline Pyrrole
M199	Medium 199
Mg-Asp	Magnesium Aspartate
MMC	Mitomycin C
NE	Norepinephrine
NO	Nitric Oxide
ODC	Ornithine Decarboxylase
PAF	Platelet Activating Factor
PAI	Plasminogen Activator Inhibitor
PAP	Pulmonary Arterial Pressure
PAS	Antiserum Directed Against Platelets
PBS	Phosphate Buffered Saline
PCPA	p-Chlorophenylalanine
PDGF	Platelet-Derived Growth Factor
PEC	Porcine Pulmonary Artery Endothelial Cell
PECAM	Platelet-Endothelial Cell Adhesion Molecule
PG	Prostaglandin
PGI_2	Prostacyclin
PMN	Polymorphonuclear Cell (Neutrophil)
PPH	Primary Pulmonary Hypertension
PVR	Pulmonary Vascular Resistance

List of Abbreviations (cont.)

PZA	Pyrrolizidine Alkaloid
RBC	Red Blood Cell
REC	Rat Pulmonary Endothelial Cell
RVH	Right Ventricular Hypertrophy
SAM-DC	S-Adenosyl Methionine Decarboxylase
SAT	Spermine/Spermidine Acetyltransferase
SDS	Sodium Dodecyl Sulfate
SFM	Serum-Free Medium
SMC	Smooth Muscle Cell
TCA	Trichloroacetic Acid
TF	Tissue Factor
TGF	Transforming Growth Factor
TM	Thrombomodulin
TNF	Tumor Necrosis Factor
tPA	Tissue-type Plasminogen Activator
TXA_2	Thromboxane
uPA	Urokinase-type Plasminogen Activator
V-CAD	Vascular Cadherin
VCAM	Vascular Cell Adhesion Molecule
VLDL	Very Low-density Lipoprotein
V_{O2}	Oxygen Consumption
vWF	von Willebrand Factor

Chapter I

INTRODUCTION

I. Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids (PZAs) are a group of structurally related compounds produced by a wide variety of plant species. The PZAs share a common nucleus, consisting of two fused, five-membered rings joined with a nitrogen atom at the bridgehead (McLean, 1970). To date, PZA-containing species have been identified in over 60 plant genera belonging to 13 unrelated families. More than 150 alkaloids of this class have been isolated and characterized (Bull *et al.*, 1968; Huxtable, 1979; Mattocks *et al.*, 1986; Huxtable, 1989; Smith and Culvenor, 1981; Mattocks, 1986). Many occur as free alkaloids and others as alkaloidal N-oxides; cycling between these states may provide a redox system for the plant (Huxtable, 1980).

PZA-containing plant species are found world-wide (Bull *et al.*, 1968; Cheeke, 1989; Smith and Culvenor, 1981), and poisoning of livestock (Bull *et al.*, 1968; McLean, 1970) as well as of humans (Hill *et al.*, 1951; Huxtable, 1989) occurs as a result of accidental or intentional ingestion of these plants. In the United States, PZA-containing plants such as *Senecio jacobaea*, which contaminates millions of acres of cropland in the Pacific Northwest, and several *Crotalaria* spp., prevalent in the southeast, are responsible for significant loss of livestock (Heath, 1969; Snyder, 1972; Huxtable, 1980). The toxic effects of PZAs, as well as target organ specificity, appear to vary with animal species, age and sex, plant species consumed, duration of exposure and total dose of alkaloid consumed (McLean, 1970; Culvenor *et al.*, 1976; Huxtable, 1979; Shull *et al.*, 1976).

Toxic PZAs may affect a number of organ systems, but hepatic changes are most commonly identified. These include veno-occlusive disease, hepatomegaly and megalocytosis (Hill *et al.,* 1951; Schoental and Head, 1955; Bull *et al.,* 1968; Bras *et al.,* 1957; McLean, 1970; Petry *et al.,* 1984). Several of the PZAs are mutagenic and genotoxic in a variety of systems (Williams and Mori, 1980; Yamanaka *et al.,* 1979; Griffin and Segall, 1986; Petry *et al.,* 1986; Takanashi *et al.,* 1980; Styles *et al.,* 1980), and liver cell carcinomas (Cook *et al.,* 1950; Schoental *et al.,* 1954; Schoental, 1968) and rhabdomyosarcomas (Allen *et al.,* 1975; Huxtable *et al.,* 1978), and cardiopulmonary effects have been noted with some frequency under these conditions (Turner and Lalich, 1965; Kay and Heath, 1969; Chesney and Allen, 1973d; Chesney and Allen, 1973b; Chesney *et al.,* 1974a). In many cases, the subtlety of the lesions and delayed development of toxicity may complicate the diagnosis of PZA toxicoses (Huxtable, 1979; Huxtable, 1989), however, neurological (Hooper *et al.,* 1974; Mattocks *et al.,* 1986), renal (Schoental and Head, 1955; Masugi *et al.,* 1965; Hayashi and Lalich, 1967), pancreatic (Putzke and Persaud, 1976) and gastrointestinal (Hsu *et al.,* 1974; Hooper, 1975) effects have been documented. The PZAs are potent antimitotic agents, affecting liver cells and tissues with rapid cell turnover (Hsu *et al.,* 1973). Teratogenicity and embryotoxicity have also been reported (Peterson and Jago, 1980)

Monocrotaline (MCT) is a PZA found in the seeds, leaves and stems of plants of the genus *Crotalaria* in the family *Leguminosae* (Neal *et al.,* 1935); *Crotalaria spectabilis* is the species most commonly encountered in the United States (Heath, 1969; Kay and Heath, 1969). Of the many PZAs currently under

investigation, MCT is perhaps the most intensively studied, and the remainder of this dissertation will be confined to a discussion of MCT.

II. Monocrotaline

A. General

Crotalaria spectabilis (common name: rattlebox) and other MCT-containing plant species are found throughout the tropical and subtropical regions of the world, including the southeastern United States. Human exposure to MCT occurs when these plants are eaten as vegetables, or when decoctions or extracts of the leaves and stems are taken as herbal teas or remedies. Inadvertent exposure also occurs when food grains contaminated with seeds of MCT-containing plants are consumed. Livestock may ingest *Crotalaria* spp. while grazing or encounter them as contaminants in grain and forage (Huxtable, 1989; Kay and Heath, 1969).

Epidemiologically, it is often difficult to demonstrate a direct connection between consumption of MCT-containing plants and toxicity; however, liver, lung and cardiotoxicity have been associated with ingestion of these plants by a number of animal species, including horses (Stalker, 1884; Rose *et al.,* 1957; Cox *et al.,* 1958), cattle (Sippel, 1964; Sanders *et al.,* 1936), pigs (Emmel *et al.,* 1935; Peckham *et al.,* 1974), goats (Dickinson, 1980), poultry (Thomas, 1934; Allen *et al.,* 1960; Allen *et al.,* 1963; Sippel, 1964) and people (Bierer *et al.,* 1960; Huxtable, 1989; Heath, 1969). Administration of MCT to experimental animals such as rats (Heath, 1969; Allen and Carstens, 1970; Kay *et al.,* 1967a; Lalich and Merkow, 1961; Lalich, 1964; Masugi *et al.,* 1965; Roth *et al.,* 1981a), rabbits (Gardiner *et al.,* 1965), dogs

(Miller *et al.,* 1978) and non-human primates (Allen *et al.,* 1965; Raczniak *et al.,* 1979) also results in toxicity. Mice are less sensitive (Miranda *et al.,* 1981b; Molteni *et al.,* 1989a),and guinea pigs, gerbils and hamsters appear to be resistant, to MCT toxicity (Chesney and Allen, 1973a; Cheeke and Pierson-Goeger, 1983). Young animals are generally more sensitive than older animals (Chesney and Allen, 1973b; Stuart and Bras, 1957; Todd *et al.,* 1985).

Chemically, MCT consists of a branched-chain, dicarboxylic acid, monocrotalic acid, esterified to a retronecine nucleus [Figure 1] (Adams and Rodgers, 1939). The alkaloid has a molecular weight of 325.3, and is a colorless crystal with a melting point of 202-203°C (IARC Monograph). The structure of MCT has been confirmed by infrared spectroscopy, nuclear magnetic resonance and mass spectrometry (Culvenor and DalBon, 1964; Bull *et al.,* 1968).

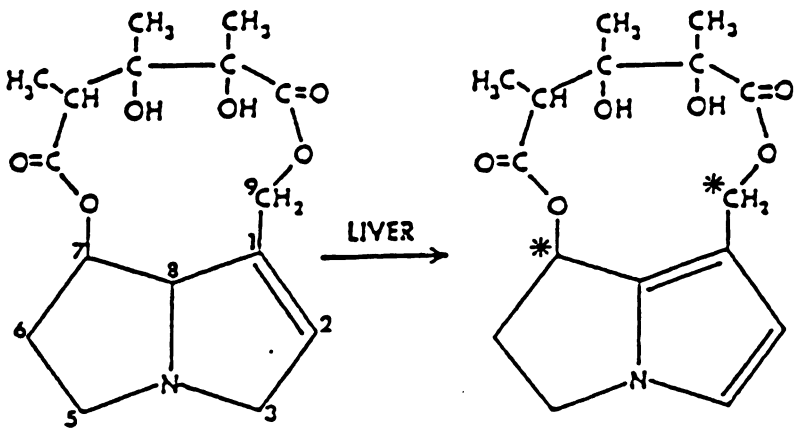

Figure 1: Structures of monocrotaline and monocrotaline pyrrole.
(* potential reactive centers)

B. Metabolic Activation

As is the case with other PZAs, the parent alkaloid, MCT, is not toxic but must undergo metabolic bioactivation (Mattocks, 1968). Mattocks and White (1971) identified the mixed-function oxidase (MFO) system of the liver as the site of metabolism of PZAs to hepato- and pneumotoxic species. Bioactivation is dependent on the presence of NADPH and oxygen, is inhibited by carbon monoxide and SKF-525A, and is enhanced by phenobarbital pretreatment of animals (Mattocks and White, 1971; White and Mattocks, 1971; Chesney *et al.*, 1974b). The 1:2 double bond of the pyrrolizidine nucleus, a structural requirement for toxicity, is necessary for dehydrogenation of the parent alkaloid to reactive, pyrrolic derivatives (Mattocks, 1969; Mattocks, 1968; Culvenor *et al.*, 1969; Huxtable, 1990a). In the case of MCT, monocrotaline pyrrole (MCTP; dehydromonocrotaline) is the primary pyrrolic metabolite [Figure 1]. Recently, cytochrome P_{450} isozyme IIIA-2 (PCN-E) has been identified as the form of P_{450} responsible for conversion of PZAs to reactive pyrroles (Williams *et al.*, 1989). The hepatic MFO system (cytochrome P_{450} isozyme IIC11 [UT-A]) also produces N-oxide derivatives of the PZAs; however, these metabolites do not appear to be responsible for the toxic effects of PZAs (Mattocks and White, 1971; Culvenor *et al.*, 1970; Culvenor *et al.*, 1976; Chesney *et al.*, 1974b; Williams *et al.*, 1989). Liver microsomes of the guinea pig are high in esterase activity and ester hydrolysis accounts for >90% of MCT metabolism in this species; dehydroretronecine (DHR), rather than MCTP, is the primary pyrrolic metabolite

generated by this species, and may explain the guinea pig's resistance to PZA intoxication (Dueker *et al.,* 1992).

Although the liver is not the only organ to possess MFO activity, it is thought to be the primary organ involved in conversion of MCT and other PZAs to pyrrolic metabolites. Microsomes prepared from lung are unable to bioactivate PZAs (Mattocks and White, 1971). Isolated, perfused livers and liver slices produce toxic pyrroles when exposed to PZAs *in vitro*, but isolated, perfused lungs and lung slices do not (Mattocks, 1968; LaFranconi *et al.,* 1984; Gillis *et al.,* 1978; Hilliker *et al.,* 1983c; LaFranconi and Huxtable, 1984). This has recently been confirmed in tandem liver-lung preparations (Pan *et al.,* 1991). Finally, pretreatment of animals with phenobarbital preferentially induces hepatic, but not pulmonary, MFOs; phenobarbital induction increases the lung and liver toxicities of MCT and other PZAs, supporting the concept that the liver is the important locus for bioactivation (Mattocks, 1968).

Until recently, little was known about the toxicokinetics of MCT. In an early study, Hayashi (1966) followed the metabolism and excretion of [^{3}H]MCT after administration of a single, subcutaneous injection to rats and found rapid accumulation of radioactivity in urine and bile. The parent alkaloid was the predominant species identified in urine, whereas the biliary form appeared to be a metabolite (Hayashi, 1966). More recently, Segall *et al.* (1990) administered a single, i.v. injection of [^{14}C]MCT to male rats and found rapid elimination of radioactivity, with 90% of the injected dose appearing in urine and bile by 7 hr. Most of the urinary material was parent alkaloid; little N-oxide was identified. Most of the

biliary radioactivity was in the form of pyrrolic metabolites. Plasma levels of radioactivity dropped rapidly by 7 hr, but red blood cell (RBC) radioactivity remained relatively high. Enterohepatic recirculation was not a major feature of MCT metabolism (Estep *et al.*, 1991).

Hepatic metabolism of [^{14}C]MCT was examined in the isolated, perfused rat liver *in situ* (Lame *et al.*, 1991). No retronecine and little N-oxide were identified in liver perfusate, supporting the contention that ester hydrolysis and N-oxidation are not major metabolic pathways for MCT. Dehydrogenation to MCTP was identified as the major metabolic pathway. Secondary reaction of MCTP with nucleophiles such as glutathione (GSH) resulted in the production of water-soluble conjugates (e.g. 7-glutathionyldehydroretronecine [GSH-DHR] or glutathionyldihydropyrrolizine [GSH-DHP]) and free monocrotalic acid. A number of investigators have identified pyrrolic glutathione conjugates in the bile of rats treated with MCT (LaFranconi *et al.*, 1985; Buhler *et al.*, 1990; Lame *et al.*, 1990; Huxtable *et al.*, 1990b) and an N-acetylcysteine conjugate has been identified in rat urine (Estep *et al.*, 1990).

Segall and coworkers also examined tissue distribution and covalent binding of a dose of [^{14}C]MCT injected subcutaneously (Estep *et al.*, 1991). Four hours after injection, binding of radioactivity was evident in liver, RBCs, lung and kidney, with maximum binding in liver. By 24 hr, bound radioactivity was decreased in all organs, but substantial levels remained in RBCs, liver, lung and kidney.

Most evidence suggests that pyrrolic derivatives are the proximate toxic metabolites of MCT and other PZAs. Pyrrolic metabolites of the PZAs are highly

reactive, bifunctional electrophiles, capable of binding to cellular macromolecules (Mattocks, 1969; Mattocks and Bird, 1983b; Mattocks, 1972; White and Mattocks, 1972; Robertson *et al.*, 1977). Covalently bound pyrroles have been identified in tissues of animals treated with MCT and other PZAs (Mattocks and White, 1970; Allen *et al.*, 1972a; Mattocks, 1972; Lame *et al.*, 1991; Mattocks and Jukes, 1990), and the quantity of pyrrole found in tissues correlates with the degree of tissue injury (Mattocks, 1972). Induction of the hepatic MFO system by pretreatment of animals with phenobarbital or other inducers results in enhanced production of pyrrolic metabolites and more severe hepato- and pneumotoxicity following MCT administration, while inhibition of hepatic MFO does the opposite (Allen *et al.*, 1972a; Tuchweber *et al.*, 1974; Chesney *et al.*, 1974b). Finally, administration of chemically synthesized pyrroles (e.g., MCTP) to animals results in toxicity which is similar to that caused by the parent alkaloid (Mattocks, 1968; Butler, 1970a; Butler *et al.*, 1970b; Bruner *et al.*, 1983).

Although MCTP formed by the liver is a convincing proximate toxicant for this organ, a number of investigators are less convinced of its role in MCT pneumotoxicity. MCTP is highly reactive, and has a half-life of 3.5-5 seconds in aqueous solution (Mattocks and Bird, 1983a; Bruner *et al.*, 1986). Mattocks *et al.* (1990) demonstrated that a small amount of reactive MCTP does escape from the liver into the circulation; whether MCTP can then survive the trip from the liver to the lung in the aqueous milieu of the blood with its alkylating functions intact is unknown. It has been proposed that some of the toxic effects of PZAs are due to secondary metabolites or breakdown products of primary pyrroles. However, MCTP

rapidly becomes non-toxic in aqueous media, and its pneumotoxic effects are not influenced by the presence of hepatic MFO inducers or inhibitors (Bruner *et al.*, 1986). Also, although the secondary pyrrolic metabolite, DHR, has been identified in urine of MCT-treated animals, it lacks the potency required to be the pneumotoxic metabolite (Hsu *et al.*, 1973).

Conjugation with GSH or mercapturic acid could allow reactive metabolites of MCT to survive in the aqueous environment of the bloodstream (Huxtable *et al.*, 1990b; Estep *et al.*, 1990). GSH-DHR appears to be pneumotoxic to rats when administered at doses comparable to MCT (75 mg/kg) (LaFranconi *et al.*, 1985; Huxtable *et al.*, 1990b). However, these doses are much higher than pneumotoxic doses of MCTP (1-5 mg/kg) (Bruner *et al.*, 1986; Pan *et al.*, 1992). Doses of 12-24 mg of the GSH conjugate of MCT/kg or 12 mg of the cysteine conjugate/kg are not pneumotoxic in the rat (Pan *et al.*, 1992). It has also been suggested that RBCs serve as transporters for reactive MCT metabolites. RBCs appear to sequester MCT-derived [^{14}C], which is then capable of covalent interaction with lung tissue. In addition, RBCs stabilize and augment the transport of reactive MCT metabolites from liver to lung (Estep *et al.*, 1991; Pan *et al.*, 1991).

In summary, MCT is metabolized by the hepatic MFO system to a reactive, pyrrolic metabolite(s). At least one primary metabolite is MCTP. It has been proposed that MCTP enters the bloodstream and proceeds to the lung, perhaps bound to RBCs, where it then binds and causes injury. Indeed, MCTP injected intravenously can reproduce the pneumotoxic effects of MCT itself. Some of the MCTP formed from MCT in the liver is also converted to GSH-conjugated forms.

These are secreted into bile; they also enter the bloodstream, since mercapturic acid conjugates have been identified in urine. GSH-conjugated metabolites of MCT are also pneumotoxic but only at relatively high doses.

C. Biological Effects (General)

The pathophysiology of MCT has been studied extensively. MCT has been administered in a variety of forms, ranging from the addition of crushed *C. spectabilis* seeds to the diet, to injection of chemically synthesized MCTP. MCT has been given in food and drinking water, applied to the skin, and injected subcutaneously and intravenously. It has been given as a single administration, or chronically over the course of several weeks. In many cases, the total dose of chemical administered can only be roughly estimated. Such diversity in experimental design makes detailed comparison of the findings difficult. Although the form of MCT and mode of administration influence the time course and extent of the toxicity, the pathologic changes seen are qualitatively quite similar. Therefore, to facilitate discussion of the biological effects of MCT, 1.) specific experimental parameters will not be described unless they provide clarification, and 2.) the designation "MCT" will refer to *C. spectabilis* as well as the purified alkaloid, unless otherwise noted. Studies utilizing the metabolite, MCTP, will be incorporated as deemed appropriate, but will be discussed more specifically in Section II.E.

1. Hepatotoxicity

The acute response of the liver to MCT treatment is centrilobular hemorrhagic necrosis with dilation, congestion and thrombosis of portal

hepatic veins. The injury progresses to a veno-occlusive lesion (Bras and Hill, 1956), with consequent development of portal hypertension (Harris *et al.,* 1942; Schoental and Head, 1955; Turner and Lalich, 1965; Merkow and Kleinerman, 1966b; Allen and Carstens, 1968). Hepatic fibrosis, cirrhosis and ascites have also been reported (Peckham *et al.,* 1974; Allen *et al.,* 1963; Emmel *et al.,* 1935). Liver function is impaired, as indicated by decreased total serum proteins, a shift in albumin:globulin ratio (Allen *et al.,* 1963; Allen and Chesney, 1972b), increased prothrombin time (Rose *et al.,* 1945; Schoental and Head, 1955), and depressed excretion of indocyanine green (Roth *et al.,* 1981a); however, hepatic bile production is not altered (Roth *et al.,* 1981a). Plasma glutamic pyruvic transaminase activity is increased (Roth *et al.,* 1981a). With severe, chronic liver disease, ammonia levels in blood may increase, resulting in hepatic encephalopathy (Rose *et al.,* 1957). Megalocytic hepatic parenchymal cells, a hallmark of chronic PZA intoxication (Jago, 1969), are also evident in MCT-treated animals (Turner and Lalich, 1965; Allen and Chesney, 1972b).

2. Nephrotoxicity

MCT treatment results in injury to the renal vasculature of rats, primarily in the glomerular region of the kidney, and is characterized by glomerular swelling and necrosis. A single injection of MCT produces hyaline thrombosis of glomerular capillaries and afferent arterioles, and medial hypertrophy is evident in interlobular arteries (Hayashi and Lalich, 1967). Similarly, chronic administration of *C. spectabilis* seeds results in marked vascular and glomerular changes but also causes degenerative changes in the proximal tubules of the kidney (Masugi *et al.,*

1965). Renal hemosiderosis occurs, perhaps as a result of intravascular hemolysis associated with microvascular damage (Hayashi and Lalich, 1967; Schoental and Head, 1955). Glomerular and tubular lesions have also been reported in swine (Peckham *et al.*, 1974; Emmel *et al.*, 1935).

In addition, several indices of renal function are altered with MCT treatment. Blood urea nitrogen is increased in animals receiving MCT in the drinking water, suggesting impaired glomerular filtration. Kidney slices from these animals also demonstrate altered ability to accumulate organic anions and cations, a change consistent with proximal tubular damage (Roth *et al.*, 1981a).

3. Carcinogenicity

MCT is mutagenic in several strains of *Drosophila* (Cook and Holt, 1966), and is a hepatic carcinogen in rats (Newberne and Rogers, 1973; Shumaker *et al.*, 1976). Administration of MCT's secondary pyrrolic metabolite, DHR, also results in neoplastic transformation (Shumaker *et al.*, 1976), and causes rhabdomyosarcomas in rats (Allen *et al.*, 1975). Both MCTP and DHR serve as initiators in mouse skin tumor formation when croton oil is used as a promotor (Mattocks and Cabral, 1982). MCTP, when administered at high doses, causes a marked inflammatory response and may serve as a complete carcinogen (Mattocks and Cabral, 1982; Hooson and Grasso, 1976). Interestingly, the metabolites of MCT are potent antiproliferative agents in cultured hepatocytes (Mattocks and Legg, 1980) and other cell lines (Reindel and Roth, 1991; Reindel *et al.*, 1991), and this characteristic of MCT toxicity may tend to inhibit tumor formation *in vivo*.

Cancer metastasis is enhanced in lungs of MCT-treated animals (Vincic *et al.*, 1989). This has been attributed to alterations in the pulmonary vasculature compatible with increased tumor cell retention.

4. Other Effects

Although the major effects of MCT intoxication involve the liver and lung, a number of other pathologic features have been reported. Whereas some of these changes may be due to direct effects of MCTP or other metabolites, others are secondary responses to changes in hepatic or pulmonary function.

Animals treated with MCT fail to thrive and do not gain weight like paired control animals (Schoental and Head, 1955; Merkow and Kleinerman, 1966a; Peckham *et al.*, 1974; Roth *et al.*, 1981a; Meyrick and Reid, 1979). Necrosis of the thymic cortex has been reported in mice treated with a single, high dose of MCT (Harris *et al.*, 1942). Generalized atrophy of lymphoid tissue and gastric ulceration are seen following oral administration of *C. spectabilis* seeds to pigs (Peckham *et al.*, 1974); gastric mucosal erosions are also reported in rats (Turner and Lalich, 1965). Mice receiving MCT by oral gavage for 14 days show suppressed immune function in the absence of marked liver or lung toxicity (Deyo and Kerkvliet, 1990). Vascular lesions of the mesentery and pancreas occur infrequently in animals receiving MCT for an extended period of time (McLean, 1970; Schoental and Head, 1955).

D. Cardiopulmonary Effects

Although a broad spectrum of lesions are associated with MCT intoxication, the cardiopulmonary effects of this compound are perhaps the most notable (McLean, 1970). Animals treated with high doses of MCT generally experience massive liver injury. However, when certain species (including the rat) are given lower doses of this alkaloid, liver damage is minimal but the lungs exhibit progressive injury (Valdivia *et al.*, 1967a; Roth *et al.*, 1981a). As is the case in both the liver and kidney, a prominent feature of MCT pneumotoxicity is vascular injury. *C. spectabilis* has been dubbed the "pulmonary hypertension plant" in acknowledgment of the frequent development of chronic pulmonary vascular disease following exposure to either the plant itself or its purified alkaloid, MCT (Kay and Heath, 1969).

The pulmonary toxicity of *C. spectabilis* was first reported by Schoental and Head in 1955. However, a systematic investigation of the pneumotoxic effects of this plant by Lalich and Merkow in 1961 really set the stage for future work in this arena. Chronic cardiopulmonary changes in response to *C. spectabilis* or MCT have been reported in swine (Emmel *et al.*, 1935; Peckham *et al.*, 1974), dairy calves (Pringle *et al.*, 1991) and non-human primates (Chesney and Allen, 1973b), but the vast majority of reports on MCT pneumotoxicity describe studies performed in rats. As the pulmonary changes follow a similar pattern in all affected species, the following discussion will be confined to findings in the rat model.

1. Pathology

a. Macroscopic changes

Rats treated with pneumotoxic doses of MCT experience tachypnea and dyspnea at rest. They become weak and listless, have poor appetites, and fail to gain weight. Respiratory distress and cyanosis are evident in the terminal stages of intoxication. The lungs of these animals are heavy and edematous, and subpleural and parenchymal hemorrhage and regional atelectasis are evident. Pulmonary vessels are dilated and congested, and occasional thrombi are noted. Pulmonary lymphatic vessels are also dilated. (Schoental and Head, 1955; Masugi *et al.,* 1965; Merkow and Kleinerman, 1966a; Lalich and Merkow, 1961; Turner and Lalich, 1965; Merkow and Kleinerman, 1966b)

Cardiac enlargement occurs later in the course of toxicity. This cardiomegaly involves only the right ventricular free wall, and does not affect the septum or left ventricle (Kay and Heath, 1966; Kay *et al.,* 1967a; Hislop and Reid, 1974; Meyrick *et al.,* 1980; Roth, 1981b; Ghodsi and Will, 1981; Hilliker *et al.,* 1982; Molteni *et al.,* 1985; Gillis *et al.,* 1978; Roth *et al.,* 1981a).

b. Microscopic changes

As stated previously, MCT causes injury to the vasculature of the lung. Alterations in pulmonary endothelial cells (ECs) are among the earliest changes noted in vessels of MCT-treated animals, occurring as early as 24 hours (Valdivia *et al.,* 1967a). ECs are prominent, often protruding into the vascular lumen, and vesicular "blebbing" is reported (Butler, 1970a; Valdivia *et al.,* 1967a; Merkow and Kleinerman, 1966b; Valdivia *et al.,* 1967b; Turner and Lalich,

1965; Rosenberg and Rabinovitch, 1988; Kay *et al.,* 1969). ECs from treated animals have marked nuclear enlargement, an increased number of cytoplasmic organelles and prominent pinocytotic vesicles (Meyrick and Reid, 1982; Kay *et al.,* 1969; Rosenberg and Rabinovitch, 1988). Capillary ECs show alternate areas of thickening and thinning, and swollen, hypertrophic endothelial cells occasionally appear to occlude capillaries (Valdivia *et al.,* 1967b; Rosenberg and Rabinovitch, 1988; Lalich and Merkow, 1961; Meyrick and Reid, 1982; Turner and Lalich, 1965; Hislop and Reid, 1974; Vincic *et al.,* 1989; Plestina and Stoner, 1972; Chesney and Allen, 1973c). ECs in alveolar wall vessels incorporate increased amounts of [^{3}H]thymidine at 7 and 21 days in rats receiving *C. spectabilis* seeds in their diet (Meyrick and Reid, 1982); vessels were not examined for this phenomenon earlier in the course of treatment.

Extension of smooth muscle into small (<25 μ) pulmonary arterioles which are normally nonmuscular is another early change, noted as soon as 3 days after treatment with MCT (Hislop and Reid, 1974; Turner and Lalich, 1965; Meyrick and Reid, 1979; Langleben and Reid, 1985). Medial hypertrophy of the small pulmonary arteries is consistently observed in lungs of MCT-treated animals (Heath and Kay, 1967; Turner and Lalich, 1965; Masugi *et al.,* 1965; Hislop and Reid, 1974; Hayashi *et al.,* 1967; Merkow and Kleinerman, 1966b; Kay and Heath, 1966; Kay and Heath, 1969; Lalich *et al.,* 1977). Increased thickness of the media is primarily due to increased smooth muscle and an increased number of elastic laminae; however, vasoconstriction has occasionally been suggested as a contributor as well. Extension of smooth muscle into small arterioles and thickening of the media in small pulmonary arteries are changes commonly associated with

pulmonary arterial hypertension (Harris and Heath, 1962). Medial smooth muscle cells (SMCs) contain increased numbers of mitochondria, increased rough endoplasmic reticulum and prominent golgi complexes, suggesting enhanced metabolic activity consistent with cellular hypertrophy (Merkow and Kleinerman, 1966a). Medial SMCs also show a small increase in [^{3}H]thymidine uptake indicative of increased DNA synthesis (Meyrick and Reid, 1982). This may represent SMC proliferation, but could also represent DNA repair or increased DNA synthesis without subsequent cell division.

Diffuse, necrotizing arteritis of small muscular pulmonary arteries and arterioles is described by Merkow and Kleinerman (1966a). They differentiate this lesion from that reported by Turner and Lalich (1965) as being fibrinoid in nature, without the presence of leukocytes. A number of investigators describe arteritis and periarteritis of small, medium or larger pulmonary arteries (Lalich and Merkow, 1961; Turner and Lalich, 1965; Merkow and Kleinerman, 1966a; Masugi *et al.*, 1965; Lalich, 1964; Lalich and Ehrhart, 1962; Merkow and Kleinerman, 1966b). These inflammatory lesions exhibit edema of the vascular wall, with lymphocytes, plasma cells, eosinophils and polymorphonuclear leukocytes (PMNs) present in some cases. Proliferation of fibroblasts frequently accompanies the inflammation. While this lesion is often associated with pulmonary hypertension, the occasional absence of this inflammatory change suggests it may be related instead to a sudden increase in pulmonary vascular pressure (Kay and Heath, 1966; Heath, 1969). Bronchial arteries are not typically affected by MCT treatment (Merkow and Kleinerman, 1966a).

Reported changes in the main pulmonary artery include thickening of the media, widening of the subendothelial space (with fluid, cell debris and collagen) and focal muscle necrosis and elastolysis. Alterations in polarity, shape and organelle content of smooth muscle cells, suggestive of a change from a contractile to a secretory phenotype, occur as well. The adventitia is also thickened with increased collagen and variable amounts of cardiac muscle (Guzowski and Salgado, 1987).

Pruning of the pulmonary vascular tree follows treatment with *C. spectabilis* seeds (Hislop and Reid, 1974; Meyrick and Reid, 1979). Blockage of small, peripheral vessels by swollen ECs or thrombi is thought to contribute to a decrease in cross-sectional area for pulmonary blood flow, thus resulting in increased pulmonary pressure. Kay *et al.* (1982c) disagree with these findings, and suggest that the techniques used to enumerate vessels in the preceding studies led to misleading results. Kay and coworkers utilized MCT in their studies, rather than *C. spectabilis* seeds, and this difference might account for the difference in their results. However, a recent cast corrosion study performed by Schraufnagel and Schmid (1989) reported a reduced pulmonary capillary density in MCT-treated animals.

MCT-induced angiogenesis has also been reported in the lung (Schraufnagel, 1990). New vessels are found primarily at the pleural surface and in the perivascular connective tissues of the bronchial vessels, areas reported to accumulate mast cells following MCT treatment (Takeoka *et al.*, 1962). Neovascularization does not occur in the alveolar capillary beds.

Prominent fibromuscular pads are evident in the intima of small pulmonary veins of animals receiving *C. spectabilis* seeds in the diet. These may be large enough to impede pulmonary venous outflow (Kay and Heath, 1966). The SMCs in these vessels swell, evaginate into the intima, and press into the ECs (Smith and Heath, 1978). Such changes have not been reported in MCT-treated rats and could reflect differences in the rate or character of the development of toxicity or may be due to other contaminating substances present in *C. spectabilis* seeds.

Blockages of vessels by intraluminal thrombi and fibrin are associated with MCT pneumotoxicity, and pulmonary capillaries may be occluded with platelets (Turner and Lalich, 1965; Lalich and Ehrhart, 1962; Merkow and Kleinerman, 1966b; Masugi *et al.,* 1965; Merkow and Kleinerman, 1966a; Valdivia *et al.,* 1965; Valdivia *et al.,* 1967a; Kay and Heath, 1966). Vascular thrombosis has been reported both early (Valdivia *et al.,* 1967a; Valdivia *et al.,* 1967b) and late (Turner and Lalich, 1965; Merkow and Kleinerman, 1966b; Hayashi *et al.,* 1967) in the course of MCT pneumotoxicity, and its role in the development of hemodynamic changes is controversial.

In addition to vascular alterations, changes occur in the parenchymal epithelium of the lungs with MCT treatment. Valdivia *et al.* (1967a) have shown that MCT treatment affects all cells and the elastic membranes of the alveolar wall, and these changes precede the appearance of many of the vascular changes. Hyperplasia of the epithelial cells of the alveoli and terminal bronchioles has been reported (Schoental and Head, 1955; Valdivia *et al.,* 1967a; Kay *et al.,* 1969; Sugita *et al.,* 1983a), and extension of bronchiolar epithelium to the alveolar ducts

and alveoli occurs as well (Kay and Heath, 1966). Wilson and Segall (1990) found cyto- and karyomegaly of alveolar type II pneumocytes in the absence of obvious cytolytic changes. Type II cells were increased in volume and nuclear diameter, but decreased in number. They also had an increased thymidine labeling index. This is reminiscent of changes seen in hepatic parenchymal cells following PZA administration (McLean, 1970) and may be related to an inability of these cells to divide in the face of a stimulus for proliferation.

There are accumulations of mast cells (Kay *et al.*, 1967b) and enlarged macrophages with foamy cytoplasm (Kay and Heath, 1966; Sugita *et al.*, 1983a) in the interstitium of the lungs of treated animals. Edema fluid is evident in the interstitium and in the alveolar spaces as early as 4 hours after treatment with MCT and hemorrhage into the alveoli is occasionally seen (Masugi *et al.*, 1965; Schoental and Head, 1955; Valdivia *et al.*, 1967a). Interstitial fibrosis is a later development (Kay *et al.*, 1969; Butler, 1970a; Meyrick and Reid, 1979; Meyrick *et al.*, 1980). These are collectively referred to as exudative lesions, and their presence suggests a loss of vascular integrity. Exudative lesions typically occur when capillary permeability and vascular pressure are increased (Wilson and Segall, 1990; Heath, 1969; Valdivia *et al.*, 1967b; Molteni *et al.*, 1984; Sugita *et al.*, 1983b).

The left ventricle of the heart is morphologically normal in MCT-treated animals. In the right ventricle, changes consistent with hypertrophy and increased energy production are evident. The number and size of mitochondria increases by 14 days posttreatment (Kajihara, 1970). As right ventricular enlargement progresses, most cardiac myocytes become hypertrophic with large

nuclei. Increased RNA synthesis is evidenced by changes in the golgi apparatus, dilation of the endoplasmic reticulum, and an increased number of free ribosomes in the cytoplasm. An increase in RNA is also indicated by a decrease in the DNA/RNA ratio in the right ventricle (LaFranconi *et al.,* 1984). Protein synthesis, and protein and collagen content, of the right ventricle increase with the ventricular enlargement (Huxtable *et al.,* 1977; LaFranconi *et al.,* 1984).

Small accumulations of lymphocytes and monocytes between myocardial fibers and interstitial edema have been reported (Lalich and Merkow, 1961; Hayashi and Lalich, 1967; Werchan *et al.,* 1989). However, right ventricular myocytes show no histologic evidence of failure; the arrangement and mean diameter of myofilaments are not different from control animals (Loskutoff *et al.,* 1983).

A report by Blaustein *et al.* (1965) identified coronary vascular changes. Intimal atheromatous plaques were evident in Wistar rats treated with MCT. Coronary vessel effects as well as subendocardial fibrosis of the right ventricle have also been reported in monkeys (Chesney and Allen, 1973b and c).

2. Pulmonary Mechanics

The effects of MCT on pulmonary function were examined by Gillespie *et al.* (1985). 20 days after receiving a single injection of MCT, rats showed evidence of mechanical, ventilatory and gas exchange deficits. Total lung capacity and residual volume were decreased, as were respiratory frequency, tidal volume, and dynamic and quasistatic compliance. There was a marked increase in pulmonary

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resistance, and the coefficient of diffusion for carbon monoxide was reduced. These changes in function were associated with morphologic changes in the alveolar wall: alveolar septae were thickened, with edema fluid, cellular debris and many mast cells present in the interstitial space, and alveolar integrity was compromised. Functional and morphological findings suggested a severe, combined restrictive and obstructive airway disorder.

Further work in this area demonstrated that changes in the lung parenchyma and the resultant ventilatory dysfunction occurred early in the course of MCT intoxication, preceding the development of pulmonary vascular changes (Lai *et al.*, 1991). Although these findings suggest a potential causative role for hypoventilation and hypoxia in the development of subsequent vascular alterations, the observation that arterial oxygen tension is reduced only very late in the course of toxicity would argue that this is not the case (Meyrick *et al.*, 1980). It may be that the changes in the pulmonary parenchyma and vasculature are coincident, both contributing to the more terminal cardiopulmonary changes seen following MCT administration.

Werchan *et al.* (1989) evaluated right ventricular function in animals with MCT-induced pulmonary hypertension, which produces a chronic pressure overload on the right heart. The hypertrophic right ventricle demonstrated enhanced performance with no evidence of failure at 2-5 weeks posttreatment, which is in agreement with previous morphologic findings. In addition, Bruner *et al.* (1983) found that although MCTP causes changes in the electrocardiogram consistent with

right ventricular enlargement, there is no evidence of dysfunction at 14 days posttreatment.

3. Vascular Alterations

The angiotoxic effects of MCT have been emphasized previously. The pulmonary lesions of MCT characteristically center on the vasculature, and include a vascular injury phase and a subsequent vascular remodeling phase. The structural alterations which follow MCT treatment have been discussed above (Section II.D.1.b.). This section will deal with the hemodynamic changes which are associated with MCT pneumotoxicity, including alterations in vascular reactivity.

a. Hemodynamics

The characteristic pathology of the pulmonary vasculature observed after MCT treatment, together with the consistent development of right ventricular enlargement late in the course of intoxication, were accepted as evidence of elevated pulmonary arterial pressure long before hemodynamic studies were performed in this model. Further indirect evidence of pulmonary hypertension was added with reports of increased right ventricular pressure in MCT- and MCTP-treated animals (Kay *et al.*, 1967a; Chesney *et al.*, 1974a). Eventually, direct measurement of pulmonary arterial pressure (PAP) in *C. spectabilis*-treated rats confirmed the delayed development of pulmonary hypertension and its relationship to right ventricular hypertrophy (RVH) in the MCT model (Huxtable *et al.*, 1977).

A thorough study of the hemodynamic changes in conscious rats following ingestion of *C. spectabilis* was performed by Meyrick *et al.* (1980). These authors found significant increases in oxygen consumption (Vo_2) and

cardiac index (CI) after 7 days of treatment; only the latter change was persistent. Changes in V_{O_2} and CI were correlated with the muscularization of pulmonary arterioles, and the increase in CI was attributed to dilatation and recruitment of vessels in response to vascular inflammation and damage.

PAP was increased by 14 days, coincident with the increase in medial thickness of small pulmonary arteries. Pulmonary vascular resistance was markedly increased by 33 days. Changes in the larger vessels of the lungs occurred late in the course of toxicity, coincident with a loss of peripheral vessels and evidence of RVH. No evidence of arterial hypoxemia was evident until long after the development of pulmonary hypertension. From this work, the authors suggested that precapillary vascular remodeling was responsible for the increased PAP seen after treatment with MCT.

b. Vascular Reactivity

It has been hypothesized that altered pulmonary vascular responsiveness could contribute to the increases in PAP seen in MCT pneumotoxicity. Vasoconstriction is thought to be the primary vascular change in some models of pulmonary hypertension (Voelkel and Reeves, 1979). Although this may not be the case with MCT, several investigators have examined changes in vascular reactivity in response to vasoactive mediators. Gillespie *et al.* (1986) found an early, transient increase in pressor response of isolated, perfused lungs from MCT-treated animals to both angiotensin II (Ang II) and hypoxia, but concluded that this was unlikely to contribute to sustained increases in arterial pressure. This course is somewhat different than that found by Hilliker and Roth (1985) using perfused lungs

from rats treated with MCTP (see below). Altiere *et al.* (1986) found vessels from MCT-treated rats demonstrated an early, transient increase in responsiveness to Ang II, norepinephrine (NE) and KCl and reduced responsiveness by 14 days after MCT treatment; relaxation in response to acetylcholine (ACH) and isoproterenol were also decreased late in the course of injury. Shubat and coworkers (1987) found a progressive decrease in contractility of isolated pulmonary vessels in response to 5-hydroxytryptamine (5HT), NE and K+ which became significant with the establishment of pulmonary hypertension. A generalized reduction in responsiveness may represent direct damage to the vascular wall, or could reflect compensatory changes in response to increased PAP. Decreased vascular responsiveness coincides with thickening of the small pulmonary arteries, and with greater vascular compliance and reduced ability to generate force (Altiere *et al.*, 1986; Langleben *et al.*, 1988).

Recent work by Ito and coworkers (1988a) confirmed the decreased responsiveness to ACH, and reported enhanced contraction in response to $PGF_{2\alpha}$. However, these investigators found no difference in response to Ang I or Ang II, nor did they detect a change in responsiveness to NE or K+.

Differences in the pathology of large and small pulmonary vessels after MCT treatment have led to investigation of differences in the SMCs of these vessels during pulmonary hypertension. SMCs of the main pulmonary artery are depolarized as compared to non-hypertensive controls, due to increased chloride conductance of the cell membrane. SMCs of smaller pulmonary arteries (100-200 μ) are hyperpolarized, and this is attributed to an increase in Na+/K+ pump activity. SMCs of small pulmonary arteries are those which undergo

hyperplasia and/or hypertrophy during MCT toxicity, and the hyperpolarization may be linked to co-transport of Na+ and amino acids in the process of cell growth (Gillespie *et al.,* 1986).

Subsequent studies by Shubat *et al.* (1990) on larger (500-800 μ) arteries isolated from MCT-treated animals demonstrated a diminished contractile response to NE and 5HT and evidence for dysfunction of the Na+/K+ pump by 4-5 days posttreatment. This pump may be a relatively early target of MCT toxicity in SMCs, and alterations in activity may be a function of vessel diameter.

4. Biochemical Changes

Evidence exists for altered biochemical function in the lung after MCT treatment. Although studies emphasizing pulmonary pathology reported changes consistent with altered function, it was not until the late 1970s that the nonrespiratory function of the lung was quantitated in MCT-treated animals.

A number of investigators have demonstrated that clearance of 5HT by the lungs is reduced, and this reduction is accompanied by decreased release of metabolite. This may reflect a specific decrease in 5HT uptake by pulmonary ECs rather than a defect in metabolism, as monoamine oxidase (MAO) activity is not impaired; however, changes in the distribution of pulmonary blood flow can also result in decreased 5HT uptake (Huxtable *et al.,* 1978; Gillis *et al.,* 1978; Roth *et al.,* 1981a; Hilliker *et al.,* 1983c). Changes in 5HT clearance occur coincident with markers of pulmonary vascular leak, such as increased lung weight-to-body weight ratio and increased release of lactate dehydrogenase (LDH) into cell-

free, bronchoalveolar lavage fluid (BALF) (Roth *et al.,* 1981a; Roth, 1981b; Roth *et al.,* 1981a).

Removal of NE by the pulmonary vasculature of MCT-treated animals is more controversial. Uptake of NE is also a carrier-mediated uptake process at the endothelial surface, and NE is metabolized by the same MAO system as is 5HT (Roth, 1985). Gillis *et al.* (1978) and Hilliker et al. (1984) found decreased removal of NE by lungs isolated from MCT-treated animals; however, Huxtable and coworkers reported that NE transport was unaffected by MCT treatment (Huxtable *et al.,* 1978). Discrepancies in these results may be due to different lung perfusion techniques.

There is reduced degradation of prostaglandins in the pulmonary circulation after MCT treatment (Ito *et al.,* 1988b; Ito *et al.,* 1988a). This is true for exogenously administered PGE_2 and $PGF_{2\alpha}$ and, presumably, for endogenous prostaglandins as well. Prostaglandin clearance is also a function of the pulmonary endothelium and requires uptake into the cell. Whether the uptake mechanism is impaired or metabolism is inhibited is not known.

The loss of relaxation of pulmonary vessels from MCT-treated animals in response to ACH is suggestive of a defect in generation of endothelium-derived relaxing factor (EDRF) (Ito *et al.,* 1988a). The reactivity of vascular smooth muscle is unimpaired in these vessels, suggesting that an endothelial defect may be responsible for the lack of response to ACH. Generation of EDRF has not been investigated by direct means in the MCT model of lung injury.

The lung vasculature, specifically the endothelial surface, is important in generation of a number of factors involved in hemostasis. Molteni *et al.* (1984,1989b) found increased production of prostacyclin (PGI_2) and decreased fibrinolytic activity in lung homogenates from MCT-treated rats. Although the decrease in fibrinolytic activity occurred relatively late in the course of lung injury, PGI_2 levels rose progressively throughout the treatment period. The authors concluded that progressive EC damage in the lungs of MCT-treated animals resulted in these alterations, but they were uncertain whether these were primary effects of the toxicant or were secondary changes occurring in response to increased PAP. The production of thromboxane A_2 (TXA_2) is also increased in MCT-treated animals (Molteni *et al.*, 1988a; Molteni *et al.*, 1989b).

Synthesis of RNA and protein is increased in the lungs and right ventricle of MCT-treated animals, and the DNA:RNA ratio is decreased (LaFranconi *et al.*, 1984). There is an increase in absolute protein content of these organs (LaFranconi *et al.*, 1984; LaFranconi and Huxtable, 1983). In the lung, the largest increase in mass is in the cytoplasmic proteins; whereas in the heart, the largest increase is in matrix proteins, including collagen. Biochemical changes of this nature support the observation that these organs respond to MCT treatment with hypertrophy, but whereas the lungs respond to direct chemical damage, the right ventricle of the heart responds to an increased work load caused by pulmonary hypertension (LaFranconi *et al.*, 1984).

In contrast to the functions mentioned above, 5'-nucleotidase activity is unaffected by MCT lung injury (Huxtable *et al.*, 1978), and the effects on

angiotensin-converting enzyme (ACE; kininase II) are controversial. [See Chapter II for a discussion of this subject.]

E. Monocrotaline Pyrrole Pneumotoxicity

The metabolic requirement for MCT toxicity complicates the investigation of its effects. Species differences in hepatic metabolism (Cheeke and Pierson-Goeger, 1983; Shull *et al.*, 1976) and individual differences in level of activating enzyme (Allen *et al.*, 1972a; Tuchweber *et al.*, 1974; Chesney *et al.*, 1974b) can influence the type and amount of metabolites produced; therefore, administration of a carefully calculated dose of purified MCT could theoretically produce variable effects *in vivo*. In an effort to circumvent these difficulties, some investigators have used chemically synthesized MCTP in their studies.

MCTP, a primary pyrrolic metabolite of MCT, is chemically reactive and, when injected into animals, causes injury in the first vascular bed it encounters. Subcutaneous injection of MCTP results in local transient necrosis and subsequent endothelial injury (Hooson and Grasso, 1976). Administration via the mesenteric vein results in liver injury (Butler *et al.*, 1970b). Whereas a single, large dose of MCTP (>10 mg/kg) injected into the tail vein of rats results in acute, fulminant pulmonary edema and death (Hurley and Jago, 1975; Plestina and Stoner, 1972), similar administration of a low dose of MCTP (2-5 mg/kg) results in pulmonary vascular injury which is similar to that seen after administration of MCT (Butler *et al.*, 1970b; Bruner *et al.*, 1986; Bruner *et al.*, 1983). Administration of a low dose of MCTP via the tail vein does not cause detectable injury to the liver or other organs.

The time course of MCTP pneumotoxicity is somewhat more rapid than that seen after MCT but follows a similar pattern (Bruner *et al.*, 1986; Schultze *et al.*, 1990). Early injury is characterized by progressive vascular leak, pulmonary edema and congestion (Butler *et al.*, 1970b; Plestina and Stoner, 1972; Reindel *et al.*, 1990; Schultze *et al.*, 1990). Changes in endothelium, alveoli, the pulmonary interstitium and vessel walls have been reported which are analogous to MCT-induced changes (Butler, 1970a; Chesney *et al.*, 1974a; Reindel *et al.*, 1990; Butler *et al.*, 1970b; Raczniak *et al.*, 1979; Lalich *et al.*, 1977). Similarly, fibrin and platelet thrombi have been noted in pulmonary arteries, arterioles, capillaries and veins (Chesney *et al.*, 1974a; Lalich *et al.*, 1977).

Increased LDH activity and protein are evident in BALF from MCTP-treated rats (Bruner *et al.*, 1983; Hilliker *et al.*, 1984; Reindel *et al.*, 1990). Removal of biogenic amines is impaired in isolated lungs and lung slices from MCTP-treated animals, suggesting similar changes in biochemical functions of the lung (Hilliker *et al.*, 1983a; Hilliker *et al.*, 1984). Development of pulmonary hypertension ensues as a result of earlier vascular changes and is followed within a few days by the development of RVH (Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Bruner *et al.*, 1983; Hilliker *et al.*, 1984; Reindel *et al.*, 1990; Schultze *et al.*, 1990).

Hilliker and Roth (1985b) were able to produce injury in isolated, perfused lungs with MCTP administered *in vitro*. The injury was characterized by increased vascular leak, increased perfusion pressure, and decreased removal of 5HT after injection of a moderate dose of MCTP into the pulmonary artery. Plestina and Stoner (1972) showed that more than half of the MCTP administered to the isolated

lung in this way is retained within the lung in the initial 30 seconds of perfusion, most likely on its first pass through the pulmonary vasculature. Ehrlich-positive material (pyrroles) was distributed throughout the lung following perfusion, but was most concentrated in the larger pulmonary vessels.

Minor differences in the response to MCTP vs. MCT exist. Platelet numbers do not decrease after MCTP treatment (Bruner *et al.*, 1983; White and Roth, 1988) as they do after MCT (Hilliker *et al.*, 1982). The transient, enhanced response to vasoconstrictors that is evident soon after MCT treatment is more persistent after MCTP treatment (Gillespie *et al.*, 1986; Hilliker and Roth, 1985a). Type I pneumocytes are affected with MCTP but not with MCT (Butler, 1970a). These differences may be due to metabolites of MCT (other than MCTP) which are produced by the liver, differences in the amount of active material which reaches the lung, or modification of some of the pyrrolic effects of MCTP by the vehicle used for its administration. However, the general character of the pneumotoxicity caused by MCTP is similar to that caused by the parent alkaloid, and the advantages its use provides are great. Therefore, for the purpose of inducing chronic pulmonary vascular injury in the rat and carrying out investigations on the mechanisms of this injury, MCTP serves as a useful alternative to MCT. Chemically synthesized MCTP has an added benefit, as its use permits studies *in vitro* with tissues unable to generate the necessary reactive metabolite.

F. Progression of Cardiopulmonary Alterations

One of the more interesting features of MCT(P) pneumotoxicity is the delayed and progressive nature of the injury. Whether MCT is administered chronically at low levels in the food or water, is given as a single, bolus injection, or is administered as the pyrrolic metabolite, overt damage to the lung is not evident immediately after treatment but requires several days to manifest itself (Valdivia *et al.,* 1967a; Valdivia *et al.,* 1967b; Raczniak *et al.,* 1979; Wilson and Segall, 1990; Meyrick *et al.,* 1980; Shubat *et al.,* 1990; Bruner *et al.,* 1983; Hilliker *et al.,* 1983a).

EC changes in small pulmonary vessels are among the first noted and are evident as early as 1-3 days after treatment with MCT(P) (Valdivia *et al.,* 1967a; Valdivia *et al.,* 1967b; Rosenberg and Rabinovitch, 1988; Reindel *et al.,* 1990). Evidence of vascular leak, consequent pulmonary edema, and inflammatory changes also are reported relatively early in the course of intoxication (Valdivia *et al.,* 1967a; Valdivia *et al.,* 1967b; Miller *et al.,* 1978; Roth *et al.,* 1981a; Sugita *et al.,* 1983b; Reindel *et al.,* 1990), as are alveolar changes and alterations in lung respiratory function (Valdivia *et al.,* 1967a; Gillespie *et al.,* 1985a; Lai *et al.,* 1991). This may be considered the "lung injury phase" of MCT toxicity, and it typically precedes vascular remodeling and the "pulmonary hypertensive phase" (Roth *et al.,* 1989).

Vascular remodeling involves thickening of precapillary resistance vessels of the lung (Lalich *et al.,* 1977; Merkow and Kleinerman, 1966b; Hislop and Reid, 1974) and, in combination with increased fluid and fibrous tissue in the lung parenchyma (Valdivia *et al.,* 1967a), leads to increased PAP (Huxtable *et al.,* 1977; Kay *et al.,* 1967a; Chesney *et al.,* 1974a). RVH follows, in response to the increased

work of pumping against a more resistant pulmonary vascular bed, and death of the animal usually results (Werchan *et al.*, 1989; Meyrick *et al.*, 1980).

This delayed and progressive development of injury suggests that lung cells are sublethally but irreversibly damaged by even a single exposure to the reactive metabolite(s) of MCT. The reactivity and lability of MCTP (Bruner *et al.*, 1986) suggest that the initial event in the toxicity occurs rapidly (ie. covalent binding of MCTP to the cell); however, the lag period implies cellular alterations which are slow to develop but which are progressive and trigger a cascade of molecular changes and pathophysiological events that result ultimately in chronic pulmonary vascular disease.

Hayashi *et al.* (1979) examined the effects of diet restriction on MCT toxicity in rats. They found that rats receiving a single injection of MCT and fed *ad libitum* (Group 1) gained weight initially, developed typical lung pathology, and died between 22 and 40 days posttreatment. MCT-treated rats that were diet-restricted (Group 2) did not gain weight, had minimal pulmonary changes, and survived the entire experimental period. A third group of rats was treated with MCT and diet restricted for 30 days, during which time they behaved like the animals in Group 2. After 30 days post-MCT, they were fed an *ad libitum* diet, began to gain weight, rapidly developed pulmonary lesions, and proceeded to die between 45 and 85 days post-MCT. This interesting study supports the idea of early, irreversible injury which is delayed in its expression; additionally, expression of the pulmonary injury appears to be dependent on either adequate nutrition or actual growth of the animal. Similar effects of diet restriction were later reported for MCTP by Ganey *et al.* (1985),

indicating that the effect is not a consequence of reduced food intake on MCT bioactivation.

Once begun, the vascular and parenchymal changes are progressive, and rarely is the injury caused by this compound reversible. Hislop and Reid (1974) reported a regression of the arterial medial thickening and RVH when feeding of *C. spectabilis* seeds was discontinued after 34 days, but they saw no recovery of lost peripheral vessels. Adult rats were used in these studies and their age may account for their recovery and survival, as young animals are typically more sensitive to PZAs than are older animals (McLean, 1970; Schraufnagel, 1990). Recovery has not been reported previously, and animals are typically moribund and/or dead by this point in similar investigations (Kay and Heath, 1966; Kay *et al.,* 1967a).

However, it does appear that there is a minimum cumulative dose of MCT necessary for the development of pulmonary hypertension and RVH (Shubat *et al.,* 1989), and discontinuation of a chronic dosing regimen before this critical dose is reached may result in regression of some lesions. Shale *et al.* (1986) gave MCT in the drinking water at 20 mg/liter for 3, 7 or 15 days, then examined a number of parameters 21 days after initiation of treatment. Three or more days of MCT resulted in an increased wet lung-to-body weight ratio, 7 or more days of MCT resulted in increased lung protein content and dry weight, but RVH occurred only after 15 or more days of MCT feeding, thus separating the development of pulmonary hypertension from increases in lung mass. The investigators estimated that rats in the 7 day group received 14.1 mg MCT/kg, suggesting that the critical dose of MCT is slightly higher (Shale *et al.,* 1986; Shubat *et al.,* 1989).

III. MCT Pneumotoxicity as a Model of Pulmonary Hypertension in People

Little is known about the mechanisms underlying certain forms of chronic pulmonary hypertension in people, and few good animal models exist for the study of this condition. MCT can be characterized as an angiotoxin which causes lung vascular injury that is delayed in onset and progressive in nature, and results in the development of pulmonary hypertension. Chronic pulmonary vascular disease in people and the pulmonary vascular toxicity produced in rats given low doses of MCT have many similar features. Study of MCT pneumotoxicity in the rat may therefore provide valuable insights with regard to pathogenesis of pulmonary vascular disease in people.

A. Primary Pulmonary Hypertension

Primary pulmonary hypertension (PPH) is a heterogeneous disease of the pulmonary vasculature, and more than one causative factor is probably involved in the pathogenesis. Few cases of PPH are diagnosed early in the course of the disease. This makes it difficult to determine the etiology of the condition and seriously limits the benefits of therapeutic intervention. One of the biggest stumbling blocks investigators have faced is a lack of good animal models with which to study this problem. Although not identical, many aspects of the pathology seen in the rat treated with pneumotoxic doses of MCT are similar to those seen in patients with PPH.

PPH is characterized by increased precapillary vascular resistance in the lungs which ultimately results in RVH and failure. The clinical definition of PPH requires an elevated PAP in the absence of a discernible cause. Also by definition,

the left atrial (or pulmonary wedge) pressure is normal (Dresdale *et al.*, 1951; Hatano and Strasser, 1975). In its purest form, the histopathological pattern which emerges is a plexogenic pulmonary arteriopathy, characterized by concentric intimal fibrosis, necrotizing arteritis and dilatation lesions.

The most severe pathological changes occur in the muscular arteries of 50-500 μ diameter. There is marked medial hypertrophy, with hyperplasia and hypertrophy of smooth muscle, and duplication of the elastic laminae. Concentric intimal proliferation is prominent in 100-200 μ diam. vessels. It has been suggested that these vascular changes are a response of the pulmonary vasculature to endothelial damage as a result of shear stress (Brenner, 1935; Clowes *et al.*, 1983).

Lesions in the pulmonary arterioles are less common but resemble those of the muscular arteries. In capillaries, swelling of ECs and thickening of the basement membrane have been noted; occasionally, capillaries may be obliterated (Pietra and Schloo, 1986). Embolic or *in situ* thrombosis is seen with plexogenic arteriopathy and is thought to occur in response to the disease process. Hyaline thrombi are occasionally found occluding small pulmonary arteries (Pietra *et al.*, 1987), accounting for further pruning of the vascular bed.

Plexiform lesions occur in 70% of PPH cases but are also seen in a variety of other chronic pulmonary vascular diseases in humans (Pietra and Schloo, 1986). These lesions occur rarely in rats treated with MCT (Watanabe and Ogata, 1976). Plexiform lesions are thought to be a remodeling response of the vasculature to very chronic elevations in vascular pressure, and they may occur less often in

laboratory animals due to their short lifespan and limited survival time after the onset of pulmonary hypertension.

Necrotizing arteritis is seen in cases with very high PAP. This is characterized by transmural vasculitis with fibrin deposition and necrosis, and occurs in response to rapid increases in pressure (Pietra *et al.*, 1987).

The general pattern of hemodynamic changes noted with PPH includes high PAP and pulmonary vascular resistance, normal pulmonary wedge pressure, and low cardiac output (CO) and CI. Systemic pressure is typically normal or low (Voelkel and Reeves, 1979). These findings are not unique to PPH, but are seen with many forms of PH, including that caused by administration of MCT to animals.

There is limited respiratory impairment with PPH, but alterations in pulmonary mechanics have been reported (Fernandez-Bonett, 1983). Biochemical function of the lung is altered as well, and the inability to clear circulating biogenic amines is suggestive of endothelial dysfunction (Heath *et al.*, 1987; Peach *et al.*, 1987). A number of EC alterations may underlie the altered reactivity of the pulmonary vasculature in PPH, including a decreased ability to generate endogenous relaxants or metabolize vasoconstrictors, increased interaction with circulating blood components, altered response to vasoactive substances, increased production of mitogens and chemotaxins, or liberation of inflammatory mediators, to name a few (Geggel *et al.*, 1987; Hogg, 1988; Barst and Stalcup, 1985).

A common feature of many of the proposed etiologic factors for PPH is their ability to cause pulmonary vasoconstriction (Hogg, 1988). Vasoconstriction of the precapillary resistance vessels is thought by some to play a role early in the

disease process, and the occasional success of vasodilator therapy supports this hypothesis (Hogg, 1988; Voelkel and Reeves, 1979). With the advance of disease, there is focal obstruction of pulmonary arterioles as a result of severe intimal fibrosis. These vascular segments are occluded and ultimately lost, leaving a small fraction of functional pulmonary arterioles. The burden of vessel obliteration adds to the vasoconstriction, and together these are thought to account for the increased vascular resistance typically seen with PPH (Wagenvoort and Wagenvoort, 1970).

PPH is not a disease that affects millions of people each year; in fact, not more than a thousand cases have been reported to date. Nevertheless, it continues to hold the interest of physicians and biomedical scientists, because, despite years of scrutiny, PPH remains a disease that is difficult to diagnose, untreatable, progressive, and typically fatal.

B. Pulmonary Hypertension in Response to Injury

Although rare cases of PPH occur, pulmonary hypertension is seen more frequently as a sequel to a number of pathological or toxicological conditions. At first glance, the primary conditions have little in common aside from the eventual obstruction of pulmonary blood flow which results in increased PAP. However, some common themes have been emerging with study of these conditions, including the concept of vascular cell injury as an underlying factor in the development of increased pulmonary vascular resistance. Again, the similarity in the development and progression of pathological changes in the MCT model to those seen in cases of secondary pulmonary hypertension is marked. Study of the rat treated with MCT(P)

may provide clues as to the common features underlying this diverse group of conditions.

1. Adult Respiratory Distress Syndrome

Adult respiratory distress syndrome (ARDS) occurs as a complication of a number of seemingly unrelated clinical conditions, including sepsis, chemical injury, shock, thermal injury or trauma (Hyers and Fowler, 1986). ARDS is a progressive condition, which is characterized initially by disruption of the pulmonary microvasculature and increased capillary permeability. Endothelial injury is evident on histopathology, and EC function is impaired, as evidenced by decreased removal of 5HT (Morel *et al.*, 1985). Thromboembolism and inflammation are evident, and PAP is increased. The vascular alterations are accompanied by pulmonary parenchymal cell injury and altered pulmonary mechanics (Snow *et al.*, 1982; Tomashefski *et al.*, 1983; Cochrane *et al.*, 1983). These acute pulmonary changes resemble the alterations seen in rats treated with high doses of MCTP (Plestina and Stoner, 1972; Hurley and Jago, 1975).

The earliest manifestations of ARDS may resolve, but in some cases the condition progresses to a more chronic form, characterized by pulmonary interstitial fibrosis, vascular remodeling and pulmonary hypertension (Snow *et al.*, 1982). The vascular lesions seen in this chronic stage are similar to those seen in rats treated with low doses of MCT(P). There may be a common denominator involved in the initiation of vascular lesions in ARDS, such as injury to cells of the microvasculature by mediators released during sepsis or traumatic injury. Study of

the MCT model may provide insights as to how these vascular cell alterations lead to the development of pulmonary hypertension.

2. Other Forms of Pulmonary Hypertension

Pulmonary hypertension also occurs as a result of exposure to chronic hypoxia (Abraham *et al.*, 1971; Meyrick and Reid, 1978; Rabinovitch *et al.*, 1979; Barer *et al.*, 1982), radiation (Phillips, 1966; Gross, 1981; Gross, 1977; Perkett *et al.*, 1986) or chemotherapeutic agents (see Chapter VI). The mechanisms involved in the development of chronic vascular alterations under these conditions are still undefined, but a direct, injurious effect(s) on vascular cells has been proposed as the initiating factor in each case.

Brief exposures to hypoxia result in immediate and reversible increases in PAP and PVR (Fishman, 1980). Sustained hypoxia prolongs these changes and leads to structural remodeling of the pulmonary vasculature and eventual RVH (Abraham *et al.*, 1971; Meyrick and Reid, 1978). EC alterations are evident within hours of exposure to hypoxia, and platelet adherence is enhanced (Meyrick and Reid, 1978).

Enhanced vasoconstriction is thought to be responsible for both the increased PAP and vascular remodeling in hypoxic pulmonary hypertension (Stanbrook *et al.*, 1984); however, it may be that early changes in vascular cells are responsible for both vasoconstriction and the initiation of remodeling. Hypoxia has numerous effects on the endothelium, including perturbation of its barrier and anticoagulant functions (Ogawa *et al.*, 1990b; Ogawa *et al.*, 1990a), enhanced release

of mitogens (Vender *et al.*, 1987) and decreased ACE activity (Schweigerer *et al.*, 1987; Shanahan and Korn, 1984).

Exposure to radiation rapidly results in increased capillary permeability, pulmonary inflammation and altered pulmonary blood flow. These changes are persistent, and pulmonary lesions progress to a chronic, fibrotic state with pronounced vascular remodeling and increased PAP (Perkett *et al.*, 1986). Changes in type II pneumocytes and capillary endothelium are evident early in the course of injury, and ECs irradiated *in vitro* respond with an inhibition of proliferation, hypertrophy, enhanced adherence for PMNs and platelets, increased release of PGI_2, increased permeability, and altered ACE activity (Rubin *et al.*, 1984; Dunn *et al.*, 1986; Rosen *et al.*, 1989).

Repeated exposure to hyperoxia, (Jones *et al.*, 1983; Crapo *et al.*, 1980), endotoxin (Meyrick and Brigham, 1983; Meyrick and Brigham, 1986), and chemicals such as α-naphthylthiourea (ANTU) (Hill *et al.*, 1984) can also result in pulmonary vascular remodeling and hypertension. The endothelium has been implicated as a primary site of lung injury by these agents as well (Crapo *et al.*, 1980; Meyrick *et al.*, 1989; Rutili *et al.*, 1982). Although the early changes which follow acute endothelial cell injury by these agents differ somewhat from those seen in hypoxic or MCT-induced lung injury, the chronic alterations they produce after prolonged or intermittent exposure are very similar.

Initial EC damage may be the shared event linking a diverse group of etiologic factors to a common pathophysiological endpoint. Chronic exposure to

some agents (eg. hypoxia, ANTU) is necessary to induce persistent vascular alterations, whereas, a single administration of MCT, or a single dose of radiation, is sufficient to produce similar injury. If endothelial injury is central to the development of subsequent alterations, then MCT(P) treatment might be expected to produce more persistent EC damage than does hypoxia. It has been proposed that persistent endothelial injury is the crucial event in the development of chronic pulmonary hypertension (Barst and Stalcup, 1985; Meyrick *et al.*, 1987).

These models of pulmonary hypertension share other common features which have been proposed to play a role in the development of chronic pulmonary vascular disease, including persistent pulmonary vascular leak (Sugita *et al.*, 1983b), chronic inflammation (Meyrick *et al.*, 1987) and altered pulmonary mechanics (Lai *et al.*, 1991). Investigation into the potential mechanisms which underlie MCT(P)-induced lung injury could increase our understanding of how vascular injury can lead to chronic pulmonary alterations.

IV. Mechanisms of MCT(P) Pneumotoxicity

Despite intensive study, the mechanisms by which MCT(P) produces delayed and progressive cardiopulmonary toxicity are not yet understood. On the basis of the progression of lesions, the sequence in which pathophysiological alterations occur, and the response to a number of pharmacological interventions, several potential mechanisms of action have been proposed for MCT(P). It is possible to group these into six broad categories: 1.) Vasoconstriction, 2.) Thrombosis, 3.) Platelets, 4.) Inflammation, 5.) Cell Growth and Proliferation, and 6.) Alkylation. Although these

categories overlap to a certain extent, they represent recurrent themes in the MCT(P) literature and provide a structure to facilitate discussion of this complex subject.

A. The Role of Vasoconstriction

Pulmonary vasoconstriction, with a resultant increase in pulmonary arterial pressure, is a primary defect in a number of chronic lung diseases, including hypoxic pulmonary hypertension (Abraham *et al.*, 1971; Stanbrook *et al.*, 1984) and some cases of primary pulmonary hypertension (PPH) (Wagenvoort and Wagenvoort, 1977; Hogg, 1988; Voelkel and Reeves, 1979). In these conditions, vascular remodeling occurs secondarily in response to increased pulmonary vascular pressure. Direct vasoconstriction of pulmonary vessels by MCT(P) was proposed as a mechanism for increased PAP by Smith and Heath (1978) based on morphologic changes they saw in pulmonary veins. The reactive nature of the pyrrolic metabolites of MCT and the delayed development of pulmonary hypertension in the rat argues against this theory, although intravenous administration of MCT does result in rapid increases in PVR (Miller *et al.*, 1978; Czer *et al.*, 1986) and PAP (Czer *et al.*, 1986) in the dog. This response may be due to a direct vasoconstrictive effect of MCT metabolites in this species, but further study will be required to confirm this theory.

Secondary vasoconstriction in response to hypoxemia has been suggested in the development of MCT-induced pulmonary hypertension (Turner and Lalich, 1965; Chesney *et al.*, 1974a). Similarities in the end-stage pathology of MCT- and hypoxia-induced hypertension led to this theory before the delayed and progressive nature of MCT pneumotoxicity was well understood. Subsequent

investigations have clearly established that vascular remodeling is evident prior to the development of pulmonary hypertension in this model (Meyrick *et al.*, 1980; Ghodsi and Will, 1981), and marked hypoxemia does not occur until pulmonary hypertension and RVH are well established after ingestion of *C. spectabilis* seeds (Meyrick *et al.*, 1980). However, hypoxic vasoconstriction continues to receive interest in the MCT model.

Gillespie and coworkers have reported that ventilatory dysfunction precedes the development of vascular changes in MCT-injected rats, suggesting that hypoventilation, impaired gas exchange, and consequent hypoxemia may be important in the evolution of pulmonary hypertension (Gillespie *et al.*, 1985a; Lai *et al.*, 1991). Hill *et al.* (1989) detected a mild hypoxemia 21 days (but not 10 days) after injection of MCT. In addition, they found that exposure to 35% oxygen on days 10 - 21 after MCT administration reversed the hypoxemia and significantly reduced vascular remodeling, right ventricular systolic pressure and right ventricular hypertrophy, but supplemental oxygen during the first 10 days following MCT was not protective. Thus, it appears that hypoxemia may be involved in the development or maintenance of MCT-induced pulmonary hypertension, perhaps by potentiating smooth muscle cell proliferation (Vender *et al.*, 1987) or vasoconstriction (Meyrick and Reid, 1978); however, it is not a factor in the early injury caused by MCT.

After MCT treatment, changes in pulmonary vascular cells which result in altered vascular responsiveness may also contribute to increased vasoconstriction. [Please refer to sections D.3.b. and D.4.] Treatment with MCT results in some of the same alterations in vascular reactivity as does endothelial denudation, including

decreased response to the vasodilator ACH and increased response to $PGF_{2\alpha}$ (Ito *et al.*, 1988a). Decreased clearance of biogenic amines (Gillis *et al.*, 1978; Hilliker *et al.*, 1983c; Hilliker *et al.*, 1984) impaired degradation of prostaglandins (Ito *et al.*, 1988b; Ito *et al.*, 1988a), decreased generation of EDRF (Ito *et al.*, 1988a) and other dysfunctions of pulmonary endothelium which occur following MCT treatment may contribute to abnormal vascular tone and responsiveness.

Vascular smooth muscle changes in response to MCT treatment which could support the vasoconstriction hypothesis are less consistent. Vessels demonstrate enhanced responsiveness to vasoconstrictors relatively early in the course of injury, but it is uncertain whether this effect is sustained (Gillespie *et al.*, 1986; Altiere *et al.*, 1986; Hilliker and Roth, 1985a). The pulmonary vasculature exhibits a heightened response to hypoxia, but this is seen either very early (Gillespie *et al.*, 1986) or quite late, after vascular remodeling is evident (Rosenberg and Rabinovitch, 1988). Most investigators believe that these changes are incompatible with a major role for vasoconstriction in the development or maintenance of pulmonary hypertension (Gillespie *et al.*, 1986; Petry *et al.*, 1986) , Meyrick *et al.*, 1980).

The response to several pharmacological interventions in MCT toxicity suggests that vasoconstriction may play a modulatory role, at least in the later cardiopulmonary alterations, and perhaps in some of the earlier changes as well. The vasodilator, hydralazine, reduces the degree of RVH and the increased BALF protein concentration in MCTP-treated rats (Hilliker and Roth, 1984b). This drug has other

effects, including inhibition of platelet thromboxane synthesis, but part of its protective effect may be in reducing pulmonary vascular resistance.

The calcium channel blocking agent, verapamil, attenuates MCT-induced pulmonary hypertension by limiting the extent of vascular remodeling of the small and large pulmonary vessels, but has no effect on the earlier lung injury phase (Mathew *et al.*, 1990). Interference with a number of calcium-dependent processes in endothelium and smooth muscle may account for these findings. β-blockade with propranalol reduces PAP and RVH (Huxtable *et al.*, 1977), and chemical sympathectomy with 6-hydroxydopamine also reduces the degree of RVH after MCT administration. Sympathetic input to the heart and vasculature may be enhanced in MCT-treated animals (Tucker *et al.*, 1983) and may thus contribute to both the increased vascular resistance and the cardiac response to increased pressure. It may be that generally reducing vascular tone early in the course of injury can modulate the extent of vascular remodeling and limit the development of *cor pulmonale*.

Concurrent administration of magnesium aspartate (Mg-Asp) also reduces arterial pathology, attenuates the increases in PAP and reduces RVH in MCT-treated rats (Mathew *et al.*, 1988). Mg++ has a number of functions in vascular cells, acting in part by regulating calcium movement; it also serves as a cofactor in many enzyme systems. Mg++ blocks vasoconstriction and enhances vasodilation in response to many agonists, modulates prostaglandin production, and inhibits coagulation and platelet adherence. Although it is not clear how Mg-Asp limits the toxic effects of MCT, many of its actions would be consistent with a relaxant effect on constricted vessels.

An important function of the pulmonary vascular endothelium is regulation of a number of circulating vasoactive mediators. ACE, or kininase II, found on the surface of ECs, converts Ang I to the potent vasoconstrictor, Ang II, and cleaves bradykinin to an inactive form (Yang *et al.,* 1970; Erdos, 1975). It has been proposed that agents which result in pulmonary endothelial injury (e.g. MCT) may alter the distribution or activity of ACE (Dobuler *et al.,* 1982; Catravas *et al.,* 1988b), thus influencing local and systemic levels of the vasoactive substances it regulates. Response to Ang II is variable in vessels from MCT-treated animals (Gillespie *et al.,* 1986; Ito *et al.,* 1988a); nevertheless, altered amounts of this potent pressor agent in the pulmonary vasculature may influence the degree of vasoconstriction. In addition to its vasoconstrictive effects, Ang II has a number of other effects on vascular cells that could affect the development of MCT-induced injury (Bell and Madri, 1990) (see Chapter II).

A number of investigators have evaluated ACE activity in lungs of rats treated with MCT in an effort to correlate changes in enzyme activity to some aspect of the development or progression of MCT-induced cardiopulmonary changes. Some investigators report a decrease in lung ACE activity which corresponds to an increase in pulmonary pressure (Kay *et al.,* 1982b; Keane *et al.,* 1982), whereas others contend that apparent changes in lung ACE activity are due to a dilution of its activity by the increased lung mass seen in this model (Huxtable *et al.,* 1978; LaFranconi and Huxtable, 1983). Yet another group reports an increase in lung ACE activity shortly after MCT treatment, with a subsequent, sustained decline in activity later on (Molteni *et al.,* 1984). A fourth group reports a sustained decline in ACE activity

which precedes the onset of pulmonary hypertension and which occurs at doses that do not produce RVH (Shale *et al.*, 1986). From these reports, it is difficult to draw definitive conclusions about ACE activity in lung vasculature injured by MCT.

Captopril and a number of other thiol and non-thiol ACE inhibitors reduce MCT-induced pulmonary vascular remodeling, interstitial fibrosis and RVH (Molteni *et al.*, 1985; Molteni *et al.*, 1986; Molteni *et al.*, 1987; Molteni *et al.*, 1988a). Interestingly, their protective effects are apparently not attributable to ACE inhibition, thiol effects or sparing of the endothelium in the early lung injury phase (Molteni *et al.*, 1987). It is possible that the anti-inflammatory or systemic hypotensive actions of these compounds are responsible for their salutary effects, or they may alter the pharmacokinetics of MCT itself. Captopril and certain other ACE inhibitors also inhibit weight gain (Molteni *et al.*, 1986), which has been shown to be similarly protective (Hayashi *et al.*, 1979; Ganey *et al.*, 1985). ACE inhibition in other forms of vascular injury and hypertension is currently receiving a lot of attention in the literature. ACE inhibitors strongly suppress neointimal proliferation following balloon catheter injury (Powell *et al.*, 1990). They are antifibrotic (Ward *et al.*, 1989), and enhance endothelium-dependent relaxation by increasing release of EDRF (Goldschmidt and Tallarida, 1991; Clozel, 1991). This is an area which merits further study in the MCT(P) model. [See Chapter II for a more complete discussion of this subject.]

In summary, it appears unlikely that vasoconstriction plays a major role in the early vascular alterations caused by MCT(P). There is evidence for increased tone in the vasculature, and relaxation of vascular smooth muscle and relief

of sympathetic input seems to attenuate the cardiopulmonary response to the early injury. However, extensive vascular remodeling, which is seen in this and other models of chronic pulmonary hypertension, results in serious reductions in arterial diameter and elasticity that must be severely limiting to vascular responsiveness. It seems likely that these fixed changes ultimately eclipse those due to altered vascular reactivity in this model of lung injury.

B. The Role of Thrombosis

Although there is no question that MCT-induced pulmonary vascular injury is associated with platelet and fibrin thrombi [see section D.1.b.], it remains controversial whether thrombosis plays an active role in the development of lung injury or merely occurs in response to the injury. Vascular thrombosis has been reported early in the course of MCT pneumotoxicity (Valdivia *et al.*, 1967a; Lalich *et al.*, 1977), but this is not a consistent finding. Pruning of the peripheral vasculature has been attributed, in part, to occlusion of small vessels and capillaries with thrombi (Schraufnagel and Schmid, 1989; Hislop and Reid, 1974), and this contributes to the decrease in cross-sectional area available for blood flow in the lungs of these animals.

The presence of vascular thrombi is suggestive of either excessive procoagulant activity or defective fibrinolysis (Bauer and Rosenberg, 1987). The endothelium is an important component of the hemostatic system, generating both anti- and procoagulent factors. [Please refer to Section V.] Injury to the endothelium as a result of MCT(P) treatment appears to result in alterations

consistent with a hypercoagulable state, including increased platelet adherence in the pulmonary vasculature (White and Roth, 1988) and decreased fibrinolytic activity (Molteni *et al.*, 1988a; Molteni *et al.*, 1986).

Unfortunately, few studies have been directed at this aspect of MCT pneumotoxicity. Fasules *et al.* (1987) found that anticoagulant heparin inhibited clotting in rats treated with MCT but did not protect against early lung injury, vascular remodeling, increased PAP or the development of RVH. Heparin administered at similar doses did attenuate hypoxia-induced pulmonary hypertension in rats (Geggel *et al.*, 1986). It is possible that localized thrombus formation in the lung could still occur in heparin and MCT-treated animals, as the lungs were not scrutinized closely for the presence of thrombi. However, it is difficult to reconcile these results with a major role for vascular thrombosis in MCT lung injury.

Direct examination of the coagulation system in MCT-treated rats is currently under investigation. It appears that vascular thrombosis is not mediated by an excess of procoagulent activity as evaluated in the peripheral blood (Schultze *et al.*, 1991); however, preliminary results suggest that there is decreased fibrinolytic activity in the lungs of rats treated with MCTP (Schultze and Roth, 1992).

C. The Role of the Platelet

Platelets were originally noted as components of the thrombi seen in the pulmonary microvasculature after administration of MCT (Turner and Lalich, 1965; Valdivia *et al.*, 1967a; Chesney *et al.*, 1974a). Subsequent to these observations, it was noted that numbers of circulating platelets are moderately decreased in MCT-

treated rats at 2, 5, and 10 days posttreatment (Hilliker *et al.*, 1982); this is a relatively early change following MCT administration. Thrombocytopenia is not evident in rats treated with MCTP (Bruner *et al.*, 1983), however platelet sequestration in the lungs is evident by 8 days posttreatment (White and Roth, 1988). These findings sparked interest in the platelet as a potential effector in this model, and its role in mediating MCT-induced cardiopulmonary injury has been investigated in some detail.

Hilliker *et al.* (1984) used antiserum directed against rat platelets (PAS) to create 48-hour windows of moderate thrombocytopenia during the course of development of MCT-induced lung injury. PAS administered 12 hours prior to MCTP was not protective; however, thrombocytopenia during days 3-5 or days 6-8 posttreatment reduced the development of RVH by 19% or 41%, respectively, but did not alter any of the markers of lung injury examined. The three intervals of thrombocytopenia were designed to bracket the initial lung injury (0-2 d), the development of major lung injury (3-5 d), and the increase in PAP (6-8 d) seen after MCTP administration. Thus, it appears that platelets do not mediate the lung injury but do contribute to the development of pulmonary hypertension (Hilliker *et al.*, 1984a; Ganey *et al.*, 1988).

Injury to vascular endothelium can result in enhanced platelet-endothelial cell interactions (Ratliff *et al.*, 1979) and may account for platelet sequestration and activation. Activated platelets can, in turn, release a number of potent vasoactive substances which result in further platelet aggregation and stimulation (adenosine diphosphate [ADP], TXA_2), altered vascular permeability

(5HT), vasoconstriction (5HT and TXA_2) and proliferation of mesenchymal cells (platelet-derived growth factor; PDGF) (Holmsen *et al.*, 1969; Longenecker, 1985; Niewiarowski and Holt, 1985).

Hilliker *et al.* (1983b) investigated the effects of MCT on the response of platelets to aggregating agents and found that platelets isolated from MCTP-treated rats 7 or 14 days posttreatment were less sensitive to stimulation than platelets from control animals. The authors proposed that this effect might be due to endothelial alterations which could influence the platelet milieu, such as increased production of PGI_2. Nevertheless, direct hyperresponsiveness of the platelet to aggregatory stimuli was not observed. In addition, Ganey and Roth (1987a) found that MCTP treatment of rats did not affect platelet 5HT content, nor did it alter basal or stimulated release of TXA_2 *in vitro*. Hence, platelet function and content appear to be essentially unaltered by MCTP treatment, with the exception of depressed aggregation quite late in the course of injury, suggesting that external stimuli present in MCT(P)-treated animals may trigger platelet responses in this model.

Which of the platelet's many activities are important in the development of MCTP-induced pulmonary hypertension? In an effort to begin to address this question, Hilliker *et al.* (1983) compared the responses of Sprague-Dawley and Fawn-Hooded (FH) rats to MCTP. FH rats have a congenital platelet defect which consists of a reduced ability to take up 5HT and to store 5HT and ADP, decreased release of these substances when stimulated, and reduced aggregatory responses to collagen and arachidonate (Raymond and Dodds, 1975).

MCTP caused similar pneumotoxic injury in both strains of rats, suggesting that 5HT and ADP may not be essential to the pathogenesis. However, FH rat platelets do generate TXA_2 and PDGF normally, and may have a small amount of residual dense granule function; perhaps these may be adequate for them to fulfill their role in MCTP pneumotoxicity (Hilliker *et al.*, 1983a).

5HT has received quite a lot of attention as a potential mediator of the pneumotoxic effects of MCT, and not only as a component of platelets; mast cells, which also release 5HT, have been identified in the lungs of rats fed *C. spectabilis* seeds. 5HT causes increased vascular permeability in vessels and is a vasoconstrictor as well; both of these effects could contribute to MCT pneumotoxicity (Sugita *et al.*, 1983b; Hilliker and Roth, 1985a). Damage to the pulmonary endothelium by MCT(P) results in a decreased ability to take up and metabolize this vasoactive mediator (Gillis *et al.*, 1978;(Hilliker *et al.*, 1982; Hilliker *et al.*, 1983c), which might lead to increased levels of 5HT in the pulmonary vasculature; the pressor response to 5HT is also increased in the lungs of MCTP-treated rats (Hilliker and Roth, 1985a). Finally, p-chlorophenylalanine (PCPA), a 5HT synthesis inhibitor, attenuated the increased PAP and RVH seen following MCT treatment (Carillo and Aviado, 1969; Tucker *et al.*, 1983; Kay *et al.*, 1985); however, this may be due to effects on metabolism or body weight gain rather than a result of decreased 5HT synthesis. Ganey *et al.* (1986) gave the 5HT receptor antagonists, metergoline and ketanserin, to MCTP-treated rats. These agents had no impact on either the lung injury or the pulmonary hypertensive effects of MCTP (Ganey *et al.*, 1986), suggesting that 5HT

does not play an important role in development of the cardiovascular changes in this model.

The platelet-derived, vasoactive mediator, TXA_2, has also received its share of attention in the MCT literature. Initial studies utilized a group of agents which shared the ability to inhibit platelet prostaglandin synthesis. Hydralazine, dexamethasone and sulphinpyrazone all reduced the degree of RVH which resulted from MCTP treatment, and hydralazine and dexamethasone also reduced BALF protein content, suggesting that platelet-derived vasoconstrictors could play a role in the development of MCTP-induced pulmonary hypertension (Hilliker and Roth, 1984b). However, these compounds could have a number of other potential effects on the lungs as well, including vasodilation and generalized anti-inflammatory effects, and the results may have been explained in a number of ways.

Arachidonic acid metabolites, particularly TXA_2, are thought to be important in endotoxin-induced pulmonary hypertension (Hales *et al.*, 1981). Interest focused on this mediator in the MCT model when it was discovered that lungs from rats treated with MCT (Stenmark *et al.*, 1985) or MCTP (Ganey and Roth, 1987b) release increased amounts of TXA_2 late in the course of injury. The source of the TXA_2 has not been identified and could be either platelets or lung tissue. However, subsequent studies with the thromboxane synthesis inhibitor, dazmegrel (Langleben *et al.*, 1986; Ganey and Roth, 1986), the cyclooxygenase inhibitor, ibuprofen, and the TXA_2 receptor antagonist, L-640,035 (Ganey and Roth, 1986) have shown that interference with TXA_2 synthesis or action does not attenuate the cardiopulmonary effects of MCT(P). Thus, another platelet mediator was eliminated from the running.

The role of the mesenchymal cell mitogen, PDGF, has also been investigated. This growth factor, released from platelets or ECs (Ross *et al.,* 1986), could play a role in the vascular remodeling phase of MCT(P) pneumotoxicity. However, PDGF neutralizing antibody was not protective when administered daily for 14 days following MCTP treatment (Ganey *et al.,* 1988). It is possible that systemically-administered anti-PDGF would not block the paracrine effects of PDGF in the microenvironment of the vascular wall, therefore it is difficult from this result to eliminate PDGF (or other growth factors) as important players in the vascular remodeling seen with MCT(P) lung injury.

In summary, platelets appear to be important after MCT(P)-induced lung injury is established but prior to the development of pulmonary hypertension and RVH. Although a few of the potential mechanisms for platelet involvement have been eliminated, there are still several to explore, including a number of other platelet-associated growth factors such as transforming growth factors α and β and epidermal growth factor (Assoian and Sporn, 1986; Oka and Orth, 1983).

D. The Role of Inflammation

A majority of investigators report a distinct inflammatory component to MCT(P) pneumotoxicity which is progressive throughout the course of the lung injury. Inflammatory infiltrates are most evident in the alveolar walls and perivascular interstitium, and consist primarily of mononuclear cells, including mast cells, macrophages, lymphocytes and plasma cells (Takeoka *et al.,* 1962; Kay *et al.,* 1967b; Valdivia *et al.,* 1967a; Sugita *et al.,* 1983b; Kay and Heath, 1966; Sugita *et al.,*

1983a; Stenmark *et al.,* 1985; Dahm *et al.,* 1986). There is also a slight, transient increase in polymorphonuclear cells (PMNs) in the BALF at 24 hr, and progressively increasing numbers are recoverable from the lungs after 5 days posttreatment with MCTP or 21 days after MCT treatment (Stenmark *et al.,* 1985; Ilkiw *et al.,* 1989; Czer *et al.,* 1986; Schultze *et al.,* 1990).

Although these inflammatory cells are generally present, their role in the development of pulmonary lesions in this model have not been established. These cells respond to and release a number of mediators which could be important in the vasoactive and structural changes which develop in the lungs of MCT(P)-treated animals, and anti-inflammatory drugs such as dexamethasone and methylprednisolone protect against the development of RVH (Hilliker and Roth, 1984b); however, these drugs also decreased weight gain and the results could be explained on this basis. Exudation of fluid and protein from the pulmonary vasculature occurs relatively early in the course of MCT(P)-induced lung injury (Wilson and Segall, 1990; Heath, 1969; Valdivia *et al.,* 1967b; Molteni *et al.,* 1984; Sugita *et al.,* 1983b; Schultze *et al.,* 1990), and may contribute to or arise as a consequence of pulmonary inflammation. Increased collagen in the adventitia of pulmonary arteries (Merkow and Kleinerman, 1966a; Guzowski and Salgado, 1987; Meyrick and Reid, 1982) and pulmonary interstitial fibrosis (Kay *et al.,* 1967b; Kay *et al.,* 1969; Butler, 1970a; Meyrick and Reid, 1979; Meyrick *et al.,* 1980) have been reported as later developments, and are likely sequelae to chronic inflammation.

Mast cells accumulate in the lungs of *C. spectabilis*-treated rats (Takeoka *et al.,* 1962; Kay *et al.,* 1967b; Valdivia *et al.,* 1967a). These cells release

a number of vasoactive mediators, including 5HT (discussed above with platelets), heparin, histamine and leukotrienes D_4 and E_4. It appears that the accumulation of mast cells correlates with the severity of pulmonary exudative changes rather than with the extent of pulmonary hypertension (Kay *et al.*, 1967b), suggesting that they do not play a critical role in the development of the hypertensive lesions. However, when mast cells are present, the mediators they release could contribute to the disease process. In the discussion of the platelet above, 5HT was eliminated as a major player in MCT(P) pneumotoxicity. However, mast cells and other inflammatory cells also generate leukotrienes (LTs), and a case has been made for their involvement in MCT(P) pneumotoxic injury. These arachidonic acid metabolites are potent vasoconstrictors and cause increased vascular permeability as well (Samuelsson, 1983). They also interact with the cyclooxygenase pathway, inducing the release of TXA_2 (Omini *et al.*, 1982). LT synthesis inhibitors block hypoxic pulmonary vasoconstriction, and are proposed to play an important role in hypoxia-induced pulmonary hypertension (Morganroth *et al.*, 1984).

Stenmark *et al.* (1985) found increased levels of LTs B_4, D_4 and E_4 in the BALF of MCT-treated rats, and treatment with the 5-lipoxygenase inhibitor, diethylcarbamazine (DEC), was protective against both the early lung injury and the later development of pulmonary hypertension in MCT-treated animals. However, Bruner and Roth (1984) found that whereas DEC protected against early lung injury in MCTP-treated animals, it did not influence the development of later lung injury and pulmonary hypertension. DEC also retarded weight in these studies, and this effect may have been responsible for any protective effects. Nevertheless, this area

merits further investigation. More specific LT synthesis inhibitors are currently available, which should make it possible to define better the role of LTs in MCT(P)-induced cardiopulmonary toxicity.

The lipid autacoid, platelet-activating factor (PAF), can stimulate LT synthesis and can induce pulmonary hypertension when infused chronically into rabbits (Ohar *et al.*, 1991). This inflammatory mediator was evaluated in MCT-treated rats by Ono and Voelkel (1991). PAF levels were increased in the lungs of treated animals at 1-3 weeks posttreatment, and administration of specific PAF antagonists reduced pulmonary vascular leak at 1 week posttreatment and inhibited the development of pulmonary hypertension and RVH. The authors propose that injury to endothelial cells or activation of macrophages may result in release of this mediator during the early phases of MCT-induced lung injury, which could in turn stimulate LT synthesis and activate platelets. This may be an important area to pursue.

The cytokines have received little attention as possible mediators in this model. Interleukin 1 (IL-1) is transiently increased in the BALF of MCT-treated rats at 4 days posttreatment, and is increased again on days 14 and 21 (Gillespie *et al.*, 1988). It is not certain what role, if any, these mediators play in MCT-induced lung injury, but macrophage and PMN products might be involved in the development of the toxicity.

Myeloperoxidase activity is increased in BALF at 21 days posttreatment (Gillespie *et al.*, 1988), suggesting that PMNs are present in the lungs after MCT(P) treatment. Granulocytes and phagocytic cells are capable of generating reactive

oxygen species (Fantone and Ward, 1982; Repine *et al.*, 1979), and it has been postulated that these could play a role in MCT pneumotoxicity. Dahm *et al.* (1986) examined the ability of cells recovered from the BALF of MCTP-treated rats to generate superoxide anion. Cells collected from MCTP-treated rats on days 7, 10 and 14 posttreatment generated less superoxide than cells from control rats when stimulated *in vitro*. These cells may have already been stimulated to generate superoxide *in vivo* so that their ability to generate more *in vitro* was exhausted, or they may have been down-regulated in this capacity. Bruner *et al.* (1987b) pursued the role of reactive oxygen species in this model by attempting to intervene with drugs which prevent formation of toxic species or degrade them. Cotreatment with deferoxamine, dimethyl sulfoxide or catalase was not protective in MCT-induced lung injury, suggesting that toxic oxygen metabolites do not play an important role in this model. It could be argued that these drugs were unable to block the effects of oxygen metabolites generated at the inflammatory cell/lung tissue interface; however, this regimen of treatment is effective in other models of neutrophil-mediated injury (Ward *et al.*, 1983; Till *et al.*, 1985).

Another agent released by activated PMNs is elastase. Neutrophil elastase degrades elastin and types III and IV collagen and causes damage to ECs in certain models of vascular injury (Mainardi *et al.*, 1980; Smedley *et al.*, 1986). Increased fragmentation of the internal elastic lamina of larger muscular arteries has been observed 4 days after MCT administration, and elastases have been proposed to play a role in MCT pneumotoxicity (Todorovich-Hunter *et al.*, 1988). Co-administration of the neutrophil serine elastase inhibitor, SC39026, attenuated the

vascular remodeling and reduced the PAP and RVH in MCT-treated animals; however, it did not protect against early lung injury, including EC changes, or reduce the inflammation (Ilkiw *et al.*, 1989; Molteni *et al.*, 1989b). When administration of the inhibitor is discontinued at 8 days posttreatment, pulmonary changes are not present at 2 weeks but are evident at 3 weeks posttreatment (Ilkiw *et al.*, 1989). Thus, neutrophil elastase appears to be important in the initial remodeling of pulmonary vessels and in the maintenance of vascular changes that result in pulmonary hypertension. Platelets (James *et al.*, 1986) and macrophages (Banda and Werb, 1981) also produce elastases, and these may be important in MCT-induced vascular remodeling as well.

A number of agents prevent pulmonary fibrosis in MCT-treated animals. Penicillamine, which inhibits collagen crosslinking, and the ACE inhibitors, captopril and CL242817, all decrease lung hydroxyproline content and attenuate the development of pulmonary hypertension and RVH in this model (Molteni *et al.*, 1985; Molteni *et al.*, 1986). None of these compounds affords protection against the early lung injury caused by MCT, however penicillamine and captopril do ameliorate the decrease in fibrinolytic activity. It has been proposed that sparing of this EC function may account for the antifibrotic effect of these compounds (Molteni *et al.*, 1985; Law, 1981).

CL242817 prevents weight gain, and could influence MCT toxicity via this mechanism (Hayashi *et al.*, 1979; Ganey *et al.*, 1985); however, the other 2 agents do not affect body weight. Penicillamine does not inhibit ACE activity but does chelate copper, which is elevated in serum in MCT(P)-treated rats (Molteni *et al.*,

1985; Ganey and Roth, 1987; Molteni *et al.*, 1988b). Although it is possible that this ability to decrease serum copper concentration is a mechanism for the protective effects of penicillamine, it seems unlikely. In general, ACE inhibitors do not chelate copper, but serum copper is also lower when CL242817 is co-administered with MCT. Serum copper concentration appears to vary with the severity of the cardiopulmonary damage induced by MCT, and agents which reduce the injury may indirectly reduce the hypercupremia (Molteni *et al.*, 1988b). Pulmonary fibrosis is a common sequel to many types of lung injury and frequently occurs as a result of chronic inflammation. By limiting the development of fibrosis, many of the later events in MCT toxicity appear to be prevented; the mechanisms of this protection remain uncertain.

The possibility that an immune-mediated response may account for some of the effects of MCTS(P) has also been explored (Bruner, 1986; Bruner *et al.*, 1987a). Immunosuppression has been achieved by either administration of an antiserum directed against rat lymphocytes (LAS) or the administration of cyclosporin A. Administration of LAS has no effect on MCT pneumotoxicity. Cyclosporin A is protective against both the early lung injury and the development of RVH, however, this compound also restricts weight gain and may protect in this way (Hayashi *et al.*, 1979; Ganey *et al.*, 1985). MCTP does not activate complement *in vivo* or *in vitro*, and complement depletion is not protective (Bruner *et al.*, 1988). From these results, the participation of either cell-mediated or humoral immunity in MCT pneumotoxicity seems unlikely.

Shubat *et al.* (1987) reported the development of vascular alterations and RVH after treatment with MCT in the absence of inflammation, suggesting this feature of MCT toxicity is not essential to the development of vascular injury. However, MCT(P)-induced inflammation, when present, may influence the progression of the injury. It is still unclear which of the specific cells and mediators are essential to the progression of lung injury and which are secondary contributors. Leukotrienes and PAF are still viable candidates, and elastases seem to be quite important as well; these will presumably receive further attention as more selective inhibitors are identified. The antifibrotic agents are also quite interesting, as they may represent a relatively late intervention which affords some protection against chronic pulmonary vascular injury.

E. The Role of Cell Growth and Proliferation

Several lines of evidence suggest that alterations in cell growth and proliferation are fundamental to the expression of MCT(P) cardiopulmonary toxicity: young, growing animals are more susceptible to the effects of MCT than older animals (Allen and Chesney, 1972b; Todd *et al.,* 1985), proliferative vascular alterations precede the development of MCT-induced pulmonary hypertension (Hislop and Reid, 1974; Meyrick and Reid, 1982; Masugi *et al.,* 1965; Kay and Heath, 1966; Heath and Kay, 1967), and hypertrophy is evident in a number of cell types in the lung and other organs (Turner and Lalich, 1965; Masugi *et al.,* 1965; Valdivia *et al.,* 1967a; Wilson and Segall, 1990). Perhaps most convincing are the protective

effects of diet restriction and the involvement of polyamines in the expression and progression of the MCT-induced pulmonary vascular changes.

Diet restriction of MCT-treated animals is almost completely protective against the lung injury and lethality of the toxicant. However, without further exposure to MCT, return to a normal diet (and consequent resumption of growth) results in the rapid development of pulmonary injury and death (Hayashi *et al.*, 1979). Diet restriction of MCTP-treated rats is also somewhat protective, although less markedly so, suggesting that protection is not due solely to decreased bioactivation of MCT (Ganey *et al.*, 1985). It is not clear whether a particular aspect of diet restriction or general suppression of weight gain is most critical to the protective effects; however, altered sodium intake has been ruled out as a potential factor (Ganey *et al.*, 1985). In any event, it appears that suppression of the growth of the animal, or alteration of its nutritional status, results in suppression of whatever mechanism it is that causes initial, irreversible changes in target cells to progress to overt pulmonary vascular lesions.

Diet restriction increases mortality from hyperoxia, and this has been attributed to compromised repair (i.e. inhibition of proliferation) in the lungs (Elsayed *et al.*, 1988). Among its myriad effects, diet restriction decreases polyamine content and protein, lipid and DNA synthesis in the lung (Gail *et al.*, 1977; Faridy, 1970; Elsayed *et al.*, 1988). Refeeding after food restriction accelerates polyamine and DNA synthesis above normal levels in the liver (Domschke and Soling, 1973), and increases ODC activity in the lung as well (Elsayed *et al.*, 1988). Polyamines are necessary for optimal cell growth and proliferation, under normal circumstances and

following injury, and agents which alter PA metabolism have been associated with reduced cell proliferation (Heby, 1981; Pegg and McCann, 1982). It may be that these small, organic cations are the link between diet restriction and protection against MCT pneumotoxicity.

The polyamines have been implicated as important mediators in the development of MCT-induced pulmonary hypertension. There is a sustained increase in the activity of lung ornithine decarboxylase (OCD) in rats treated with MCT (Olson *et al.*, 1984b). This precedes the development of pulmonary hypertension and right ventricular hypertrophy (Olson *et al.*, 1984b). Lung S-adenosylmethionine decarboxylase (SAM-DC) activity is also increased by MCT treatment. ODC and SAM-DC are the rate-limiting enzymes in the polyamine synthetic pathway; they regulate production of these organic cations, which are associated with cell growth, proliferation and differentiation (Pegg and McCann, 1982). Levels of the diamine, putrescine, and the polyamine, spermidine, are elevated in whole lung and pulmonary arterial wall by day 4 and day 7, respectively, after MCT treatment; spermine content is increased in lung and vessels at 21 days posttreatment (Olson *et al.*, 1984a; Orlinska *et al.*, 1988). In addition, lung spermidine/spermine acetyltransferase (SAT) activity and levels of the secondary polyamine, N^1-acetylspermidine, are increased by MCT treatment (Orlinska *et al.*, 1989).

Continuous treatment with α-difluoromethylornithine (DFMO), a specific, irreversible inhibitor of ODC activity, decreases the levels of putrescine and spermidine in the lungs and attenuates the medial wall thickening, increased PAP and RVH associated with the pulmonary hypertensive phase of MCT pneumotoxicity

(Olson *et al.*, 1984a; Olson *et al.*, 1985). DFMO treatment also blunts the development of pulmonary edema and attenuates the early, transient increase in vascular reactivity which follows MCT treatment (Gillespie *et al.*, 1985b; Olson *et al.*, 1985).

DFMO does not alter the bioactivation of MCT, thus does not afford protection in this manner (Olson *et al.*, 1985). When animals receiving MCT and DFMO are supplemented with ornithine, the immediate precursor to the polyamines, their lung polyamine levels again increase to those seen in MCT-treated animals and the protective effects of DFMO are reversed (Olson *et al.*, 1989), suggesting that polyamines are important in the evolution of MCT-induced pulmonary hypertension. More specifically, these data support the concept that cell growth and/or proliferation is important in the expression of MCT-induced pulmonary effects: increased polyamine levels are essential for cell growth in response to any stimulus (Heby, 1981). The search for the specific stimulus (or, more likely, stimuli) for cell proliferation in the MCT model continues.

Increased epidermal growth factor (EGF)-like immunoreactivity is detectable in the perivascular regions of the lung in rats treated 4 days earlier with MCT (Gillespie *et al.*, 1989). This coincides with a time when platelet depletion protects against the pulmonary hypertensive effects of MCT, and platelets may serve as a source for this EGF. Chronic administration of exogenous EGF for 7 days results in increased medial thickening in arteries 100-200 μ in diameter, increased PAP and increased lung polyamine content. Smaller muscular arteries are not affected, but in general, the effects of EGF on the lung are quite similar to those

caused by administration of MCT. It seems likely that this smooth muscle cell mitogen may serve as one stimulus for cell proliferation after MCT treatment. Studies using EGF-neutralizing antibody may help to confirm the importance of this mediator in the MCT model.

Much of the cell proliferation which occurs after administration of MCT involves the smooth muscle cells of the pulmonary vasculature. In addition to its anticoagulant effects, heparin has several properties that can interfere with vascular remodeling. It inhibits platelet adherence and degranulation and thereby eliminates this source of PDGF in the vessel wall (Heiden *et al.*, 1977). Heparin also inhibits smooth muscle cell proliferation and migration, both *in vivo* and *in vitro* (Clowes and Karnovsky, 1977; Clowes and Clowes, 1986; Hoover *et al.*, 1980). It has been shown to reduce significantly vascular remodeling, PAP and RVH in mice exposed to chronic hypoxia (Hales *et al.*, 1983), and is useful in preventing intimal hyperplasia after balloon angioplasty as well (Clowes and Karnovsky, 1977).

Fasules *et al.* (1987) found that neither anticoagulant nor nonanticoagulant heparin attenuated MCT-induced lung injury. They attributed this lack of protection to a number of factors: increased inflammation, more widespread injury, different mediators involved in stimulation of proliferation, different smooth muscle cell responsiveness to heparin, or altered pharmacokinetics for heparin in the MCT model. These results suggest that despite certain similarities in the chronic pulmonary vascular alterations caused by hypoxia and MCT, there may be important differences in the mechanisms underlying cell proliferation in the two models.

The evidence supporting a critical role for cell growth and altered proliferation in the expression of MCT(P) lung injury is compelling. Numerous cell types and mediators may be involved, and regulation of these phenomena is likely to be quite complex. Nevertheless, this is an area that merits further investigation.

F. The Role of Alkylation

The importance of the proliferative response in the development of cardiopulmonary toxicity after MCT treatment is interesting, particularly in light of the fact that the reactive metabolite, MCTP, is itself a potent antiproliferative agent (Mattocks and Legg, 1980; Reindel and Roth, 1991; Reindel *et al.*, 1991). Many of the PZAs are potent antimitotic agents in the liver, inhibiting cell division of hepatocytes but not interfering with cell growth or DNA synthesis and resulting in the development of very large cells with large nuclei (Bull *et al.*, 1968). Similar effects have also been noted in type II pneumocytes after treatment with MCT (Wilson and Segall, 1990). The antiproliferative effects of the PZAs have generally been attributed to the ability of reactive pyrrolic metabolites to act as a bifunctional alkylating agents and to crosslink DNA and other cellular macromolecules (Culvenor *et al.*, 1969).

Alkylation involves the interaction of an electrophilic center in one molecule with a nucleophilic center in another, resulting in the formation of a covalent bond. Electron-rich sulfur, nitrogen or oxygen atoms of cellular macromolecules are frequent targets for alkylating agents *in vivo* (Robertson *et al.*, 1977; Robertson, 1982). Monofunctional alkylating agents can be toxic, perhaps

through binding to active sites in proteins and inhibiting their activity. If some of the proteins affected are those essential to cell division, monofunctional alkylation could result in an inhibition of cell proliferation. Carbon 7 (C-7) of DHR, a pyrrolic metabolite of MCT, covalently binds to thiol groups on cysteine and GSH *in vitro*, suggesting that metabolites of MCT may alkylate thiol groups on proteins within cells (Robertson *et al.*, 1977).

Bifunctional alkylating agents (eg. MCTP) have two reactive centers and can function as crosslinking agents. Mattocks and Legg (1980) compared the effects of pyrroles with mono- or bifunctional alkylating ability on a rat liver cell line. The antimitotic effect of these compounds was dependent on the chemical reactivity of the pyrroles and their mono- or bifunctional activity. Some of the highly reactive monofunctional pyrroles demonstrated weak antimitotic ability, but bifunctional pyrroles with similar reactivity were much more effective antimitotic agents. Furthermore, a number of potent agents used in cancer chemotherapy are bifunctional alkylating agents, and their cytotoxic potential has been associated with their ability to crosslink DNA (Tokuda and Bodell, 1987; Bodell, 1990). For the most part, investigators in this field equate decreased colony-forming ability with cell death; thus, the cytotoxic effect most commonly associated with DNA crosslinking is inhibition of cell proliferation.

Pyrrolic derivatives of MCT crosslink DNA *in vitro* (Robertson, 1982; White and Mattocks, 1972; Hincks *et al.*, 1991), and DNA:DNA interstrand crosslinks and DNA:protein crosslinks are detectable in livers of rats treated with MCT *in vivo* (Petry *et al.*, 1984). The initial interaction of DHR involves C-7 of the pyrrole and

N^2 of deoxyguanosine (Robertson, 1982). Recent work with MCTP demonstrates that both the C-7 and C-9 positions of MCTP can react with a number of nucleosides at their nitrogen atoms, which could indicate a much less specific interaction with DNA than was previously supposed (Niwa *et al.,* 1991).

Crosslinking in rat liver is maximal 12 hours after MCT treatment, and slowly decreases so that it is indistinguishable from controls by 96 hours. Interestingly, a single administration of PZA can result in prolonged antimitotic effects in the liver (Culvenor *et al.,* 1969; Jago, 1969). Accordingly, it is difficult to correlate inhibition of proliferation with DNA crosslinking *in vivo*, as the former effect is persistent while the latter is transient. Perhaps the antimitotic effects of MCT and other PZAs are not due to the actual presence of crosslinks in the DNA, but are due to the consequences of repair of these lesions. Alternatively, the assay may not be sensitive enough to detect a small but persistent fraction of crosslinks. Further investigation will be required to resolve this issue.

It is possible to protect against the liver toxicity caused by MCT and the PZA-containing plants, *Senecio jacobea* and *S. vulgaris*, by adding mercaptoethylamine or cysteine to the diet (Hayashi and Lalich, 1968; Buckmaster *et al.,* 1976). These agents have sulfhydryl groups which may bind the reactive PZA moieties, limiting their ability to alkylate cellular macromolecules. Cysteine may also protect by increasing cellular GSH levels. Administration of the antioxidant, ethoxyquin, is protective against the lethal effects of MCT in mice. Other antioxidants are not protective, suggesting that ethoxyquin's ability to increase

glutathione S-transferase activity may be responsible for its beneficial effects (Miranda *et al.*, 1981a).

It is possible that the interaction of a reactive metabolite of MCT with DNA or other nucleophiles in cells of the pulmonary vasculature is the most immediate consequence of exposure to this compound. The delayed consequences of such an interaction may initiate a cascade of events that results in the development of the characteristic pathophysiology we associate with MCT pneumotoxicity. The lungs of MCT(P)-treated animals have not been examined for DNA crosslinking, nor has anyone looked at the ability of cysteine and other sulfhydryl-containing compounds to protect the lung from MCT(P)-induced injury. Studies of this type would provide important information with regard to the importance of crosslinking to the development of pulmonary vascular injury. In addition, there is some question as to the nature of the MCT metabolite that reaches the lung vasculature. The identification of DNA:DNA or DNA:protein crosslinks in the lung would support the concept that MCTP or another bifunctional metabolite is directly involved in the injury.

From this discussion, it is apparent that the mechanisms involved in the induction of lung injury and the development of pulmonary hypertension in this model are neither simple nor fully understood. The complexity of the response to MCT suggests that a single mechanism is unlikely to account for all aspects of the toxicity. More realistically, the chronic cardiopulmonary changes which result may be the cumulative response to effects on a number of vascular and parenchymal cells.

A dissection of the model to its component parts may provide information about the direct effects of MCT(P) on specific cell types and the possible consequences of these effects on the development of lung vascular injury.

V. The Vascular Endothelium

Damage to the endothelium is a common feature of several models of lung injury and pulmonary hypertension. Situated at the interface of the blood and the vascular wall, the endothelium is first among lung cells to encounter blood-borne mediators and toxicants. Once thought to serve as a passive barrier, the endothelium is now recognized for the important role it plays in the maintenance of vascular homeostasis. This tissue is capable of generating, modifying and responding to a variety of bioactive substances, and alterations in endothelial cell function may be central to many forms of vascular and systemic disease.

There is a massive body of literature which focuses on the endothelial cell in both healthy and diseased states. Although it is not possible to review this work in detail, I will attempt to give a brief sketch of normal endothelial cell structure and function, as well as an overview of the response to injury, in order to set the stage for a discussion of the involvement of the endothelium in MCT(P)-induced pulmonary vascular disease.

A. Structure

The vascular endothelium covers the intimal surface of the blood vessels and consists of a single layer of flattened, polygonal cells. In profile, ECs

exhibit an attenuated cytoplasm with a bulging nucleus. They range from 10-50 μ in diameter and from 0.1-0.5 μ in thickness. *In vivo*, ECs tend to orient themselves longitudinally in the direction of blood flow (Wehrmacher, 1988). The morphologic character of the endothelial layer, like many other features of ECs, varies with the vascular source and may be continuous as in the brain or lung vessels, fenestrated as in glomerular capillaries, or discontinuous as in the spleen,liver or bone marrow (Renkin, 1988).

Electron microscopy reveals that ECs are covered with many finger-like projections and caveolae, greatly increasing their surface area (Ryan, 1982c). The luminal surface of ECs is covered with a carbohydrate-rich coating called the glycocalyx. This is a specialization of the plasma membrane consisting of glycosaminoglycans and the polysaccharide side chains of plasma membrane glycoproteins and glycolipids. The glycocalyx is typically 15-40 nm thick and gives the surface of the EC a fuzzy appearance on electron micrographs (Luft, 1966; Parson and Subjeck, 1972). A number of plasma proteins are found adsorbed to this carbohydrate surface, including albumin, α_2-macroglobulin, lipoprotein lipase, fibrinogen, antithrombin III, heparin cofactor II, protein C and protein S (Wehrmacher, 1988). The glycocalyx is proposed to play a role in regulation of endothelial permeability by influencing the distribution of anionic sites on the cell surface, and it may serve as a size, shape and charge barrier to the movement of small molecules (Simionescu and Simionescu, 1986). It also may act to mask surface receptors, thus playing a role in recognition between ECs and blood elements (Ryan, 1986).

ECs are able to synthesize their own subcellular matrix, which consists of collagen (Types I, III, IV or V), elastin, and the glycoproteins laminin and fibronectin. The composition of the subendothelial matrix varies with the state of growth, cellular environment, vessel type and species and can influence EC proliferation, migration, spreading and multicellular organization (Sage, 1984; Jaffe *et al.*, 1976; Carnes *et al.*, 1979; Madri and Williams, 1983; Jaffe and Mosher, 1978; Gospodarowicz and Ill, 1980; Form *et al.*, 1986). The abluminal surface of the EC is anchored to the subendothelial matrix by specialized matrix binding proteins known as integrins. These proteins bind to laminin, fibronectin, collagen and vitronectin and are involved in securing ECs not only to the matrix but also to adjoining ECs (Macarak and Howard, 1983; Lampugnani *et al.*, 1991).

Fibronectin is not restricted to the subendothelium but covers all surfaces of the EC and is released to the intercellular stroma and blood or culture medium, as are collagen and elastin. A layer of fibronectin is essential for EC adhesion, spreading and locomotion. In addition, it interacts with the glycosaminoglycans, dermatan sulfate, heparin sulfate and chondroitin sulfate, which are synthesized by ECs and deposited on the cell surface or released to the subendothelium and blood or medium. The glycosaminoglycans bind lipoprotein lipase and several coagulation factors to the endothelial surface, trap lipid in the vessel wall, and play a role in EC regulation of SMC proliferation (Barnes *et al.*, 1978; Buonassisi, 1973; Castellot *et al.*, 1982; Camejo *et al.*, 1985; Colburn and Buonassisi, 1982).

ECs are interconnected a three types of junctions: occluding (tight), adherens, and connecting (gap) junctions. Desmosomes are generally not present, however there are occasional desmosome-like regions evident at the abluminal plasma membrane. The presence or absence of a particular type of intercellular junction depends a great deal on the vessel being considered. For example, capillary ECs do not have gap junctions; postcapillary venular ECs are joined by modified tight junctions (Larson and Sheridan, 1982; Simionescu *et al.*, 1975; Franke *et al.*, 1988). In addition, ECs in confluent monolayers are connected by endothelial growth inhibitory protein (EGIP) and calcium-dependent vascular cadherin (V-CAD). Disruption of these intercellular attachments may result in stimulation of endothelial proliferation (Heimark and Schwartz, 1988). ECs also send processes through the subendothelial matrix and internal elastic lamina to form myoendothelial junctions with the underlying SMCs, and these two cell types communicate via gap junctions as well (Wehrmacher, 1988; Clowes *et al.*, 1989).

In addition to the integrins and cadherins mentioned previously, two other forms of EC-related cell adhesion molecules are associated with the plasma membrane. The selectins and addressins are responsible for interaction of white blood cells with the vessel wall (Lelkes, 1991). These molecules are important in the EC response to inflammation, and they will be discussed in this context later.

EC contraction, spreading, migration, proliferation, and attachment to other cells and the subendothelial matrix are all activities mediated by the cytoskeleton. Vascular ECs contain microtubules and actin microfilaments which are highly dynamic and contribute to a number of cell functions. Desmin and cytokeratin

intermediate filaments are evident in the ECs of some vascular beds in select species, but vimentin is the only intermediate filament present in all mammalian ECs. These intermediate filaments are less dynamic and may be involved in some way with the state of cell differentiation. In general, cytoskeleton-mediated activity is involved in the maintenance of endothelial integrity and control of vascular permeability; a functional cytoskeleton is also critical to the monolayer repair process (Gottlieb *et al.,* 1991; Alexander *et al.,* 1991).

B. Homeostatic Functions of Endothelium [Figure 2]

1. Regulation of Vascular Permeability

One of the earliest recognized functions of the vascular endothelium was its role as a barrier to movement of water, solutes and macromolecules between the vascular space and the interstitium. An intact layer of ECs was thought to serve as a predominantly physical barrier to fluid movement, as a loss of endothelial monolayer integrity resulted in vascular leak and its consequences. Although the basic structure of the endothelial layer is important to its ability to regulate vascular permeability, it is now recognized that this regulatory process is quite active and complex.

Transport pathways for fluids and solutes through unfenestrated microvascular endothelium (e.g., pulmonary vascular endothelium) include passive movement through the cell membrane, vesicular, small pore and large pore pathways of the cell, and passage through the intercellular junctions. Water and small, lipophilic molecules generally move through the lipid bilayer directly. Shuttling of transcytotic vesicles from all membrane surfaces results in no net fluid movement,

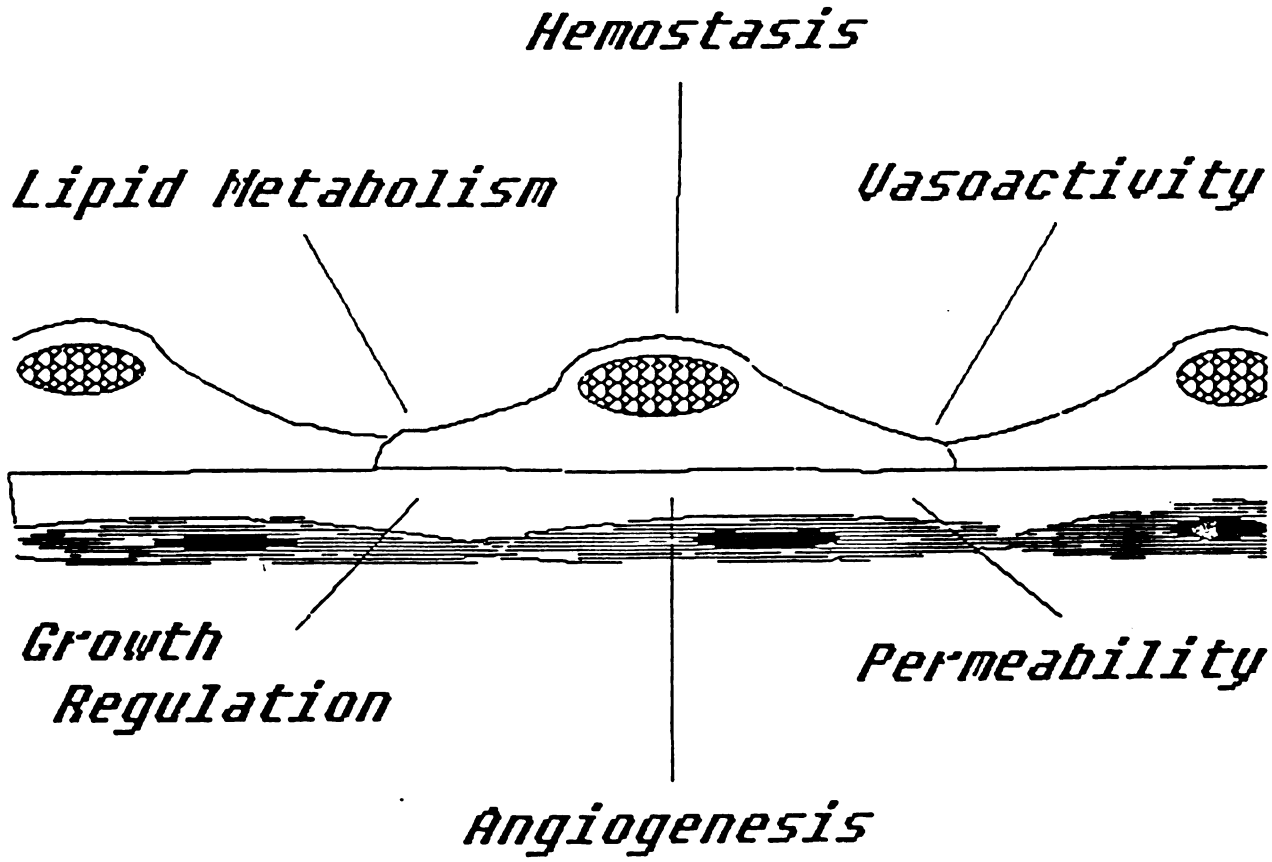


Figure 2: Homeostatic functions of endothelium.

but does contribute to the equilibration of luminal and abluminal fluids. Small and large pore channels provide a continuous route for movement of water and solutes, including plasma proteins, across the endothelial layer. Larger, lipid-soluble molecules may move through the intercellular junctions by diffusion in the membrane (Renkin, 1977; Renkin, 1988).

Fenestrated endothelial layers possess all of the pathways mentioned above, but also allow transport of much larger molecules. Open fenestrae, such as those found in the glomerular capillaries, are 700-800 Å in diameter and would thus restrict only the cellular components of the blood. However, in this organ, the basement membrane and epithelium help to limit perfusion of larger plasma proteins. In other vascular beds, the fenestrae are covered by a thin diaphragm which restricts the movement of plasma proteins but allows freer flow of water, ions and small molecules than is possible through continuous endothelium. Discontinuous endothelial layers lack a continuous basement membrane, and very large proteins and even cells are able to enter the bloodstream at these locations (Renkin, 1988; Deen *et al.*, 1983).

In addition to the passive means of transport, there are endocytotic and transcytotic receptors which bind and transport larger molecules, including low density lipoprotein (LDL), insulin, growth factors, and plasma transport proteins such as albumin and transferrin. As was mentioned previously, the endothelial surface is covered by a glycocalyx, and its proteoglycans, sialoconjugates and adsorbed proteins provide a negative surface charge. Specialized coated pits bind the more cationic ligands, but it has been proposed that charge-differentiated

microdomains exist along the surface of the cells which may serve as sites for transport of anionic molecules that would otherwise be repelled (Simionescu and Simionescu, 1986).

2. Regulation of Vasoactivity

Another important function of the endothelium involves its ability to take up and metabolize circulating vasoactive molecules, generating both active and inactive mediators. This function is especially critical in the pulmonary endothelium, as the lung receives 100% of the cardiac output and can therefore adjust the level of vasoactive substances present in the blood delivered to the systemic circulation.

The numerous caveolae present on the surface of ECs serve to increase the vascular surface area exposed to the blood and provide a specialized microenvironment for the activity of metabolic enzymes. Several endothelial ectoenzymes have been localized to these structures, including ACE, ATPase, ADPase, 5'-nucleotidase, carboxypeptidase N and carbonic anhydrase (Ryan and Ryan, 1985; Ryan, 1986). ACE is the carboxy terminal dipeptidase responsible for conversion of Ang I to the active vasoconstrictor, Ang II, and degradation of bradykinin (BK). Thus, activity of this enzyme tends to promote vasoconstriction (Yang *et al.*, 1970; Erdos, 1975; Ryan, 1982c; Ryan and Catravas, 1990). ACE activity is much greater in ECs than in other cells of the vascular wall, and has been used as a marker to identify this cell type in culture (Auerbach *et al.*, 1982).

The endothelial ectonucleotidases (ATPase, ADPase and 5'-nucleotidase) are the major regulators of circulating adenine nucleotide levels *in vivo*.

AMP is relatively inactive in the blood vessels, but ADP and adenosine have marked effects on platelets and are vasoactive as well (Smith and Ryan, 1972; Pearson *et al.*, 1980; Slakey *et al.*, 1988). Carboxypeptidase N is important in the pulmonary clearance of anaphylatoxins (Ryan and Ryan, 1985; Ryan *et al.*, 1982b), and carbonic anhydrase presumably plays a role in the exchange of CO_2 and bicarbonate in the vessels (Ryan *et al.*, 1982a).

Endothelial cells also possess monoamine oxidase activity which is responsible for degrading the biogenic amines, 5HT and NE. MAO is intracellular, thus removal of these substances necessitates facilitated transport into the cell (Hughes *et al.*, 1969; Iwasawa *et al.*, 1973; Cross *et al.*, 1974). Enzymes that metabolize histamine are also present in ECs, although not in all vascular beds or all species (Robinson-White and Beaven, 1982). In addition, the lung vasculature serves to clear Ang II, adenosine, numerous prostaglandins, and the vasoconstrictor peptide endothelin (ET) from the blood. Angiotensinases A and C have been identified in pulmonary ECs, and it is likely that the clearance of prostaglandins and ET are endothelial-mediated as well (Gillis and Roth, 1976; Piper *et al.*, 1970; Johnson and Erdos, 1977; Pearson *et al.*, 1978; Sirvio *et al.*, 1990). Although endothelial uptake and subsequent phosphorylation or deamination of adenosine occurs, it is not clear how this compound enters ECs, and the importance of endothelial clearance of adenosine *in vivo* is unknown (Catravas *et al.*, 1988a).

A number of receptors for vasoactive mediators have been identified on the surface of endothelial cells, including α and β adrenergic receptors, M_1 and M_2 muscarinic receptors, H_1 and H_2 histamine receptors, and receptors for

Ang II, 5HT and bradykinin (Buonassisi and Venter, 1976; Gryglewski *et al.*, 1988; Borsum, 1991). Mechanosensors, which respond to shear stress by opening and closing K+ channels, are also found on the endothelial cell surface (Lansman, 1988; Olesen *et al.*, 1988), and it is thought that ECs can sense changes in oxygen tension as well (Pohl, 1990). These receptors and sensors allow the endothelial cells to sense and react to changes in their environment and permit them to respond to stimulation by generating their own set of vasoactive mediators.

Endothelium-derived relaxing factors (EDRFs) are released in response to a number of stimuli, including acetyl choline (ACH; M_2), NE (α_2), thrombin, BK, histamine (H_1), 5HT ($5HT_1$), adenine nucleotides, stretch, hypoxia, shear stress, and others. As the name implies, the factors generated in response to these stimuli cause relaxation of vascular smooth muscle; they also have a disaggregatory effect on platelets (Furchgott and Zawadzki, 1980; Furchgott, 1984; Gryglewski *et al.*, 1988; Auerbach, 1981; Azuma *et al.*, 1986). Nitric oxide (NO) appears to be one of the relaxing factors released by ECs. In addition to its vasoactive properties, it may also serve as a protective chemical barrier against cytotoxic injury caused by superoxide (Palmer *et al.*, 1987; Gryglewski *et al.*, 1988). Some of the agonists (e.g., ACH on M_1 receptors) also cause ECs to release a substance which acts on K+ channels of SMCs to cause hyperpolarization. This is referred to as endothelium-derived hyperpolarizing factor (EDHF), and also results in relaxation of smooth muscle (Taylor and Weston, 1988). In addition, several of the mediators which cause ECs to release EDRFs also stimulate generation of PGI_2 by ECs, which contributes to vasodilation and inhibits platelet activation. Of the

agonists mentioned, ACH and BK may be the most physiologically relevant in maintaining the vasculature in an unconstricted and nonthrombogenic state under unstimulated conditions (Radomski *et al.*, 1987; Gryglewski *et al.*, 1988; Furchgott and Zawadzki, 1980).

In addition to relaxant substances, ECs can generate potent vasoconstrictive substances in response to stimuli. Endothelium-derived contracting factors (EDCFs) fall into three categories: 1.) vasoconstrictive arachidonic acid metabolites (eg. PGH_2 or TXA_2) are generated in response to Ang I or II, 5HT, substance P, adenine nucleotides, superoxide or stretch; 2.) an unnamed EDCF produced in response to severe hypoxia; and 3.) the potent vasoconstrictor peptide, ET, produced in response to Ang II, vasopressin, transforming growth factor β (TGF_β) or thrombin (Katusic and Shepherd, 1991; Yanagisawa *et al.*, 1988; Yanagisawa and Masaki, 1989). Several of these stimuli also release EDRFs, and it is thought that local regulation of blood flow may be modulated by a balanced generation of these endothelial-derived mediators.

3. Regulation of Hemostasis

The role of the endothelium in regulation of the hemostatic system has been investigated in detail. [For a review of this subject, please see Nawroth *et al.* (1985)] Any regulatory adjustment of this system involves a complex interplay of anticoagulant and procoagulant activities. Under normal conditions, the vascular endothelium must provide a nonthrombogenic surface for smooth, unimpeded blood flow; therefore, the balance is shifted in favor of the EC's anticoagulant functions.

In the previous section, the roles of EDRF and PGI_2 in vasorelaxation were discussed. These substances also decrease platelet binding and aggregation (Radomski *et al.*, 1987; Moncada *et al.*, 1977) and therefore decrease the thrombogenicity of the vessel wall. Vascular ECs have a variable capacity to produce a number of other arachidonic acid metabolites, including the cyclooxygenase products TXA_2, PGE_2, PGD_2, and $PGF_{2\alpha}$, and the lipid hydroperoxides, 5-, 12- and 15-hydroxyeicosatetraenoic acid (5-HETE, 12-HETE and 15-HETE) and 9- and 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE) (Nawroth *et al.*, 1985; Buchanan *et al.*, 1985; Buchanan and Bastida, 1988). Some of these products are pro- and some are anticoagulant in nature, and again a balance must be maintained in the production of these mediators.

In addition to EDRF and PGI_2, the 15-lipoxygenase product, 13-HODE, plays an imortant role in maintaining a nonthrombogenic vascular surface. This mediator is found in the cytosol of ECs but is able to inhibit platelet adherence and activity at the EC surface. It also inhibits PGI_2 synthase; thus, levels of PGI_2 vary inversely with concentrations of 13-HODE (Buchanan *et al.*, 1985). 15-HETE inhibits synthesis of PGI_2 and decreases platelet activity as well but tends to enhance vessel thrombogenicity. It appears that the relative concentrations of these compounds influence the expression of adhesive moieties on a number of cell types, including ECs and platelets. 13-HODE production is predominant under unstimulated conditions and inhibits cell adhesion; 15-HETE predominates after stimulation or injury, and enhances cell adhesivity (Hopkins *et al.*, 1984; Buchanan and Bastida, 1988).

The glycosaminoglycan, heparan sulfate, is produced by endothelial cells and then binds onto the cell surface or in the subendothelial matrix. Heparan sulfate binds antithrombin III, which inactivates thrombin and may also inactivate coagulation factors IXa, Xa and XIIa (Castellot *et al.,* 1982; Stern *et al.,* 1985).

Thrombomodulin (TM) and protein S are two more EC products with anticoagulant function. TM is located on the surface of ECs where it acts as a receptor for thrombin. Once bound to TM, thrombin loses its procoagulant ability and instead activates the anticoagulant protein C; activated protein C in turn inactivates factors Va and VIIIa. Protein S, also generated by ECs, facilitates the activation of protein C by thrombin (Esmon and Owen, 1981; Stern *et al.,* 1986).

Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are serine proteases which are generated by ECs in response to stimuli similar to those that release EDRF, including thrombin. tPA and uPA give endothelial cells their fibrinolytic capacity, and the EC surface provides a site for assembly of the fibrinolytic components (Levin and Loskutoff, 1982; Loskutoff and Edgington, 1977).

The ability of ECs to metabolize adenine nucleotides, some of which have potent effects on platelets, was discussed in the previous section. ADPase is quite important in this regard, as ADP is a powerful aggregatory stimulus for platelets (Smith and Ryan, 1972; Ryan and Ryan, 1985). The maintenance of an intact endothelial surface is also important in preventing platelet adherence to the subendothelial stroma (Fajardo, 1989).

Vascular endothelium has procoagulant activity as well, but this is either negligible in unstimulated ECs or is overbalanced by the anticoagulant activity under normal circumstances. For this reason, the procoagulant features of ECs will be covered in the discussion of the response to EC injury.

4. Regulation of Vascular Cell Growth

ECs modulate the growth of SMCs (and other mesenchymal cells) in the vascular wall by a number of mechanisms. The composition of the extracellular matrix, which is influenced by a number of factors (see Section V.A.), may in turn influence SMC phenotype, movement and proliferation (Carey, 1991). It seems that under quiescent conditions, proliferation of vascular smooth muscle is kept in check by inhibitory substances produced by ECs. Heparin inhibits smooth muscle cell proliferation *in vivo* and *in vitro* (Clowes and Karnovsky, 1977; Clowes *et al.*, 1989; Hoover *et al.*, 1980). ECs produce heparan sulfate that is deposited into the subendothelial matrix and stroma adjacent to the SMCs. Factors present in the blood release heparin-like activity from this bound heparan sulfate which could account for the growth-inhibitory effects of confluent ECs on SMC proliferation (Castellot *et al.*, 1981; Castellot *et al.*, 1982; Herman and Castellot, 1987). In addition, extracellular heparan sulfate binds proteins of the fibroblast growth factor family, and thus regulates the growth of vascular cells by modulating the availability and stability of these mitogens (D'Amore, 1990).

Growth may also be modulated to some extent by gap junctional communication between ECs and SMCs (Larson and Sheridan, 1982; Davies *et al.*, 1985; Davies, 1986). This type of intercellular communication does influence cell

proliferation in other cell types (Loewenstein, 1979). However, although these junctions exist between ECs and SMCs, their role in this process has yet to be determined.

ECs synthesize and release a number of peptide growth factors which are capable of stimulating the proliferation of SMCs and other mesenchymal cells. Again, the production of these mediators is negligible in the undisturbed vessel wall, therefore endothelium-derived growth factors will be discussed as a part of the response to injury.

5. Other Roles

The endothelium plays an active role in the uptake and handling of lipids by the vessel wall. ECs accumulate acetate and mevalonate and incorporate them into sterol (Borsum, 1991). They also have surface receptors that bind low density lipoprotein (LDL), high density lipoprotein (HDL), β-very low density lipoprotein (β-VLDL) and chylomicrons. Both transcytotic receptors and endocytotic receptors are present, thus lipids may be transported across the endothelium or may be degraded or oxidatively modified within endothelium (Reckless *et al.*, 1978; Morel *et al.*, 1984). ECs, like macrophages, also possess receptors to scavenge modified, or acetylated, LDL. This property is used in characterization of cultured ECs, but it may also contribute to EC damage in the presence of large amounts of modified lipid (Voyta *et al.*, 1984). The glycosaminoglycans present on the EC surface bind lipoprotein lipase, therefore ECs are also able to hydrolyze triglycerides (Camejo *et al.*, 1985).

Angiogenesis, both during growth and in response to injury or neoplasia, appears to be an EC-mediated process. It involves a fibrinolytic-type response of ECs (primarily venular) to a number of chemotactic and mitogenic substances, including acidic and basic fibroblast growth factors (aFGF and bFGF), EGF, PGE_1, PGE_2, tumor necrosis factor α (TNF_α) and fibrin. The angiogenic process has been reviewed in detail by Folkman (1982) and Auerbach (1981).

C. Functional Response of Endothelium to Injury or Disease

1. Alterations in Permeability

The most obvious result of endothelial injury is the development of interstitial edema as a result of increased vascular leak. A loss of the structural and functional integrity of the endothelial monolayer occurs during inflammation primarily as a result of changes in the postcapillary venules in response to inflammatory mediators (Pober, 1988; Pober and Cotran, 1990; Majno *et al.*, 1967). With discontinuous tight junctions, venular endothelium is somewhat leaky under normal circumstances, and this leakiness is enhanced during inflammation. Endothelial barrier function can also be impaired in capillaries or precapillary vessels by oxidant injury (Shasby *et al.*, 1982) or chemical damage (Meyrick *et al.*, 1989; Plestina and Stoner, 1972).

Alterations in certain cellular macromolecules represents one important mechanism leading to increased vascular permeability. For example, cytoskeletal elements are important in determining EC shape and in maintaining their connections with other cells and the subcellular matrix. Disruption of the

cytoskeleton occurs with some forms of EC injury, resulting in retraction of individual cells and a loss of intercellular contacts. These structural changes in the endothelial monolayer can contribute to a loss of vascular integrity and consequent increases in permeability (Phillips *et al.*, 1988; Hinshaw *et al.*, 1989; Molony and Armstrong, 1991). In addition, changes in the glycocalyx that covers the endothelial surface may influence permeability to a number of substances in addition to water by masking or unmasking transport receptors, or by altering the surface charge distribution (Simionescu and Simionescu, 1986).

2. Alterations in Vasoactivity

Endothelial injury may result in disruption of the balanced production of relaxing and constricting factors, leading to pathologically altered vascular responsiveness and tone. Many of the protective attributes of EC function appear to be lost or depressed with mechanical or toxic injury, resulting in a vascular bed that has little ability to protect itself from the direct effects of circulating vasoactive mediators. [Figure 3]

EC production of PGI_2 is often increased as a result of EC injury or stimulation, perhaps in an effort to modulate the vasospastic and procoagulant responses to vascular damage (see below). However, injured vessels may also lose the ability to generate EDRFs and the vasodilatory prostaglandins. This may be due to frank denuding of the luminal surface of the vessel, but it is more frequently due to a decreased endothelial capacity to generate these substances. Several of the stimuli that cause the release of EDRF also stimulate generation of EDCFs, and this ability is often maintained or increased in injured vessels. These

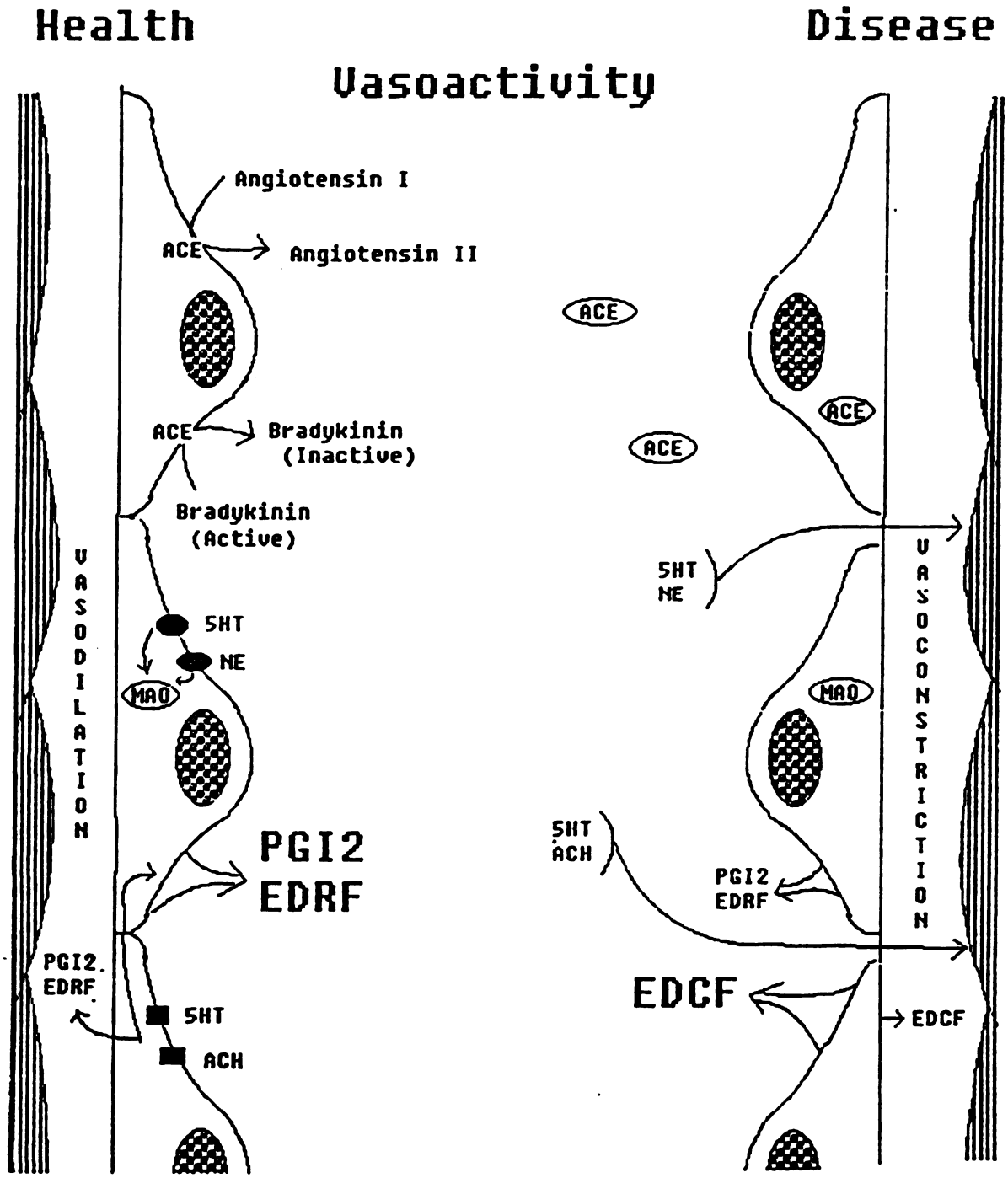


Figure 3: Endothelial modulation of vasoactivity in health and disease.

changes in EC responsiveness shift the balance to favor vasoconstriction (Rubanyi, 1991; Shepherd and Katusic, 1991; Katusic and Shepherd, 1991; Chandler and Giri, 1983; Cartier *et al.*, 1991; Adnot *et al.*, 1991; Cartier *et al.*, 1991). Also contributing to the pressor response are agents like 5HT and ACH, which stimulate the release of EDRF in healthy vessels but are themselves direct vasoconstrictors. With increased permeability or actual loss of the intervening endothelium, these mediators gain direct access to receptors on the surface of SMCs and stimulate contraction (Rubanyi, 1991; Becker, 1991; Ludmer *et al.*, 1986).

EC injury can also interfere with the metabolism of circulating vasoactive mediators, resulting in altered blood concentrations of these compounds and their metabolites. Although ACE activity and levels of Ang II are increased in some models of lung vascular injury (Brizio-Molteni *et al.*, 1984; Zakheim *et al.*, 1976), decreases in enzyme activity are more frequently reported and may serve as an indicator of pulmonary endothelial or microvascular dysfunction (Ryan and Catravas, 1990; Catravas *et al.*, 1988b). Loss of pulmonary ACE activity with a concurrent increase in plasma ACE activity has been reported after administration of agents which cause acute EC injury and may reflect sloughing of the enzyme from the surface of the EC (Hollinger *et al.*, 1980b; Hollinger *et al.*, 1980a). Decreased ACE activity may also be a compensatory response of the endothelium to chronic elevations in pulmonary blood pressure, or may occur in response to direct EC injury (Keane *et al.*, 1982; Chandler and Giri, 1983; Rounds *et al.*, 1985; Stalcup *et al.*, 1979; Bell and Madri, 1990).

Some models of lung vascular injury result in a decreased ability to remove circulating biogenic amines from the circulation, and this is considered to be an indication of EC injury *in vivo* (Block *et al.*, 1985; Cross *et al.*, 1974; Gillis, 1973; Iwasawa *et al.*, 1973; Gillis *et al.*, 1978). Inability to clear 5HT or NE from the blood could result in elevated concentrations of these mediators in the circulation, thus exacerbating the situation described above. This may be of particular importance when high concentrations of amines are released locally from platelets or as a result of inflammation.

3. Alterations in Hemostasis

The endothelium plays a critical role in modulating the hemostatic process in response to injury or disease. The balance between coagulation and fibrinolysis that results in a nonthrombogenic vascular surface under normal conditions shifts to favor a procoagulant state during injury, inflammation or neoplasia. When functioning properly, EC act not only to initiate thrombosis but also to limit the extent of this response. However, with some types of vascular injury, the hemostatic response may become excessive; under these circumstances, the hypercoagulative state can actually contribute to the disease process. [Figure 4]

Tissue factor (TF; thromboplastin, factor III) is a cellular cofactor that binds factor VII and initiates the coagulation process. The subintimal vascular wall contains high levels of TF, but quiescent ECs express very little of this protein. Thus, the EC layer provides a barrier between the blood and this potent stimulus for coagulation. Disruption of the intimal layer results in exposure of TF, and a thrombotic response is initiated rapidly (Maynard *et al.*, 1977; Nawroth *et al.*,

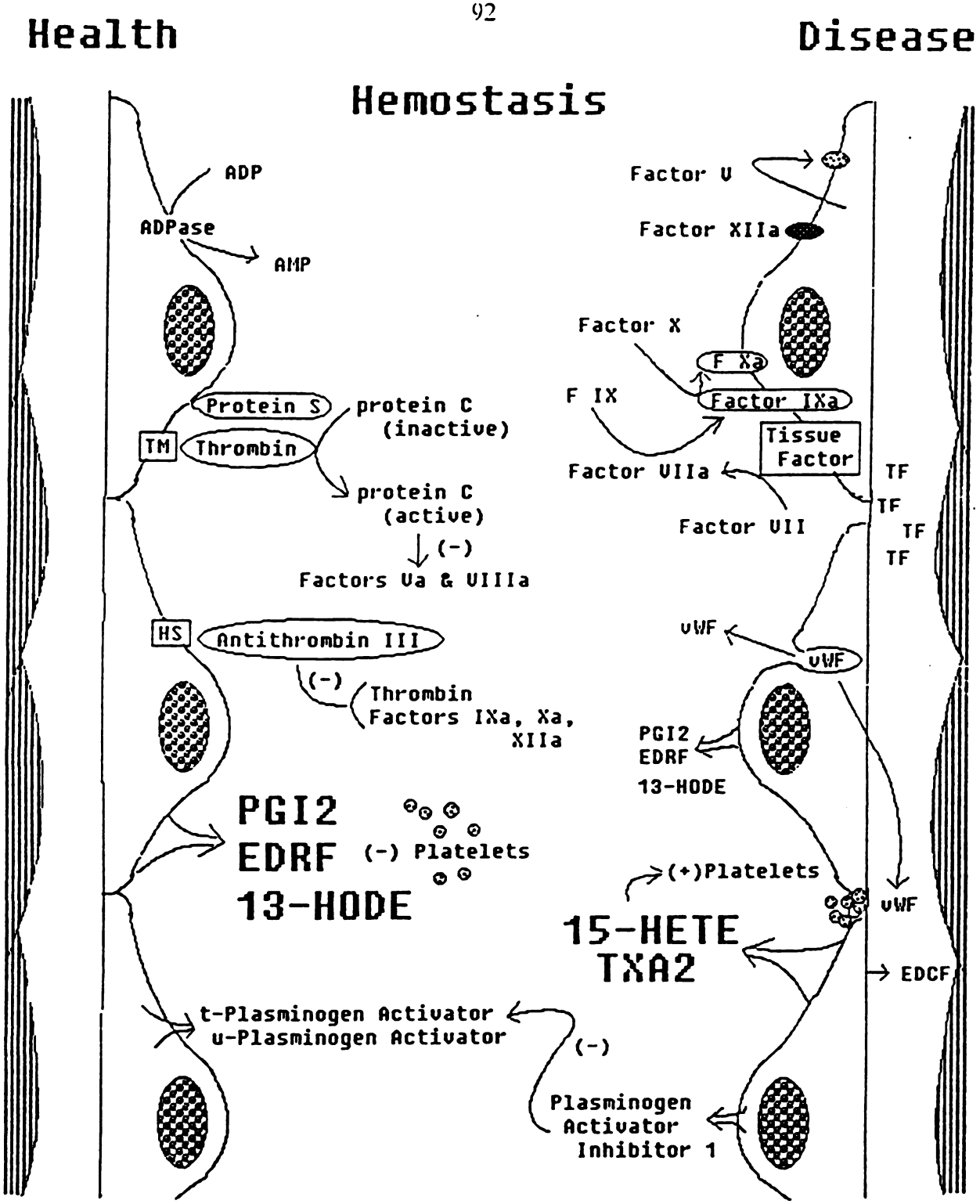


Figure 4: Endothelial modulation of the hemostatic system in health and disease.

1985). Perturbation of cultured ECs can result in increased expression of TF by these cells (Galdal, 1984; Jaffe, 1987); it is not yet known if this is a response to injury *in vivo* as well. If so, this would permit an intact but injured endothelium to initiate the coagulation process.

Just as the EC surface can serve as a site for assembly of the fibrinolytic components (see above), so can it serve as a base for propagation of the coagulation process. Factor IX is bound on the surface of ECs, where it can be activated by factor VIIa. Factor IXa then promotes activation of factor X, which can also bind to the EC surface (Stern *et al.*, 1985; Nawroth *et al.*, 1985). Bound factor Xa can then bind and activate prothrombin. ECs also synthesize and bind coagulation factor V and promote activation of factor XII (Cerveny *et al.*, 1984; Fajardo, 1989; Wiggins *et al.*, 1980).

ECs synthesize, store and secrete von Willebrand factor (vWF; factor VIII-associated antigen). This is a carrier protein for factor VIII, but vWF is also important in mediating platelet adherence to the subendothelium during denuding injury (Hoyer *et al.*, 1973; Jaffe *et al.*, 1974). In primates, cattle and rats, vWF is concentrated and stored in Weibel-Palade bodies within the EC cytoplasm. Identification of these structures, or immunolocalization of vWF in the cytoplasm, helps to confirm the identity of EC in culture (Reinders *et al.*, 1984; Wagner *et al.*, 1982). Mechanical or toxic injury to endothelium leads to increased intracellular vWF, primarily associated with the endoplasmic reticulum, and increased vWF in the subendothelial matrix (Reidy *et al.*, 1989), and circulating levels of vWF increase after irradiation injury or during renal failure. Thus, vWF may contribute to the

hypercoagulable state which exists with some forms of vascular disease, and increased plasma levels of vWF may serve as an indicator of endothelial injury (Sporn *et al.*, 1984; Warrell *et al.*, 1979).

ECs may promote hemostasis by modulation of the fibrinolytic system. ECs produce tissue plasminogen activator inhibitor 1 (PAI-1), which limits the activity of tPA and thus decreases fibrinolysis (Loskutoff and Edgington, 1977). Injured ECs may also release more of the platelet activating and vasoconstrictive lipid mediators, such as 15-HETE and TXA_2, and less 13-HODE. Stimulated ECs may continue to release PGI_2, but perhaps at insufficient levels to overcome procoagulant influences (Chandler and Giri, 1983; Buchanan and Bastida, 1988; Ingerman-Wojenski *et al.*, 1981; Cotran, 1987; Jaffe, 1987). As noted above, denuding and nondenuding injury to blood vessels can result in a decreased ability to generate EDRFs, and this would also contribute to enhanced platelet adherence and thrombosis.

4. Enhancement of Vascular Cell Growth

Quiescent ECs *in vivo* do not generate or release mitogens to an appreciable extent; in fact, the vasodilatory and antithrombotic mediators that are constitutively released by these cells (eg. EDRF or PGI_2) actually inhibit SMC proliferation (Shepherd and Katusic, 1991). However, after toxic or physical injury or when stimulated by inflammatory mediators, ECs synthesize and release a variety of substances that are potent SMC chemoattractants and/or mitogens. These may be important in initiating or exacerbating the structural remodeling which is a part of many chronic vascular diseases. [Figure 5]

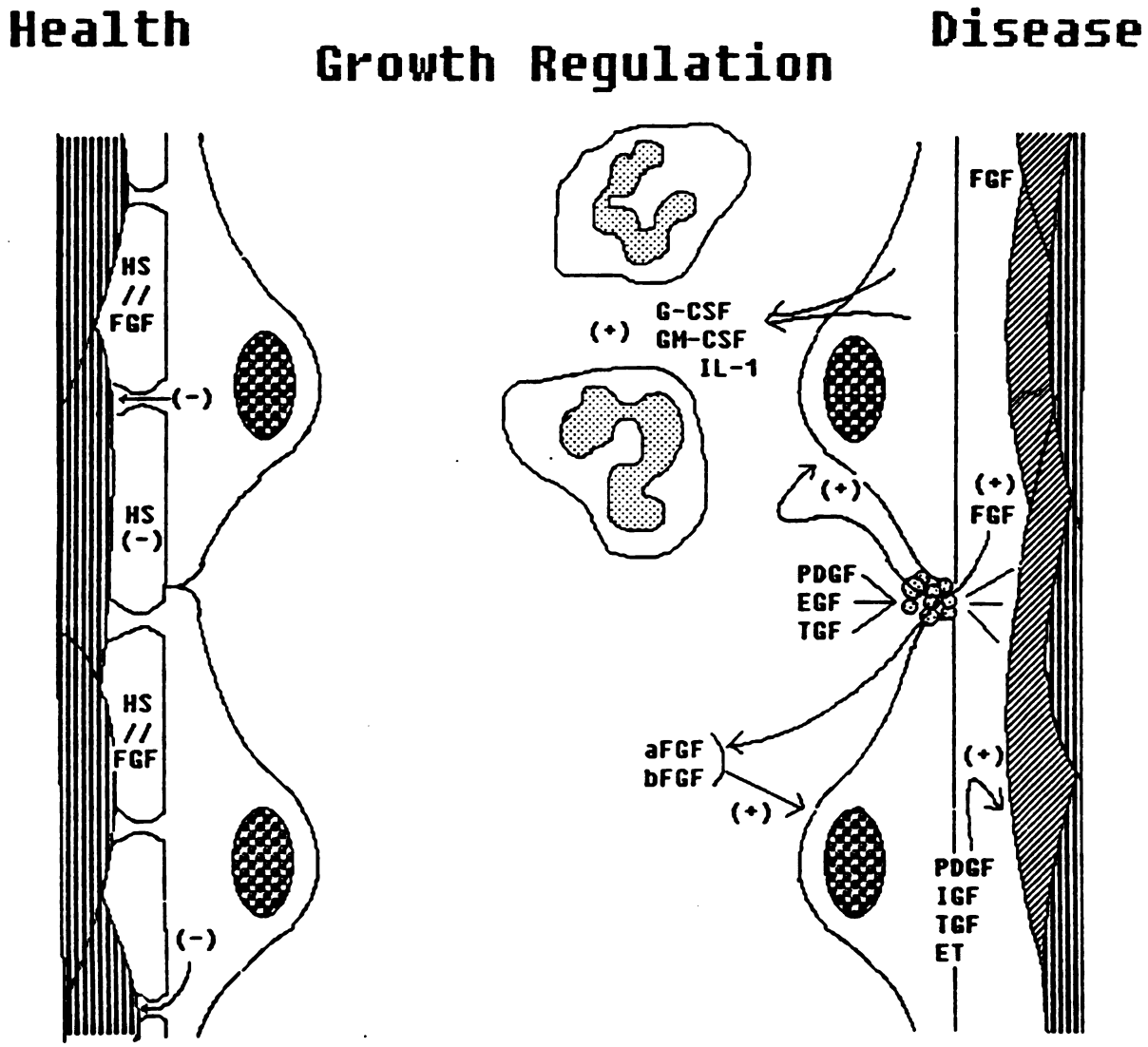


Figure 5: Endothelial modulation of vascular cell growth in health and disease.

Many of the vasoconstrictive mediators stimulate SMC proliferation. This is true for ET, Ang II, and the catecholamines and may be the case for all vasoconstrictors. Similarly, many of the peptide growth factors have vasoconstrictive properties (Nemecek *et al.*, 1986; Campbell-Boswell and Robertson, 1981; Yanagisawa and Masaki, 1989; Blaes and Boissel, 1983; Kourembanas *et al.*, 1990; Gibbons and Dzau, 1990; Shepherd and Katusic, 1991; Dzau and Gibbons, 1991). Their common ability to increase intracellular calcium may account in part for this correspondence (Ryan *et al.*, 1988; Dzau and Gibbons, 1991; Berk *et al.*, 1987; Shepherd and Katusic, 1991).

Mitogens produced by ECs include a platelet-derived growth factor-like substance (PDGF) (DiCorleto and Bowen-Pope, 1983; Fox and DiCorleto, 1984; Vlodavsky *et al.*, 1987), acidic and basic fibroblast growth factors (aFGF and bFGF) (Schweigerer *et al.*, 1987; Vlodavsky *et al.*, 1987; Burgess and Maciag, 1989), insulin-like growth factor 1 (IGF-1) (Dzau and Gibbons, 1991), endothelial cell-derived growth factor (ECGF) (Gajdusek *et al.*, 1980), granulocyte and granulocyte-macrophage colony stimulating factors (G-CSF and GM-CSF) (Jaffe, 1987; Zsebo *et al.*, 1988) and the cytokine IL-1 (Shanahan and Korn, 1984; Giri *et al.*, 1985; Mizel, 1989). All of these are mitogenic for SMCs, and some are chemotactic as well (Grotendorst *et al.*, 1982; Clowes *et al.*, 1989). A few (e.g., aFGF, bFGF) are also auto-stimulatory and cause EC proliferation (Schweigerer *et al.*, 1987; Sato and Rifkin, 1988); IL-1 inhibits EC proliferation (Cozzolino *et al.*, 1990). Stimulated ECs also release a connective tissue growth factor which is related to but distinct from PDGF, a hepatocyte growth factor which is hypothesized to be a ubiquitous factor

for tissue repair (Bradham *et al.*, 1991; Noji *et al.*, 1990), and $TGF_{\beta 1}$, which promotes angiogenesis, inhibits EC proliferation, and modulates SMC proliferation (Antonelli-Orlidge *et al.*, 1989; Assoian and Sporn, 1986) .

The release of growth factors from ECs in combination with other possible alterations as a result of injury, such as loss of cell attachments, loss of gap junctional communication, or alteration of the composition of the extracellular matrix, may result in an environment favorable to SMC migration and proliferation (Simionescu and Simionescu, 1986; Ryan, 1986; Carey, 1991). In addition, the increased platelet adherence which may occur as a result of EC injury could result in increased release by platelets of growth factors, most notably PDGF, TGF_{α}, TGF_{β} and EGF (258,422,423}, which could also contribute to the vascular remodeling process. Platelets also release thrombin, which is a potent stimulus for EC production of growth factors and other mediators (Schini *et al.*, 1989). Increased production and release of mesenchymal cell growth factors by ECs has been documented under a variety of adverse conditions (Hsieh *et al.*, 1991; Madri *et al.*, 1991; Vender *et al.*, 1987; Kourembanas *et al.*, 1990; Albelda *et al.*, 1989; Fox and DiCorleto, 1984) and may represent enhancement of the repair process involved in wound closure.

5. The "Activated" Endothelial Cell

ECs, particularly those of the postcapillary venules, respond to cytokines and other inflammatory mediators in a manner quite similar to macrophages; ECs in this stimulated state are said to be "activated" (Cotran, 1987). [Figure 6] Agents to which endothelial cells respond include endotoxin, thrombin,

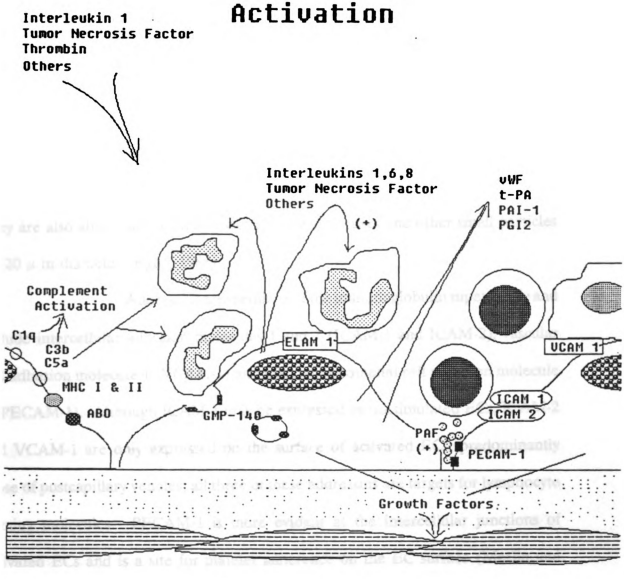


Figure 6: The "activated" endothelial cell.

IL-1, tumor necrosis factor (TNF), phorbol esters, lymphotoxin, and others. During the activation process, ECs undergo a number of structural and functional alterations. They become plumper and more prominent than quiescent ECs, and biosynthetic organelles increase. There is a reorganization of the actin and microtubule networks of the cells and a redistribution of some of the focal contact proteins, but interaction with the matrix is not disrupted (Molony and Armstrong, 1991). Thus, activated cells retract and change shape, but generally do not detach. Gaps appear between the retracted cells and vascular permeability increases. Activated cells become more mobile and they divide more rapidly than quiescent endothelial cells (Cotran, 1987). They are also able to act as phagocytes and engulf viruses and other small particles 0.1-20 μ in diameter (Ryan, 1988).

Addressins are members of the immunoglobulin superfamily and include intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule 1 (VCAM-1) and platelet-endothelial cell adhesion molecule 1 (PECAM-1). Although ICAM-1 may be expressed by unstimulated ECs, ICAM-2 and VCAM-1 are only expressed on the surface of activated ECs, predominantly those of postcapillary venules; all three of these addressins are targets for lymphocyte homing molecules. PECAM-1 is more evident at the intercellular junctions of activated ECs and is a site for platelet adherence on the EC surface (Marlin and Springer, 1987; Osborn *et al.*, 1989; Newman *et al.*, 1990; Albelda and Buck, 1990). Another set of adhesion molecules expressed on the surface of activated ECs are the selectins. These include endothelial leukocyte adhesion molecule-1 (ELAM-1) and granule-membrane protein 140 (GMP-140). ELAM-1 is expressed primarily on

venular ECs and binds PMNs. GMP-140 is present on the surface of Weibel-Palade bodies within ECs. With activation, these adhesion molecules are inserted into the cell membrane where they bind PMNs (Bevilacqua *et al.*, 1987; Johnston *et al.*, 1989).

EC activation results in the unmasking of Fc and C3b receptors on the endothelial surface, perhaps through alteration of the glycocalyx. Activated ECs express major histocompatibility Class I and II antigens and the ABO blood group antigens. The protein factor, C1q, is found on their surface as well, which binds circulating immune complexes and activates complement (Ryan *et al.*, 1981; Wedgewood *et al.*, 1988; Jaffe *et al.*, 1973).

Activated ECs not only respond to inflammatory mediators, but they release a tremendous array their own soluble mediators, including the cytokines IL-1, IL-6, IL-8, TNF and lymphotoxin, which promote chemotaxis and activation of PMNs and other leukocytes. They release factors that inhibit (tPA and PGI_2) and promote (PAI-1 and vWF) platelet activation and hemostasis. Activated ECs generate complement components, including C5a, a potent neutrophil chemotactic factor; they also produce PAF (see Section IV.D. above), a variety of growth factors (see Section IV.E. above), and collagenases that can degrade collagens I-V, thus influencing cell attachment and motility. They can also metabolize LTA_4 to LTB_4, LTC_4, LTD_4 and LTE_4, substances that attract PMNs and participate in anaphylaxis (Miossec *et al.*, 1986; Whatley *et al.*, 1988; Warren *et al.*, 1987; Cotran, 1987; Bussolino *et al.*, 1988; Ibe and Campbell, 1988; Modat *et al.*, 1990). At our present level of understanding, it is difficult to appreciate the potential consequences of this dramatic, multifaceted response to stimulation. Many of these studies were

performed *in vitro*, and in a variety of cell types; a localized response to inflammatory mediators *in vivo* may result in a more limited spectrum of effects appropriate to the stimulus.

In summary, the vascular endothelium performs a number of functions in addition to its role as a barrier to fluid movement. In fact, ECs are a dynamic, interactive component of the blood vessel wall, and their functional status must be considered as well as their structural integrity when evaluating toxic injury to the vasculature.

Study of endothelial function has revealed that the activity observed at a specific time is actually the sum of many activities which are constantly being modulated to achieve an appropriate balance for a specific set of conditions. This balance of stimulation and inhibition, coagulation and fibrinolysis, quiescence and activation, appears to allow the endothelium a broad spectrum of responses to sublethal injury. ECs in different vascular beds and in different species demonstrate heterogeneity. It has been proposed that many EC surface and metabolic properties are inducible, not constitutive, and represent an adaptation of otherwise identical cells to different vascular environments (Gimbrone, 1987). If this is true, then the response of ECs to different types of injury may also be heterogeneous. In addition, the importance of EC function to vascular homeostasis and the careful balance of activities required for homeostasis suggests that EC dysfunction might contribute to certain disease processes.

VI. Endothelial Cell Alterations in MCT(P) Pneumotoxicity

A. MCT(P)-Induced Endothelial Cell Alterations *In Vivo*

Endothelial damage occurs in the pulmonary vasculature with MCT(P) treatment *in vivo*, and the morphological and functional evidence of this damage has been discussed in previous sections. However, it may be useful to synthesize these observations and review them in light of the preceding discussion of EC function.

Morphologic alterations in pulmonary ECs are evident as early as 24 hours after administration of MCT(P), and these include swelling, nuclear enlargement, vesicular blebbing and an increase in the number of cytoplasmic organelles (Merkow and Kleinerman, 1966b; Valdivia *et al.*, 1967a,b; Rosenberg and Rabinovitch, 1988; Meyrick and Reid, 1982; Reindel *et al.*, 1990 and others). ECs in alveolar wall vessels incorporate increased amounts of [^{3}H]thymidine (Meyrick and Reid, 1982), which is indicative of an increased level of DNA synthesis associated with proliferation or DNA repair in these cells. EC hypertrophy, a rather unusual response of these cells to toxic injury, has been noted in later stages of MCT(P) lung injury. Occlusion of capillaries and small arterioles with swollen, hypertrophic ECs is thought to contribute to pruning of the peripheral vasculature (Butler, 1970a; Hislop and Reid, 1974; Raczniak *et al.*, 1979; Valdivia *et al.*, 1967b; Meyrick and Reid, 1979).

A number of other morphologic changes in the lung are suggestive of altered EC function. The presence of interstitial edema and other exudative changes suggest a loss of vascular integrity and increased capillary permeability in the lungs of MCT(P)-treated animals (Wilson and Segall, 1990; Heath, 1969; Valdivia *et al.*,

1967b; Molteni *et al.*, 1984; Sugita *et al.*, 1983b). Decreased ability of the endothelium to serve as a barrier is also reflected by an increased lung weight-to-body weight ratio, increased release of protein into BALF, and increased accumulation of circulating [^{125}I]albumin in the lungs after treatment with MCT(P) (Roth *et al.*, 1981a; Roth, 1981b; Bruner *et al.*, 1983,1986; Reindel *et al.*, 1990). In addition, the increased numbers of inflammatory cells noted in the lungs (Kay *et al.*, 1967b; Sugita *et al.*, 1983b; Stenmark *et al.*, 1985; Ilkiw *et al.*, 1989; Schultze *et al.*, 1990) may be a result of EC activation with consequent increased production of chemoattractants and altered adhesivity, as well as a loss of vascular integrity.

Intraluminal thrombi and fibrin blockages are seen both early (Valdivia *et al.*, 1967a) and late (Turner and Lalich, 1965; Merkow and Kleinerman, 1966b; Hayashi *et al.*, 1967) in the course of MCT-induced lung injury and might be indicative of an imbalance in endothelial production of pro- and anti-coagulant substances. The observation that lungs from MCT(P)-treated rats demonstrate decreased fibrinolytic ability supports this hypothesis (Molteni *et al.*, 1988a, 1989b; Schultze and Roth, 1992). Platelet thrombi are reported in the pulmonary microvasculature of MCT(P)-treated animals (Turner and Lalich, 1965; Valdivia *et al.*, 1967a; Chesney *et al.*, 1974a), and platelet sequestration occurs by 8 days after treatment with MCTP (White and Roth, 1988). Changes in platelet activity or adherence may reflect exposure of the subendothelial matrix or may occur as a result of altered EC production/release of coagulation factors or mediators such as EDRF or PGI_2.

MCT-induced angiogenesis occurs at the pleural surface and in the peribronchial region (Schraufnagel, 1990). ECs of postcapillary venules are sensitive to angiogenic stimuli and generally initiate this response. Atheromatous plaques have been reported in the coronary vasculature of MCT-treated rats, and might be a result of altered lipid metabolism by ECs in these vessels (Chesney and Allen, 1973c). Finally, the muscularization of normally nonmuscular arterioles (Hislop and Reid, 1974; Turner and Lalich, 1965; Meyrick and Reid, 1979; Langleben and Reid, 1985; Reindel *et al.*, 1990) and the increased medial smooth muscle seen in the small pulmonary arteries (Turner and Lalich, 1965; Merkow and Kleinerman, 1966b; Hislop and Reid, 1974; Heath and Kay, 1967; Kay and Heath, 1966; Reindel *et al.*, 1990) which are characteristic of MCT(P)-induced vascular remodeling may also reflect EC dysfunction, since the endothelium plays an important role in the regulation of SMC proliferation.

More direct evidence of functional change in ECs comes from biochemical studies in the lungs of animals treated with MCT(P). Uptake and clearance of biogenic amines (e.g., 5HT and NE) is reduced in isolated perfused lungs and in lung slices from MCT(P)-treated rats (Huxtable *et al.*, 1978; Gillis *et al.*, 1978; Roth *et al.*, 1989; Hilliker *et al.*, 1983c; Hilliker *et al.*, 1983a; Hilliker *et al.*, 1984). This function has been localized to the endothelium, and altered clearance of amines has been used as a marker of pulmonary endothelial dysfunction. Pulmonary clearance of prostaglandins is also reduced (Ito *et al.*, 1988b; Ito *et al.*, 1988a). Changes in ACE activity have been reported but are controversial. Changes

in biochemical function of endothelium such as these may contribute to alterated vasoactivity in MCT(P)-treated animals.

There is a loss of pulmonary vasodilation in response to ACH after MCT treatment which suggests a decreased ability to generate EDRF (Ito *et al.*, 1988a; Altiere *et al.*, 1986). PGI_2 and TXA_2 production are increased in lungs of MCT(P)-treated animals, perhaps as a result of altered endothelial synthesis (Molteni *et al.*, 1984; Ganey and Roth, 1987b).

The important structural and functional properties of the endothelium and its ability to respond heterogeneously to stimulation or injury have made this tissue a focus in a number of models of systemic vascular injury, including atherosclerosis and essential hypertension. The anatomic situation of the pulmonary endothelium, just downstream from the liver and at the interface of the blood and vessel wall, makes it a likely target for blood-borne toxic metabolites. Considering the evidence demonstrating that changes in EC structure and function occur as a result of treatment with MCT(P) *in vivo*, endothelium would seem to be a logical tissue on which to focus studies on mechanisms of MCT(P) pneumotoxicity.

B. The Endothelial Cell in Culture

The value of direct examination of an individual cell type has been appreciated for many years; it permits identification of characteristic cell functions and investigation into the direct effects of exogenous agents on those functions. Isolated cell systems are also useful in the investigation of mechanisms of toxicity, since they can facilitate the detection of very early and/or subtle alterations in cell activity after exposure to toxicants (Ramos and Cox, 1987).

Although the cumulative mass of the endothelium in the body is nearly a kilogram (Wolinsky, 1980), the fact that these cells exist in monolayers closely apposed to other vascular cells makes it difficult to study their function *in situ*. For this reason, much of what we know about EC function has been learned from work done in cultured ECs (Borsum, 1991). Studies *in vivo* are needed to confirm that similar functions are carried out by ECs *in situ*; nevertheless, the isolated EC systems currently available provide a means to study both EC biology and response to injury.

Isolated, unfixed ECs in blood vessels have been studied in "en face" Häutchen preparations or attached to cellulose acetate paper (Pastan *et al.*, 1977; Smith and Ryan, 1973a; Tomlinson *et al.*, 1991). These preparations are short-lived and fragile and therefore of limited usefulness, but they are representative of the endothelium *in situ*. A technique for the culture of ECs from the human umbilical vein was first reported by Maruyama (1963). Since that time, a number of investigators have developed methods for the isolation and culture of endothelial cells from a variety of vessels and species, ranging from the aorta of the cow to the pulmonary microvasculature of the rat (Lewis *et al.*, 1973; Habliston *et al.*, 1979; Giri *et al.*, 1985; Booyse *et al.*, 1975; Ryan and White, 1986). A variety of methods, both mechanical and enzymic, have been utilized to collect EC cells; dozens of media formulations have been advocated to sustain them; and a variety of propagation methods have been used to perpetuate them, all with a common goal: a system *in vitro* that is as representative of the situation *in vivo* as possible.

In many respects, this goal has been achieved. ECs replicate well in culture and form a contact-inhibited monolayer. Cultured ECs retain many of the

morphologic features that are characteristic of related ECs *in situ*, including the presence of Weibel-Palade bodies, pinocytotic vesicles, surface projections and caveolae, a glycocalyx and fenestrae (Ryan *et al.*, 1978; Smith and Ryan, 1972; Smith and Ryan, 1973b). ECs in culture carry out some of the biochemical and metabolic functions which have been reported *in vivo*. Among these are the conversion of Ang I to Ang II and degradation of BK by ACE (Ryan *et al.*, 1976), uptake of acetylated LDL (Voyta *et al.*, 1984), and synthesis and storage of factor VIII-related antigen (Wagner *et al.*, 1982; Ryan *et al.*, 1978), all of which are used in the identification and characterization of cultured ECs.

Limitations exist in any isolated cell system and the EC in culture is no exception. ECs in the body do not exist as an isolated mass of cells; they are in constant contact and in communication with the surrounding matrix, smooth muscle cells and blood. In addition, they are subject to physical forces such as shear stress and mechanical stretch. The importance of these contacts to the maintenance of normal cell function is becoming more evident as techniques to study the endothelium *in situ* are improved.

The proliferative rate of normal vascular EC *in vivo* is low, with average doubling times that range from months to years. In culture, these cells have a population doubling time of 18-65 hours. This rate, influenced by the culture medium and the concentration of serum and other biological additives used, is more representative of ECs after wounding or under neoplastic conditions than of quiescent cells *in vivo* (Cotran, 1965; Greenburg and Hunt, 1978; Duthu and Smith, 1980; Hobson and Denekamp, 1984). ECs *in vivo* and *in vitro* have a limited

doubling potential and, unless the cells are transformed, a finite lifespan (Duthu and Smith, 1980). Thus, a more rapid aging process occurs in cultured cells, and this should be considered when evaluating structural and functional alterations in response to treatment.

Although ECs in culture synthesize extracellular matrix components which are similar to those formed *in vivo*, it is difficult to achieve conditions that allow them to synthesize a functionally intact basement membrane (Kramer *et al.*, 1984; Huber and Weiss, 1989). This has hampered permeability studies, as well as investigation of the interactions between ECs and inflammatory cells. EC matrix characteristics also affect cell shape, spreading, motility and proliferation, and these may in turn affect cell function (Madri and Stenn, 1982; Gospodarowicz and Lui, 1981; Hennig *et al.*, 1989; Madri *et al.*, 1991).

Cultured ECs constitutively synthesize and release growth factors, most notably PDGF (DiCorleto and Bowen-Pope, 1983). They can be stimulated to produce large amounts of tissue factor, a capacity which has not been demonstrated *in vivo* (Jaffe, 1987). Cultured microvascular ECs tend to form tubes readily, thus providing a model for study of angiogenesis (Folkman, 1982; Madri and Pratt, 1986). These and other differences between ECs *in vivo* and *in vitro* may be attributed to an absence of the regulation provided by other cell types and mediators normally present in the vascular wall. Despite such differences, the benefit of being able to examine the direct effects of exogenous mediators and toxicants on a specific target cell type is great, and EC culture remains a valuable tool. Certain nuances of MCT(P) toxicity render this model a difficult one to study *in vivo* with regard to

elucidating mechanisms of action on the lung vasculature. There is evidence *in vivo* to suggest that the pulmonary endothelium is a likely target for reactive MCTP arriving via the bloodstream, and EC cultures are a simplified but useful model of certain aspects of MCTP-induced toxicity.

C. MCT(P)-Induced Endothelial Cell Alterations *In Vitro*

Reindel and Roth (1981) examined the time- and dose-dependent effects of MCTP on cultured bovine pulmonary artery endothelial cells (BECs). BEC injury in response to a single exposure to MCTP was delayed in onset and progressive in nature, much like the pulmonary toxicity *in vivo*. In the 24 hour period after exposure to MCTP, monolayers of BECs showed no evidence of cytotoxicity as evidenced by morphologic change or release of LDH. By 48 hours posttreatment, there was a subtle increase in cell detachment followed by a rise in LDH release and increased generation of PGI_2. These responses became progressively more pronounced over the duration of the study. As cells detached, a monolayer free of obvious gaps was maintained by enlargement and spreading of the remaining cells, which in some cases enlarged as much as 20-fold. These cells were bizarre in appearance, having enlarged, occasionally multiple nuclei with multiple, prominent nucleoli, dilated endoplasmic rèticulae and pronounced cytoplasmic stress fibers and vacuoles; however, they remained viable as they were able to exclude trypan blue. In addition, colony forming efficiency assays revealed that MCTP-treated BECs were unable to divide; these antiproliferative effects were evident at lower concentrations of MCTP than those needed to produce overt cytotoxicity (Reindel and Roth, 1991).

The repair processes which occur in endothelial monolayers after wounding follow a predictable sequence which involves cell spreading, migration and proliferation. Whereas the former two processes may be sufficient to repair small, discrete defects in a monolayer, cell proliferation is required to restore monolayer integrity when larger or more widespread defects are present (Wong and Gotlieb, 1985; Coomber and Gotlieb, 1990). In BEC monolayers treated with MCTP, defects in the monolayer which result from the cytotoxic effects of MCTP are compensated for initially by the spreading of adjacent cells. As cell detachment continues, the remaining cells, which are unable to divide, become dramatically enlarged. In time, when this response becomes inadequate, gaps appear in the monolayer. A similar response by the endothelium *in vivo* could result in the delayed and progressive development of vascular leak which is characteristic of MCT(P) pneumotoxicity (Reindel and Roth, 1991).

Porcine pulmonary artery endothelial cells (PECs) studied under similar conditions respond somewhat differently from BECs (Reindel *et al.*, 1991). PECs are much less sensitive to the cytolytic effects of MCTP than are BECs, exhibiting less cell detachment [Figure 7] and LDH release [Figure 8]. Although they become more spindle-like in shape, they do not enlarge markedly. However, treated PECs demonstrate a delayed and progressive increase in generation of PGI_2, and they are equally sensitive to the antiproliferative effects of MCTP [Figure 9] (Reindel *et al.*, 1991).

The more subtle morphologic response of PEC monolayers to MCTP treatment does not appear to be due to a qualitative difference in the response of

this cell type to injury. When MCTP-treated PEC monolayers are artificially wounded to mimic the loss of monolayer integrity seen in treated BECs, the adjacent PECs migrate into the defect and enlarge markedly [Figure 10] (Hoorn *et al.*, 1990). These results suggest that, whereas BECs are much more sensitive to the cytolytic effects of MCTP than are PECs, surviving cells of each species respond very similarly to a loss of monolayer integrity. Both cell types are inhibited in their ability to divide when treated with MCTP yet are viable and able to respond to a loss of cells from the monolayer by cell migration and enlargement.

Cattle and swine differ in their target organ susceptibility to ingested *C. spectabilis*; cattle typically develop veno-occlusive disease of the liver (Sanders *et al.*, 1936; Sippel, 1964), whereas swine may experience pneumotoxic changes in response to ingestion of this plant (Emmel *et al.*, 1935; Peckham *et al.*, 1974). It has been suggested that species differences in response to PZAs may be due to differences in metabolism (Shull *et al.*, 1976; Cheeke and Pierson-Goeger, 1983), but the results of studies *in vitro* suggest that there are interspecies differences in ECs,

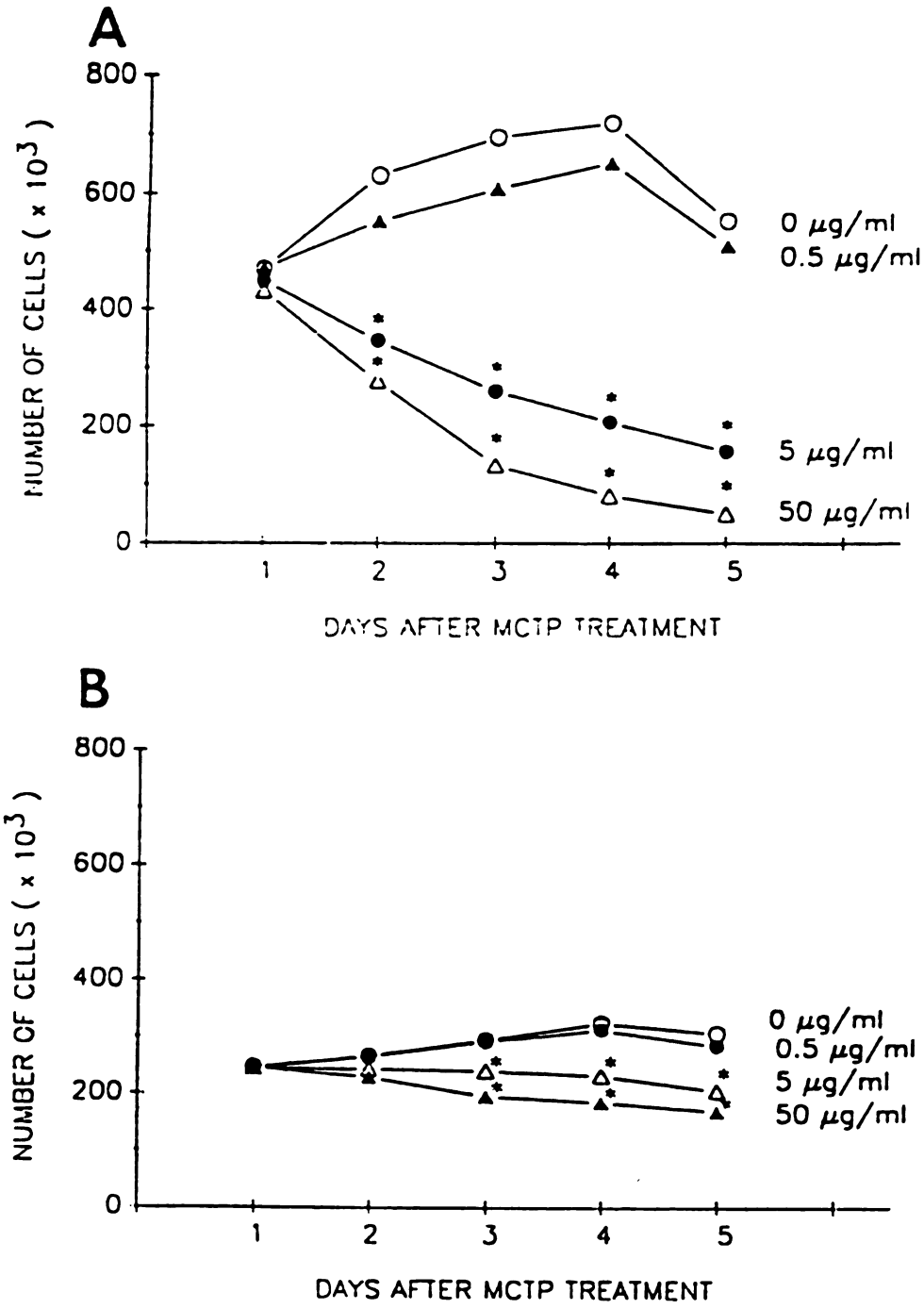


Figure 7: Effects of MCTP on monolayer cellularity.

BEC (A) and PEC (B) monolayers were exposed to a single administration of MCTP on day 0. Values represent mean +/- SE of 5 (BECs) or 4 (PECs) independent experiments. * Significantly different from control ($p < 0.05$)

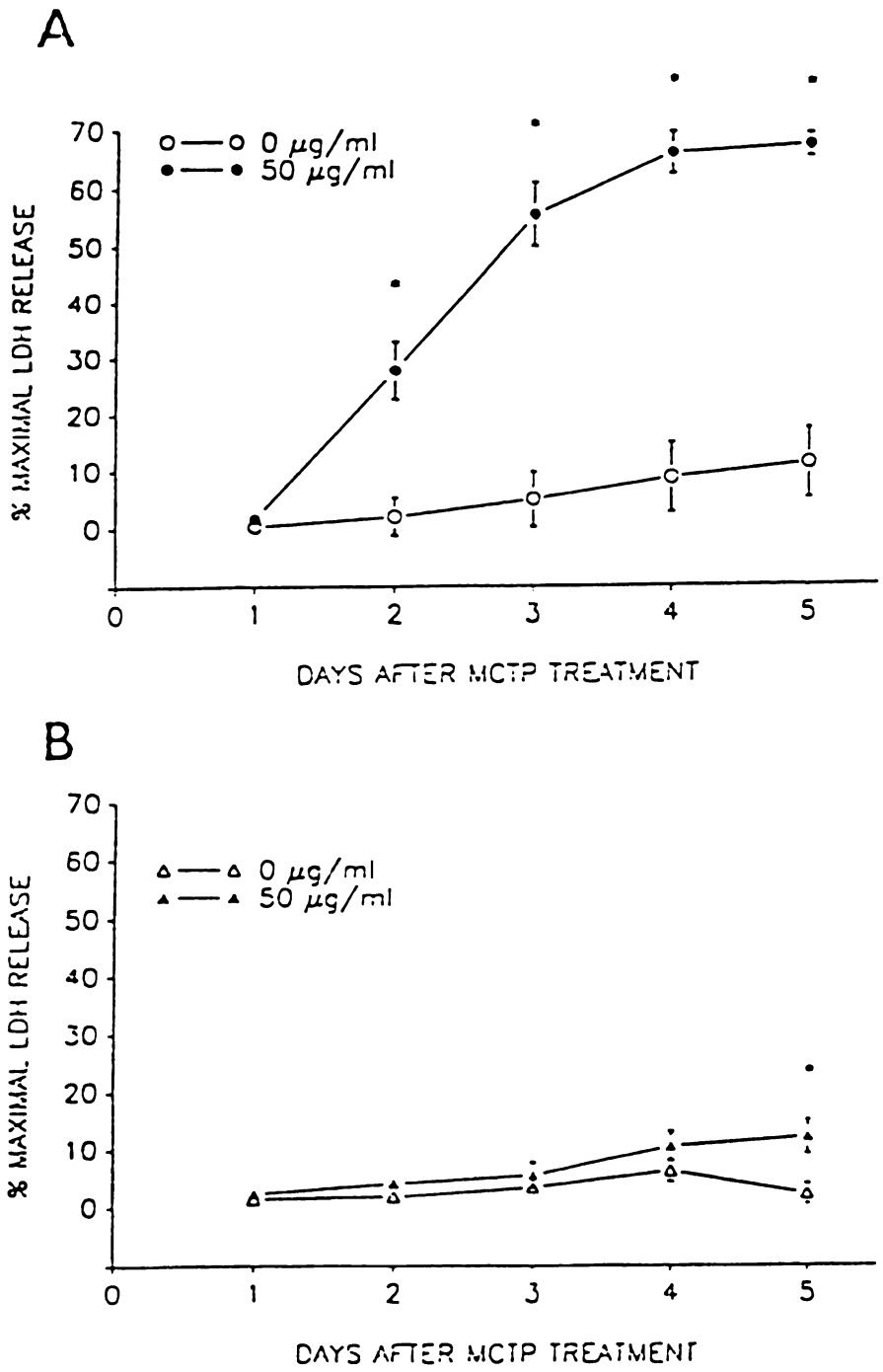


Figure 8: Release of lactate dehydrogenase from MCTP-treated monolayers.

BECs (A) and PECs (B) were exposed to 0 or 50 µg MCTP/ml medium on day 0. Each value represents mean +/- SE of 6 independent experiments. * Significantly different from controls at same time ($p < 0.05$)

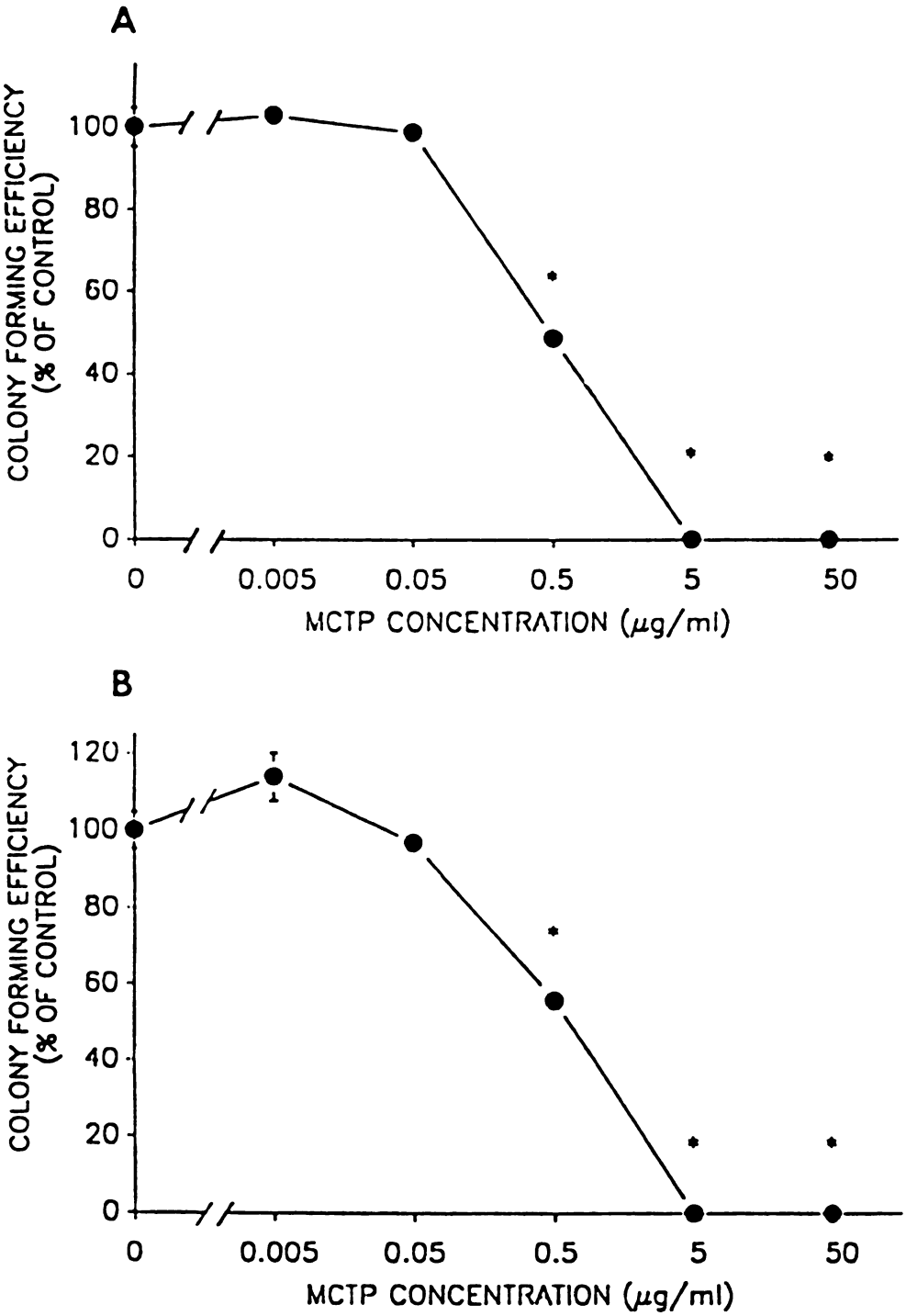


Figure 9: Effects of MCTP on colony-forming efficiency.

BECs (A) and PECs (B) were plated at low density and treated with a single adminsitration of MCTP or vehicle on day 0. Values represent means +/- SE of 4 independent experiments. * Significantly different from control ($p<0.05$)

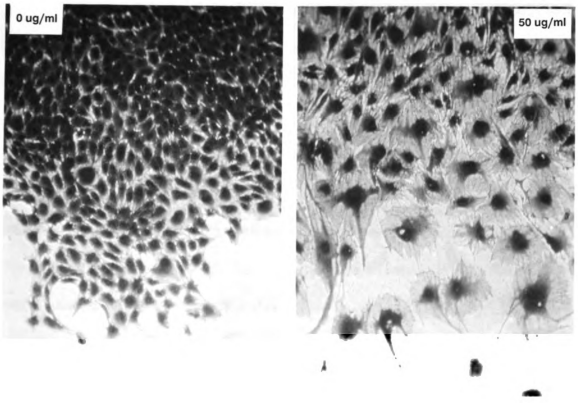


Figure 10: Effects of MCTP on PEC morphology.

PEC monolayers were treated with MCTP or vehicle and scraped 4 hours posttreatment. Fixed and stained cells were photographed at 5 days posttreatment.

at least in culture, which might also account in part for differences in sensitivity to these compounds (Reindel *et al.*, 1991). This area of investigation merits further study.

In high concentrations, the parent alkaloid, MCT, is also directly toxic to PECs and BECs in culture. Administration of millimolar quantities of MCT to ECs results in pronounced vacuolation, decreased monolayer cellularity and increased permeability to albumin within hours of treatment. The increased monolayer permeability is attenuated by pretreatment of ECs with SKF-525A, an inhibitor of the cytochrome P_{450} MFO system. Injured cells are also inhibited in their ability to proliferate; they become hypertrophic and exhibit increased quantities of DNA and increased polyamine content (Hacker *et al.*, 1991; Salone *et al.*, 1991). These results suggest that pulmonary vascular endothelium is able to metabolize MCT to a bioactive form(s). MCT-induced cytolytic changes are evident much earlier than those seen after administration of MCTP, but this may be a phenomenon related to the presence of high levels of MCT. The identity of the putative cytotoxic metabolite(s) has not been established in this system; however, the effects on cell proliferation are similar to those seen with MCTP, and preliminary studies revealed that cytotoxic concentrations of MCT also cause DNA crosslinking in PECs (Wagner *et al.*, unpublished observations), suggesting that a bifunctional pyrrole may be generated. The relevance of these observations to MCT(P) pneumotoxicity *in vivo* has not been established.

MCTP treatment inhibits the proliferation of a variety of primary and transformed cells in culture in addition to ECs, including Madin-Darby canine kidney

cells, Crandel-Rous feline kidney cells, undifferentiated human keratinocytes and bovine and porcine pulmonary artery SMCs. Although these cell types vary in their susceptibility to the cytolytic effects of MCTP, colony forming ability is inhibited to a similar extent in all cell types after exposure to identical concentrations of this compound (Reindel *et al.*, 1988; Reindel and Roth, 1991) Reindel, Hoorn and Roth, unpublished observations).

Many of the PZAs exert antiproliferative effects on the liver *in vivo*, and this has been attributed to their ability to act as a bifunctional alkylating agents which form DNA crosslinks (Bull *et al.*, 1968; Mattocks and Legg, 1980; Culvenor *et al.*, 1969; Jago, 1969; Petry *et al.*, 1986). DNA crosslinking occurs in the livers of rats treated with MCT (Petry *et al.*, 1984) and in cultured bovine kidney epithelial cells treated with MCT in the presence of a metabolic activating system (Hincks *et al.*, 1991). In this cultured kidney cell system, the ability of various PZAs to cause DNA crosslinking is associated with their ability to inhibit cell proliferation, but the exact nature of this relationship remains conjectural.

It is not known if MCT(P) treatment *in vivo* results in DNA crosslinking in the pulmonary vasculature. However, recent studies in cultured ECs have begun to address the relationship between the ability of MCTP to act as a bifunctional alkylating agent and its cytotoxic effects. Cultured PECs treated with MCTP *in vitro* manifest both DNA:DNA and DNA:protein crosslinking at concentrations that inhibit cell proliferation (Wagner *et al.*, 1991; Wagner *et al.*, 1992). Further investigation is required: 1.) to determine if there is a cause/effect relationship between DNA crosslinking and the inhibition of EC proliferation, and

2.) to ascertain if and how these alterations in vascular ECs contribute to the development of the delayed pneumotoxicity caused by MCT(P) *in vivo*.

D. Potential Role of Endothelium in the Development of Pulmonary Hypertension

It is possible to fit together what we currently know about MCT(P) pneumotoxicity and EC injury to form a tentative hypothesis to explain how early endothelial changes lead to vessel leakage and pulmonary edema [Figure 11]. ECs line the pulmonary vasculature in a monolayer. In the normal lung vasculature, occasional EC death results in a small defect in the monolayer. This stimulates cell migration and division which covers the defect quickly, before significant vascular leak occurs.

When MCTP is given intravenously *in vivo*, the endothelium of the pulmonary vasculature is probably a major site of covalent binding of this bifunctional electrophile. The effects of this binding could be lethal (e.g., cytolysis, perhaps as a result of membrane disruption or altered calcium homeostasis) or nonlethal but with profound biological consequences (e.g., DNA crosslinking). As ECs die due to normal cell turnover or as a result of MCTP treatment, a small defect forms in the monolayer. This stimulates cells to divide, but the crosslinked DNA is unable to replicate correctly; thus, cells migrate and enlarge but are unable to divide.

In time, these damaged ECs may also die, resulting in a larger defect in the monolayer. Surrounding cells are again stimulated to divide but are frustrated in the attempt; these cells enlarge and die, too. Eventually, the defect in the

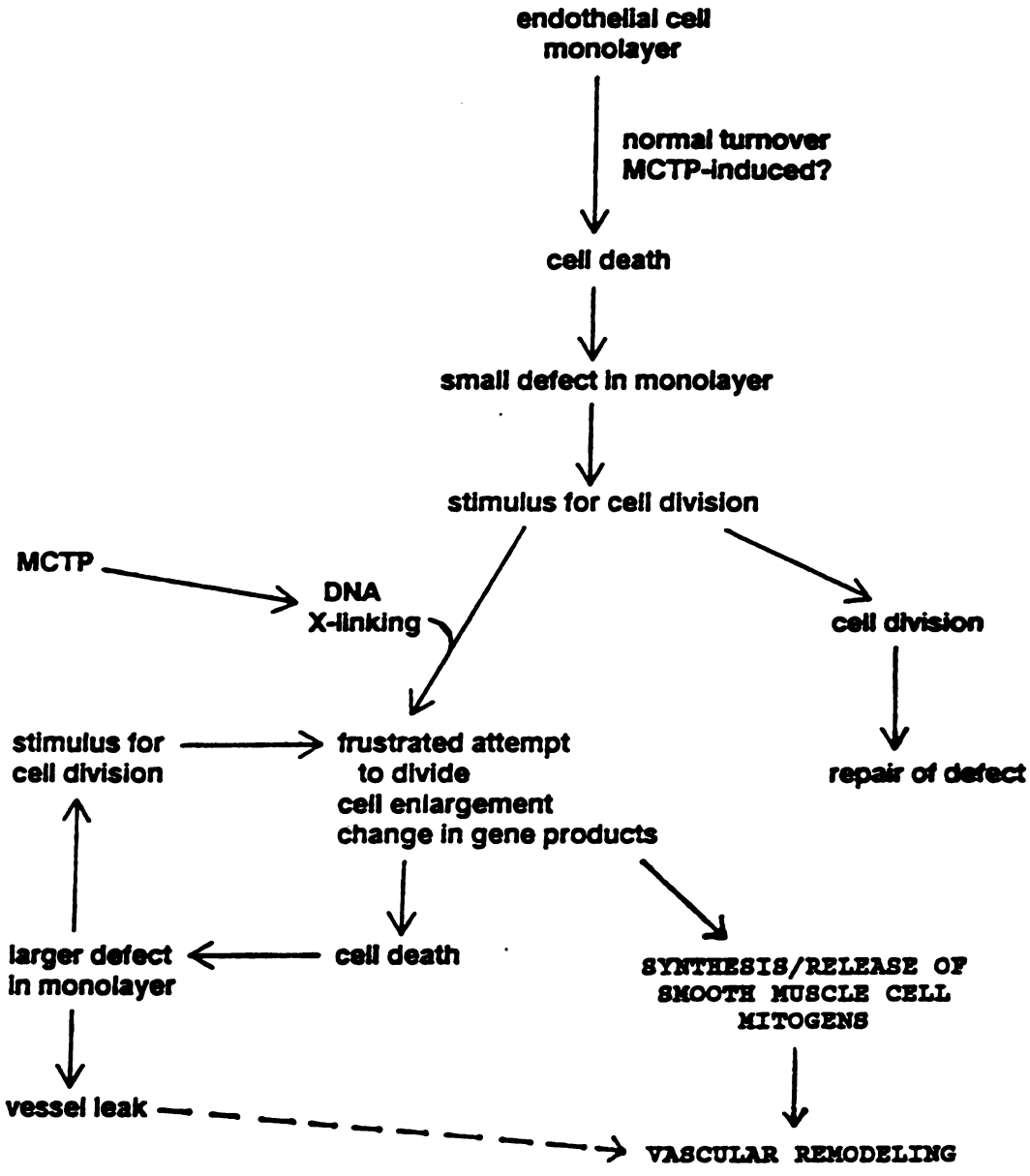


Figure 11: Role of MCT(P)-induced endothelial injury in the development of pulmonary vascular alterations: An hypothesis.

Surviving ECs, if still functional, may express an altered profile of gene products or demonstrate altered enzyme activity which could in turn influence surrounding vascular cells. For example, if there were a change in the balance of EC signals regulating SMC growth, vascular remodeling could ensue. Altered ACE activity on the surface of the endothelium could result in abnormal levels of Ang I, Ang II and BK in the pulmonary circulation, leading to altered vasoactivity. Figure 12 illustrates a number of EC changes which could contribute to the pulmonary vascular changes seen with MCT(P) pneumotoxicity.

Cultured ECs have thus far been used to examine the time- and dose-dependent effects of chemically synthesized MCTP on EC morphology and on limited aspects of cellular function. These early studies demonstrated that there are direct effects on ECs treated with MCTP and that expression of these effects appears to be delayed and progressive. Further identification and clarification of functional changes in ECs in response to treatment with MCTP may help to identify early, subtle alterations that lead to the delayed and progressive changes characteristic of MCT(P)-induced lung injury. In addition, manipulation of this model *in vitro* may help to define mechanisms which underlie the critical EC alterations.

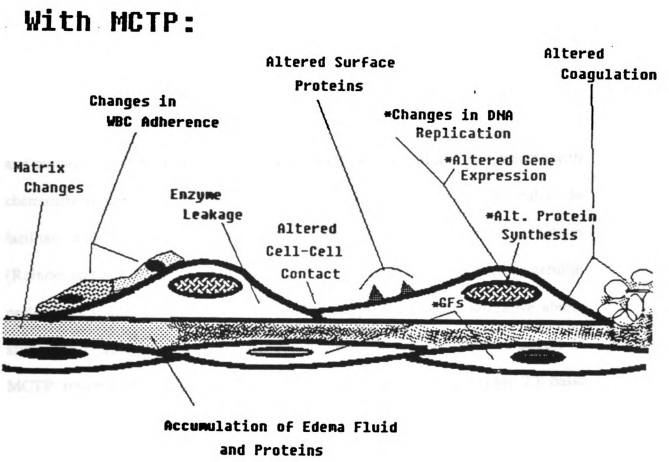


Figure 12: Potential-endothelial cell alterations after exposure to MCTP.

VII. Research Goals

It is proposed that the endothelium is a primary target of MCTP and that the functional consequences of MCTP-induced EC injury contribute to the development of pulmonary vascular lesions in this model of pneumotoxicity. A major objective of my dissertation research was to test the hypothesis that MCTP causes changes in endothelium *in vitro* that are consistent with its ability to cause lung vascular injury and pulmonary hypertension *in vivo*. To do this, I utilized cultured ECs treated with chemically synthesized MCTP. This model was developed in our laboratory to facilitate examination of the direct effects of MCTP on vascular endothelium (Reindel and Roth, 1991; Reindel *et al.*, 1991). Cultured ECs have proven useful in preliminary studies identifying changes in EC morphology and proliferative ability after MCTP treatment. I have extended these studies to examine the effects of MCTP treatment on: 1.) a surface enzyme function (ACE activity); 2.) basic intracellular function (ability to synthesize DNA, RNA and protein); and 3.) a specific aspect of EC function (regulation of mesenchymal cell proliferation).

Interspecific differences are evident in the response of cultured ECs to MCTP treatment. These differences may reflect inherent differences which are expressed when cells of various species are maintained in culture. However, if these differences exist *in vivo* as well, they may in part explain differences in target organ toxicity in response to PZAs. A second objective of my work has been to continue to investigate species differences in the response of cultured ECs to MCTP treatment in an effort to correlate these alterations with the responses seen *in vivo*. To do this, I have characterized the response of cultured rat pulmonary endothelium to MCTP

treatment and compared this response to that described in BECs and PECs treated with MCTP. This is an extremely relevant species comparison, as the rat is typically used to study MCT(P) pneumotoxicity *in vivo*. In addition, I have extended the species comparison of porcine and bovine endothelium to a functional parameter, ACE activity. Studies with ECs of various species have contributed to a better understanding of the endothelial response to injury as well, and this subject will be discussed in my concluding remarks.

My third objective was to begin to investigate DNA crosslinking as a mechanism in MCTP-induced EC injury. Damage to monolayers as a consequence of treatment with certain other EC toxicants (e.g. endotoxin, cyclosporin A), is typically much more rapid in onset (Meyrick, 1986; Paulsen *et al.*, 1989; Zoja *et al.*, 1986). Cell detachment and LDH release are evident within a few hours, and obvious monolayer disruption is evident by 24 hours. These effects are generally not progressive, and with removal of the toxicant the cells may recover and repair the damage to the monolayer. The hypertrophic response which gradually develops after a single administration of MCTP does not occur with these other toxic compounds. Thus, the delayed and progressive nature of MCTP toxicity in ECs may represent a unique response of this cell type to toxic injury, and may reflect its inability to effect repair. In PECs, MCTP causes DNA crosslinking which is quite persistent (Wagner *et al.*, 1992). These findings led to the development of a second hypothesis: crosslinking is responsible for the inhibition of proliferation seen in cultured ECs treated with MCTP, and this cytostasis results in the characteristic delayed, progressive and hypertrophic EC response to injury. In partial test of this hypothesis,

I examined the effects of another crosslinking agent, Mitomycin C (MMC), on cultured porcine and rat endothelium.

Chapter II

MONOCROTALINE PYRROLE-INDUCED CHANGES IN ANGIOTENSIN-CONVERTING ENZYME ACTIVITY OF CULTURED PULMONARY ARTERY ENDOTHELIAL CELLS

Summary

Changes in structural and functional integrity of endothelium have been recognized as relatively early features of the delayed and progressive pulmonary vascular injury caused by the pyrrolizidine alkaloid, monocrotaline (MCT). Although a number of investigators have evaluated angiotensin-converting enzyme (ACE) activity in the lungs of rats treated with MCT, the exact nature of changes in activity of this enzyme and the role they may play in MCT pneumotoxicity remain controversial. Monocrotaline pyrrole (MCTP) is a putative toxic metabolite of MCT. Treatment of cultured porcine and bovine pulmonary artery endothelial cells (PECs and BECs, respectively) with chemically-synthesized MCTP results in toxicity which is also delayed and progressive in nature. In this study, we examined the direct effects of MCTP on cultured endothelial cell ACE activity. Post-confluent monolayers of PECs or BECs were treated with a single administration of MCTP at time 0, then they were examined for their ability to degrade the synthetic peptide, [^{3}H]benzoyl-phe-ala-pro. In PECs, which are relatively insensitive to the direct cytolytic effects of MCTP, monolayer ACE activity was unchanged initially, but gradually decreased by 4 days after treatment with a high dose of MCTP. This decrease was transient, and PEC monolayer ACE activity returned to the control value by 10 days posttreatment. BEC monolayer ACE activity was also unchanged initially, but rapidly declined 4 days after MCTP treatment and remained depressed throughout the posttreatment period. BECs are quite sensitive to the cytolytic effects of MCTP, and the decline in ACE activity occurred coincident to the decrease in monolayer cellularity and

appearance of marked cytotoxicity previously reported in these cells. When ACE activity was expressed as a function of cell number, cellular ACE activity was still significantly decreased 4 days after a high concentration of MCTP in both PECs and BECs. In summary, high concentrations of MCTP appear to decrease endothelial ACE activity in a model which excludes complicating hemodynamic factors present *in vivo*. The decline in ACE activity is delayed, and the magnitude and duration of the decrease corresponds to the degree of overt MCTP-induced cytotoxicity. This suggests that altered endothelial ACE activity is unlikely to be a direct effect of MCTP on the enzyme but may be a response to the delayed cell injury which results from exposure to this compound.

Introduction

Changes in endothelial structural and functional integrity have been noted relatively early in the course of MCT pneumotoxicity *in vivo* (Reindel *et al.*, 1990; Bruner *et al.*, 1983; Hilliker *et al.*, 1982), and it has been suggested that sublethal but persistent changes in endothelial cell function may play a role in the development and/or maintenance of MCT-induced pulmonary hypertension (Reindel *et al.*, 1990; Reindel and Roth, 1991; Roth and Reindel, 1990). An important function of the pulmonary vascular endothelium is cleavage of circulating, inactive angiotensin I (Ang I) to the potent vasoconstrictor, angiotensin II (Ang II), by the action of the exopeptidase known as angiotensin-converting enzyme (ACE). This membrane-bound enzyme also serves to inactivate the vasodilator peptide, bradykinin (Yang *et al.*, 1970; Erdos, 1975). ACE has been localized to the luminal surface of endothelial cells in structures called caveolae intracellulares (Ryan *et al.*, 1975). Pulmonary conversion of these peptides appears to be a function of pulmonary vascular surface area and the time it takes for blood to transit the lung vasculature (Fanburg and Glazier, 1973), thus it has been suggested that pulmonary vascular disease might be associated with changes in pulmonary ACE activity (Gillis and Catravas, 1982; Dobuler *et al.*, 1982; Catravas *et al.*, 1988b). It is also possible that agents which result in pulmonary endothelial injury could directly alter the distribution or activity of this surface enzyme.

A number of investigators have evaluated ACE activity in lungs of rats treated with MCT in an effort to correlate changes in enzyme activity to some aspect of the

development or progression of MCT-induced cardiopulmonary changes. The results of these studies have been disparate and controversial. Some investigators report that there is a decrease in lung ACE activity which corresponds to an increase in pulmonary pressure (Kay *et al.*, 1982a; Keane *et al.*, 1982), whereas others contend that apparent changes in lung ACE activity are due to a dilution of this activity by the increased lung mass seen in this model (Huxtable *et al.*, 1978; LaFranconi and Huxtable, 1983). Yet another group reports an increase in lung ACE activity shortly after MCT treatment, with a subsequent sustained decline in activity later on (Molteni *et al.*, 1984). A fourth group finds a sustained decline in ACE activity which precedes the onset of pulmonary hypertension and which occurs at doses that do not produce right ventricular hypertrophy (Shale *et al.*, 1986). From these reports, it has been difficult to draw definitive conclusions about ACE activity in lung vasculature injured by MCT.

MCTP, a putative toxic metabolite of MCT, is toxic to cultured bovine and porcine pulmonary artery endothelial cells (BECs and PECs, respectively) (Reindel and Roth, 1991; Reindel *et al.*, 1991). This cultured endothelial cell model makes it possible to investigate the direct effects of MCTP on endothelial ACE activity. The purpose of this study was to determine whether MCTP affects the activity of this ectoenzyme in cultured endothelium and to characterize the development of any changes that occur.

Materials and Methods

Preparation of endothelial cells: Lines of PECs and BECs were derived from segments of pulmonary artery by modifications of the methods of Jaffe *et al.* (1987) and Booyse *et al.* (1975), as described by Reindel *et al.* (1991). Sections of pulmonary artery were collected aseptically from freshly killed young market animals, rinsed in sterile Hank's balanced salt solution (HBSS; Sigma Chemical Company, St. Louis, MO), and placed into sterile Puck's saline containing 3% antibiotic/antimycotic solution (300 units/ml penicillin, 300 μg/ml streptomycin, 0.75 μg/ml Fungizone; Ab/Am; GIBCO, Grand Island, NY) on ice for transport. In a laminar flow hood, the adventitia, external elastic laminae and outer medial smooth muscle layers were removed by careful dissection. Trimmed squares of artery were placed into 60 mm tissue culture plates with luminal surfaces submerged in a thin film of collagenase (Type 1A, 0.1%; Sigma). After incubation for 3 to 5 minutes at 37°C, the luminal surface of the vessel was gently brushed with a single stroke of a rubber policeman, and the brushings were resuspended in fresh, calcium- and magnesium-free HBSS (CMF-HBSS; Sigma). Under an inverted microscope (TMS; Nikon, Tokyo, Japan), small clusters of endothelial cells were collected with a micropipette and transferred to wells of 12-well tissue culture clusters (Corning, Park Ridge, IL) containing 0.5 ml of Opti-MEM (GIBCO) with 5% fetal calf serum (FCS; Biocell, Rancho Dominguez, CA) and 1% Ab/Am (PECs) or Medium 199 (M199; GIBCO) containing 10% FCS and 1% Ab/Am (BECs). Plates were incubated for 5 to 7 days under standard incubation conditions, 37°C with 7.5% CO_2/92.5% air.

At this time, wells with colonies exhibiting typical cobblestone morphology and that were free of spindle cells were passed into larger wells using 0.25% trypsin/0.01M EDTA (GIBCO). These cells were grown to confluence, and wells were selected for passage on the basis of characteristic endothelial cell morphology.

At the time of their third passage, cells were plated onto glass coverslips for further characterization, including uptake of Di-I-Ac-LDL (acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate; Biomedical Technologies, Inc., Stoughton, MA), angiotensin-converting enzyme activity (Ventrex, Portland, ME), positive staining for factor VIII-related antigen and electron microscopic examination, and were tested for contamination with mycoplasma (Hoechst Stain Kit; Flow Laboratories, McLean, VA). Cultured endothelial cell lines were maintained in Opti-MEM with 3% FCS and 1% Ab/Am (PECs) or M199 with 10% FCS and 1% Ab/Am (BECs) and were split at a ratio of 1:3 or 1:4 at weekly intervals by either enzymic (trypsin/EDTA) or mechanical dissociation. All studies were performed with cells between passages 4 and 9.

Preparation of MCTP: MCTP was prepared from MCT (Transworld Chemical, Washington, DC) via an N-oxide intermediate by the method of Mattocks (1968). MCTP isolated from this synthesis procedure has Ehrlich activity (Mattocks and White, 1971) and a structure compatible with MCTP as determined by mass spectrometry and nuclear magnetic resonance (Bruner *et al.*, 1986). MCTP was dissolved in N,N-dimethylformamide (DMF; Sigma Chemical Co., St. Louis, MO) at

a concentration of 20 mg/ml, and all dilutions of MCTP were made with DMF. A 2.5 μl volume of MCTP solutions or DMF vehicle (0 μM MCTP) per milliliter of medium was used to achieve the nominal concentrations of MCTP (0, 1.5, 15 or 150 μM) used in the study.

Assay of angiotensin-converting enzyme activity: PECs and BECs were plated into 12-well tissue culture clusters (25 mm diameter) and allowed to form confluent monolayers. Monolayers were not used for assay of ACE activity until at least 10 days after plating as ACE activity of cultured endothelial cells is not restored after dissociation and replating until cells have been maintained in a confluent state for this length of time (DelVecchio and Smith, 1981). Mature monolayers were treated with a single administration of MCTP (0, 1.5, 15 or 150 μM) on day 0. ACE activity of monolayers was analyzed at 8 hours and 2, 4 and 7 days (also at 10 days, for PEC monolayers) posttreatment using the standard radioassay protocol provided by Ventrex Laboratories, Inc. (Portland, ME). The assay involves cleavage of the synthetic peptide [^{3}H]benzoyl-Phe-Ala-Pro to form [^{3}H]benzoyl-phenylalanine and the dipeptide alanyl-proline by endothelial ACE. The tritiated portion of the peptide becomes soluble in organic scintillant when cleaved, and can be extracted, separated and counted in a liquid scintillation counter. Activity in the presence of the specific ACE inhibitor, Captopril (SQ 14,255; 10^{-6}M), was subtracted from total activity to ensure that conversion of substrate was due to the action of ACE. Calculation of ACE activity was carried out as described in the Ventrex protocol, using a formula

representing a simplified form of the integrated first order Michaelis-Menton equation:

$$E = \frac{\frac{4000(T-I)}{f_p \times S_o}}{t}$$

where, E = enzyme activity as % substrate utilized/minute/ml
t = time in minutes of reaction
T = c.p.m. of TEST monolayer
I = c.p.m. of INHIBITOR-treated monolayer
f_p = fractional partition of product into counting phase = 0.5
S_o = c.p.m. of substrate (total c.p.m.)

A unit of ACE activity is defined as that required to hydrolyze substrate at an initial rate of one percent per minute at 37°C.

Effects of MCTP on monolayer cellularity: After determination of ACE activity as described above, monolayers were washed three times with CMF-HBSS (Sigma) to remove non-adherent cells. Adherent cells were enzymically removed from the plate surface with 0.025% trypsin-0.27 mM EDTA solution (GIBCO) and counted using a Coulter counter (Model ZM; Coulter Electronics, Luton, England).

Statistical analysis: ACE activity data are presented as means of 5 (PECs) or 3 (BECs) separate experiments, each consisting of two wells per concentration of MCTP. The standard error of difference (Steel and Torrie, 1980) for each data set is presented. Data were analyzed with a blocked analysis of variance (ANOVA).

Individual comparisons between treatment groups (MCTP and DMF vehicle) were made using Tukey's omega as the *post hoc* test ($p < 0.05$).

Cell numbers are presented as means +/- SEM. These data were analyzed using a completely random ANOVA, and individual comparisons were made using the Tukey's omega test ($p < 0.05$).

Results

The effects of MCTP on cellularity of PEC and BEC monolayers are shown in Figures 13A and 13B, respectively. Cell monolayers were treated once with MCTP at zero time. Whereas PEC and BEC cell numbers continued to increase slightly with time in wells treated with vehicle or the lowest concentration of MCTP, they declined in monolayers treated with the higher doses of MCTP, beginning at day 4 posttreatment. When examined using phase contrast microscopy, all monolayers appeared to remain intact and free of obvious gaps throughout the posttreatment period. PECs and BECs treated with higher concentrations of MCTP (15 or 150 μM) demonstrated distinct morphological changes as monolayer cellularity decreased. These changes have been reported previously (Reindel and Roth, 1991; Reindel *et al.*, 1991) and include cell enlargement, vacuolization and nuclear changes in BECs and a milder enlargement with a shift toward a more spindle-like shape in PECs.

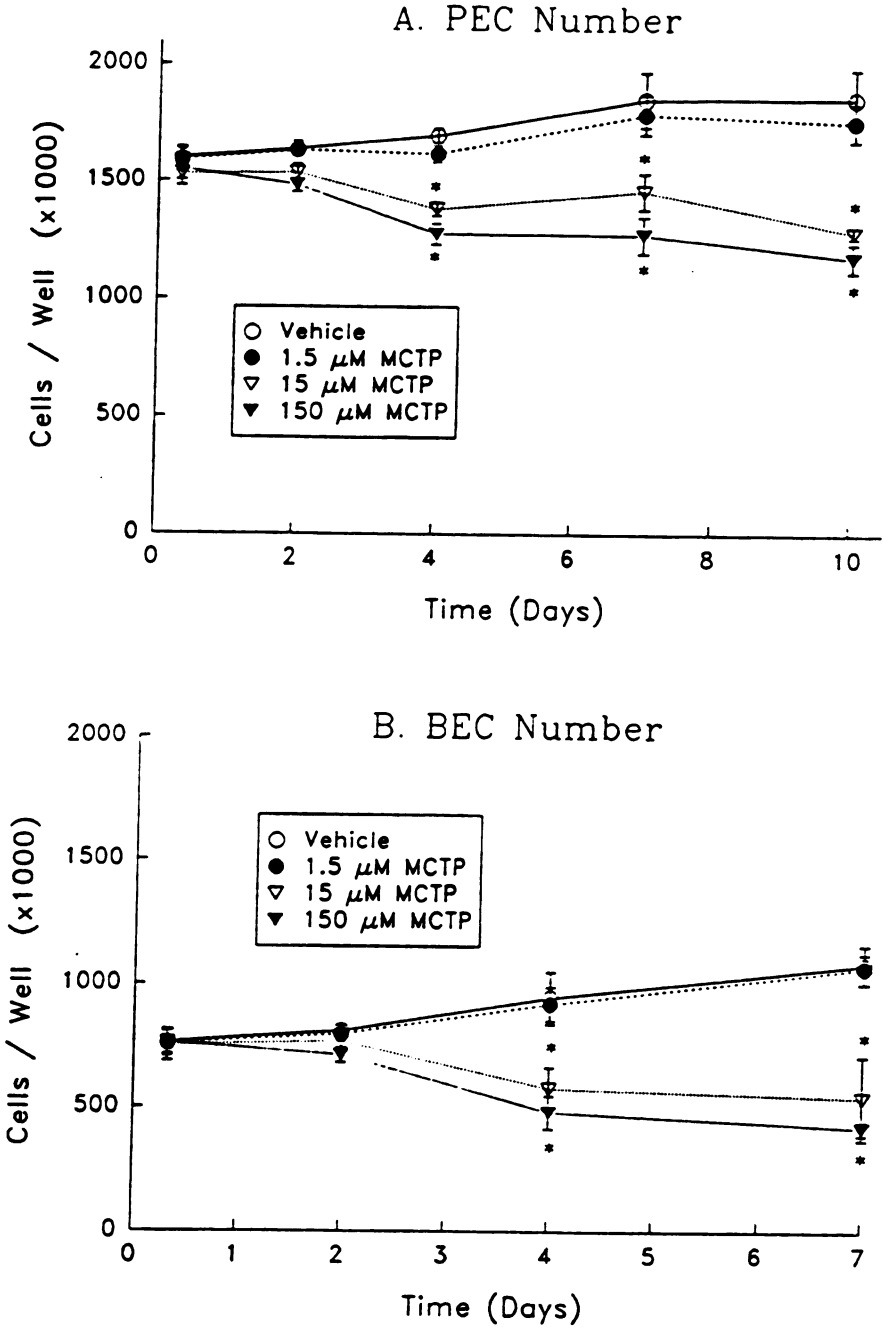


Figure 13: Effects of MCTP on cellularity of PEC (A) and BEC (B) monolayers.

Post-confluent monolayers were exposed to a single administration of MCTP on day 0. Values represent the means of replicate studies (n=5 for PECs; n=3 for BECs).
* Significantly different from vehicle control ($p < 0.05$)

Changes in ACE activity of mature monolayers of PECs and BECs after a single exposure to MCTP are shown in Figure 14. ACE activity of PEC monolayers (Figure 14A) treated with 150 μM MCTP decreased gradually, reaching a nadir at 4 days posttreatment then returning to control levels by 10 days posttreatment. BEC monolayers also showed a concentration-dependent decline in ACE activity with treatment, but the decrease was much more marked and persistent (Figure 14B). In monolayers treated with the highest concentration of MCTP, ACE activity was significantly decreased by 2 days posttreatment, and after 4 days posttreatment these monolayers demonstrated virtually no ACE activity. In BECs, the intermediate MCTP concentration also led to reduced ACE activity after day 2.

Cellular ACE activity after MCTP treatment is expressed in Figure 15 as a function of PEC and BEC number (units of activity per million cells). While the total activity of vehicle-treated PEC and BEC monolayers were similar (see Figure 14), activities normalized to cell number were quite different. BECs (Figure 15B) had about twice the ACE activity per million cells than PECs (Figure 15A). The delayed, transient decrease in ACE activity of PECs treated with 150 μM MCTP was still evident, but cellular ACE activity returned to the control value by 7 days and was increased by 10 days posttreatment. Cells treated with 15 μM MCTP showed no change in activity until day 10, at which time cellular ACE activity was elevated. In BECs, cellular ACE activity was significantly depressed by 4 days posttreatment in cells treated with the highest concentration of MCTP. However, decreases in cellular ACE activity were not evident in BECs treated with 15 μM MCTP.

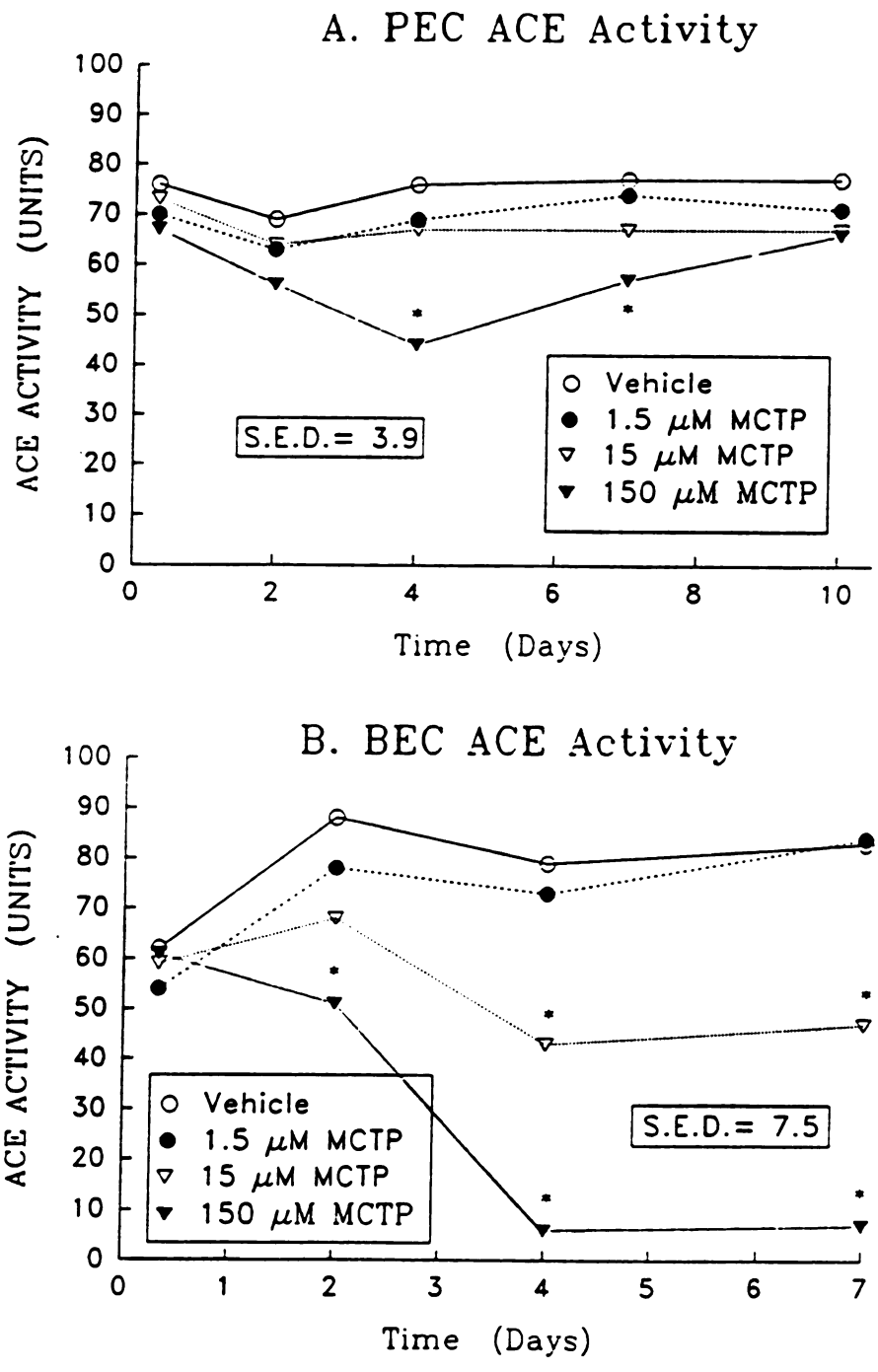


Figure 14: Angiotensin-converting enzyme activity of MCTP-treated monolayers.

Monolayers of PECs (A) and BECs (B) were exposed to a single administration of MCTP at time 0. One unit of ACE activity is defined as that required to hydrolyze substrate at an initial rate of one percent per minute at 37°C. Values represent the means of replicate studies (n=5 for PECs; n=3 for BECs). Standard error of difference = 3.89 PECs; 7.55 BECs). * Significantly different from vehicle control ($p<0.05$)

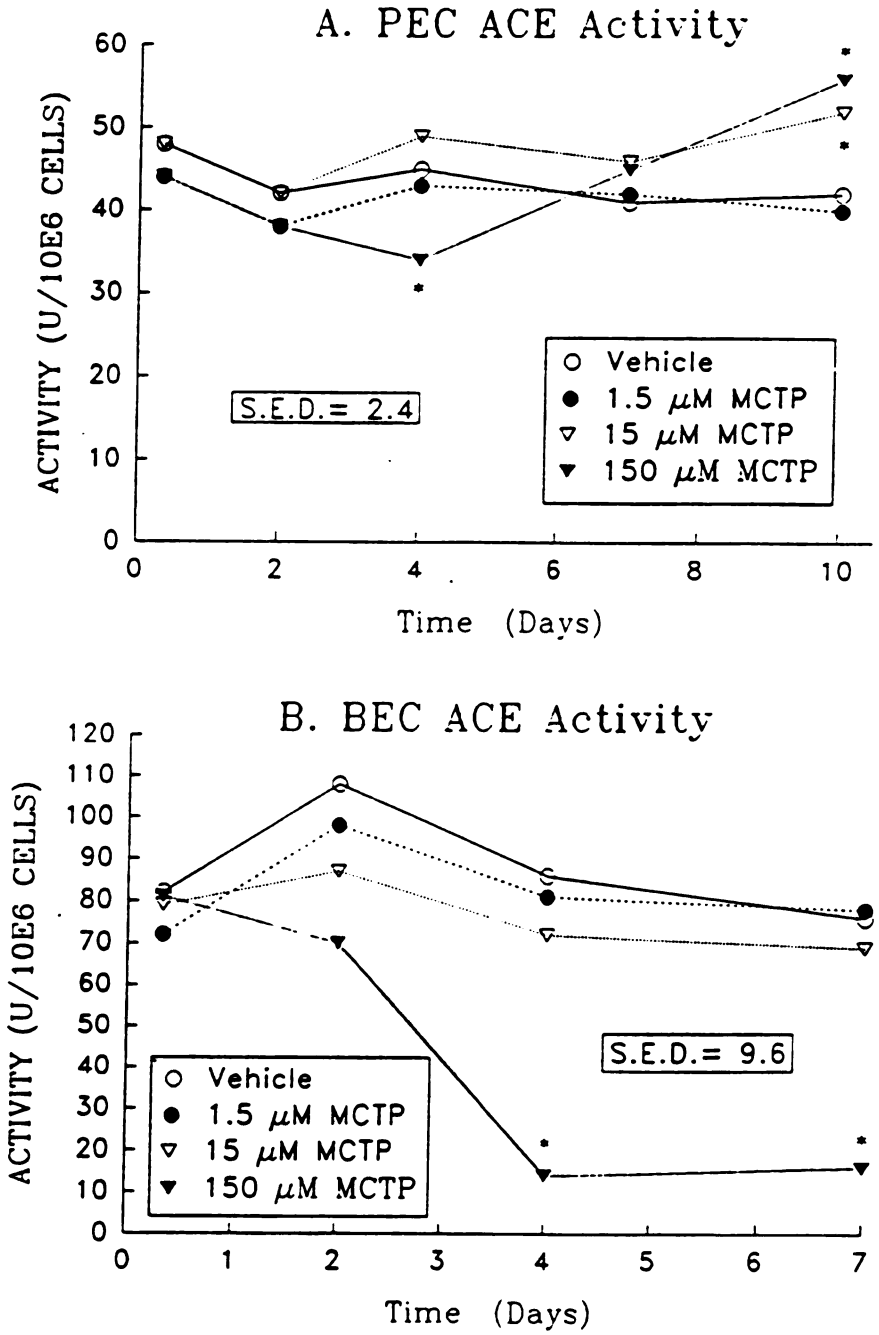


Figure 15: Angiotensin-converting enzyme activity expressed as a function of cell number.

Mature monolayers of PECs (A) and BECs (B) were exposed to a single administration of MCTP at time 0. Values represent the means of replicate studies (n=5 PECs; n=3 BECs). Standard error of difference = 2.44 PECs; 9.58 BECs.
* Significantly different from vehicle controls ($p < 0.05$)

Discussion

A number of conditions which result in pulmonary injury or alterations in pulmonary blood flow have been associated with changes in angiotensin-converting enzyme activity in lung or serum. In some cases, these changes may reflect direct damage to the vascular endothelium. For example, administration to rats of alpha-naphthylthiourea (ANTU) (Hollinger *et al.*, 1980a) or paraquat (Hollinger *et al.*, 1980b) at doses which cause acute lung injury results in a rapid, transient decrease in lung ACE and a corresponding increase in serum ACE. These changes are thought to reflect a loss of endothelial integrity, which is supported by the rapid onset of pulmonary edema.

In other cases, changes in ACE activity appear to be compensatory and occur coincident with or subsequent to the development of pulmonary hypertension. This appears to be the case in animals exposed to conditions of chronic hypobaric hypoxia (Jederlinic *et al.*, 1988; Keane *et al.*, 1982) or in the lungs of hypoxia-adapted rats (Oparil *et al.*, 1988). Fetal rats and rabbits, in which pulmonary arterial pressure is normally greater than in adults, also have low pulmonary ACE activity which increases soon after birth (Wallace *et al.*, 1979; Stalcup *et al.*, 1978). Under these conditions of increased pulmonary arterial pressure, decreased ACE activity might result in decreased conversion of Ang I to Ang II, a potent vasoconstrictor, as well as less degradation of the vasodilator, bradykinin.

A similar scenario has been described for MCT pneumotoxicity in the rat. Kay *et al.* (1982a) report that a single, subcutaneous injection of 60 mg MCT/kg

causes delayed and progressive pulmonary vascular injury which results in vascular remodeling and consequent pulmonary hypertension by 10-14 days posttreatment. These changes are accompanied by a decrease in lung ACE activity per mg protein beginning by day 10, which is supported by decreased conversion of Ang I to Ang II (Keane and Kay, 1984). Molteni *et al.* (1984) report a transient increase in pulmonary ACE activity after 1 week of MCT followed by a persistent decline in activity during weeks 2 through 6. In these studies, rats received MCT in their drinking water (20 mg/liter) throughout the experimental period. No changes in serum ACE were reported under either set of experimental conditions. Kay and Keane maintain that the alterations in ACE activity seen with MCT are a result of the pulmonary hypertensive conditions, and may be a protective mechanism to limit this physiological change (Kay *et al.*, 1982a; Keane *et al.*, 1982). This view is held by Molteni *et al.* (1984) as well, but they also suggest that the transient increase in ACE which they find in their model precedes vascular remodeling and reflects endothelial injury and dysfunction.

In a separate study, Shale *et al.* (1986) found that lung ACE (reported as nmol/min/lung and nmol/min/mg protein) decreased when MCT was given in an oral dosing regimen which did not produce pulmonary hypertension or right ventricular hypertrophy. Although this supports the idea that ACE does not play a causative role in the development of these sequelae, it also suggests that the decrease in ACE activity seen with MCT treatment is not solely a response to increased pulmonary vascular pressure. Rather, it suggests an early, direct effect of MCT on the lung. The increased pulmonary tissue mass due to inflammation and hyperplasia

after MCT treatment may in fact dilute what is really an unchanged endothelial ACE content at this stage in the injury.

The concept that a decrease in lung ACE activity may be due to increased total lung protein is one that has been maintained by Huxtable and his co-workers for some time (Huxtable *et al.*, 1978; LaFranconi and Huxtable, 1983). Although they found that MCT administered in the drinking water for 3 weeks did decrease lung ACE per gram of dried lung, total lung ACE activity was unchanged. Any decrease in lung ACE activity was attributed to dilution by an increased total lung mass. However, Kay and Keane maintain that ACE decreases in their model whether it is calculated on a per tissue weight or total lung basis, and they suggest that the difference in response may be due to differences in experimental design (Keane and Kay, 1984).

My objective in performing this study was to determine if MCTP would directly alter ACE activity in an isolated endothelial cell system. Although we found that MCTP does decrease endothelial ACE activity *in vitro*, our results raise a number of interesting points with respect to changes in ACE activity after cell injury. In both PECs and BECs, decreases in ACE activity did not occur immediately after administration of MCTP; rather, changes were delayed for several days. This suggests that the decrease in ACE activity was not due to a direct interaction of MCTP with the enzyme or to other changes which may occur at the cell surface at the time of treatment. MCTP is quite reactive and much of it probably binds to cells rapidly after administration *in vitro*. However, the inactivation of ACE does not

appear to be an early or an immediate change effected by MCTP, even at relatively high concentrations.

A more rapid decrease in ACE activity has been reported in cultured endothelial cells when they are subjected to hypoxia (Stalcup *et al.*, 1979). This decrease is rapidly reversible with restoration of normoxia, and it occurs without marked structural changes in the cells. The authors of that report suggest that the decrease in enzyme activity results from a response of the endothelial cells to the hypoxia rather than as a direct effect of hypoxia on the enzyme. Our results suggest that this may be the case with MCTP treatment of endothelial cells as well. Ryan and Catravas (1990) suggest that, in addition to enzyme dysfunction, changes in ACE activity may occur as a result of changes in the microenvironment of the enzyme. Some of the delayed cytotoxic changes which occur as a consequence of exposure to hypoxia or MCTP treatment could result in subtle alterations of the endothelial cell surface which are incompatible with ACE function.

There are clear species differences in the response of pulmonary artery endothelial cells to treatment with MCTP. Species differences have been reported with respect to cell detachment, release of lactate dehydrogenase and changes in cell morphology (Reindel *et al.*, 1991). While BECs are quite sensitive to the cytolytic effects of MCTP, PECs appear to be relatively resistent. However, both cell types are inhibited in their abilities to proliferate at the concentrations used in this study, and both show distinct changes in morphology after MCTP treatment. The differences noted with respect to ACE activity of BEC and PEC monolayers after MCTP treatment are also pronounced, and these seem to parallel the morphologic

and cytotoxic changes. For example, the transient nature of the decrease in PECs corresponds with the decline in cell numbers, and the more dramatic and persistent fall in ACE activity in BECs reflects the more marked cytotoxic response of this cell type.

ACE inhibitors, such as captopril, cilazapril or CL242817, and angiotensin receptor antagonists prevent or attenuate the vascular remodeling seen after denuding or toxic injury to the endothelium (Grotendorst *et al.,* 1982; Schweigerer *et al.,* 1987; Molteni *et al.,* 1985; Powell *et al.,* 1990; Clozel *et al.,* 1991; Zakheim *et al.,* 1975), suggesting that Ang II may be important in the pathophysiology of the vascular response to injury. It is possible that endothelial modulation of production of this potent mediator may occur at a local level under conditions which might predispose the vessel to myointimal hyperplasia. Bell and Madri (1990) have shown that ACE inhibition or angiotensin receptor blockade in cultured bovine aortic endothelial cells (BAECs) and smooth muscle cells (SMCs) results in increased BAEC migration and plasminogen activator levels, while limiting SMC migration. They suggest that blockade of this autocrine pathway might reduce vascular wall injury by enhancing wound closure, increasing the antithrombotic tendency of the area and decreasing the rate of SMC infiltration. ACE activity declined substantially in our cells after treatment with MCTP only after there was a decrease in monolayer cellularity and evidence of cytotoxicity. This suggests that the change in enzyme activity may have occurred in response to these alterations and may function to limit the local damage. In PECs, in which the cellular damage was less pronounced, the changes in ACE activity were also quite subtle and transient. These cells eventually

had increased ACE activity on a cellular basis by 10 days posttreatment, which may reflect more persistent alterations in the cells' response to injury.

It is important to point out that the most dramatic changes in ACE activity in both PECs and BECs occurred only at the highest concentrations of MCTP. The pulmonary vascular endothelium may not experience such a high exposure after a pulmonary hypertension-producing dose of MCTP *in vivo* (Reindel *et al.*, 1990), except, perhaps, in certain regions. The pattern of MCT- or MCTP-induced lung injury *in vivo* is multifocal in nature, suggesting nonuniform distribution of toxicant to the various regions of the lung vasculature. Although endothelial damage does result after treatment with MCT or MCTP *in vivo*, the damage is unlikely to be evidenced by a noticeable decrease in cellular ACE activity unless rat pulmonary endothelium is much more sensitive to these compounds than the species we have tested. Considering this, it seems likely that changes in ACE activity seen after MCT treatment *in vivo* occur either as a result of altered lung mass, pulmonary vascular surface area, or blood flow, or in response to changes in pulmonary vascular pressure, but probably do not reflect altered endothelial ACE activity as a direct result of treatment.

In summary, ACE activity in pulmonary artery endothelium is altered after MCTP treatment *in vitro*, and this alteration is clearly not secondary to hypoxia, increased pressure, or other complicating hemodynamic factors that may be present *in vivo*. These changes appparently are not due to a direct action of MCTP on the enzyme, but they more likely occur as a response to endothelial cell injury.

Chapter III

MONOCROTALINE PYRROLE ALTERS DNA, RNA AND PROTEIN SYNTHESIS IN PULMONARY ARTERY ENDOTHELIAL CELLS

Summary

Cultured porcine pulmonary artery endothelial cells (PECs) treated with monocrotaline pyrrole (MCTP) remain viable but are unable to divide and exhibit an altered morphology. Such responses raise a question about the extent to which affected cells carry out normal functions such as RNA and protein synthesis. Accordingly, the cellular activity of MCTP-treated PECs was examined in this study. PECs were treated with a single administration of MCTP or vehicle, and determinations of cell number, protein and DNA content were made at times up to 7 days posttreatment. DNA, RNA and protein synthesis were quantified by incorporation of [^{3}H]-labeled thymidine, uridine and leucine, respectively. Increases in cell number that occurred with time in the control cells were reduced in MCTP-treated cells. At 7 days post-treatment, both protein and DNA content increased above control levels. Synthesis of DNA, RNA and protein continued in all treatment groups throughout the post-treatment period, but cells treated with high concentrations of MCTP showed less synthetic activity than controls during the initial 48 hours posttreatment. By 7 days, MCTP-treated cells were producing significantly more DNA, RNA and protein. These results indicate that cells treated with MCTP continue to synthesize DNA, resulting in an increased DNA content. In addition, treated cells continue to synthesize RNA and translate RNA into protein. Thus, cellular activity is maintained but altered substantially by MCTP exposure.

Introduction

An interesting feature of MCTP pneumotoxicity is its delayed and progressive nature. There is a delay of several days after a single administration of MCT or MCTP before major lung injury is evident. This suggests that initially lung cells may be sublethally but irreversibly damaged by MCTP. Although the reactivity of MCTP suggests that the initial event in the toxicity occurs rapidly (ie, covalent binding of MCTP to the cell), the lag period implies cellular alterations which are slow to develop but which are progressive and trigger a cascade of molecular changes and pathophysiological events that result ultimately in chronic pulmonary vascular disease.

By virtue of its anatomic location, the pulmonary vascular endothelium is a likely target for binding of MCTP that is delivered to the lung via the circulation, and there are indications that endothelial cells are altered with MCTP treatment *in vivo*. *In vitro*, administration of a sublethal concentration of MCTP causes injury to cultured endothelial cells. A single treatment of endothelial cells from porcine pulmonary arteries with MCTP at noncytotoxic concentrations inhibits cell proliferation (Reindel and Roth, 1991; Reindel *et al.*, 1991). PECs treated with MCTP become spindle-shaped and show a delayed and progressive increase in release of prostacyclin (Reindel *et al.*, 1991). If PEC monolayer integrity is compromised by mechanical wounding after MCTP treatment, the remaining cells migrate into the defect, enlarge dramatically, and remain viable but do not divide as normal cells would do (Hoorn *et al.*, 1990). Although PECs treated with MCTP are unable to proliferate normally, they do remain viable in culture for long periods of

time. MCTP-treated cells may be functionally different from normal ECs, and these changes in function might contribute to the delayed and progressive development of MCTP pneumotoxicity *in vivo*. Many types of cellular stresses, such as heat shock and hypoxia, alter the amounts and types of RNA and protein which cells produce (Williams *et al.*, 1989; Thomas *et al.*, 1982; King *et al.*, 1989; Kourembanas *et al.*, 1990; Ogawa *et al.*, 1990b). This study was designed to investigate the effects of MCTP treatment on synthesis of RNA and protein by PECs to determine if MCTP treatment influences basic cellular functions.

The arrest of cell proliferation observed after MCTP treatment and the ability of this compound to act as a bifunctional alkylating agent (Robertson, 1982; Petry *et al.*, 1984) suggest that changes may occur in treated ECs at the level of DNA. This suggestion is supported by observations that DNA crosslinking occurs in the livers of rats treated with MCT (Petry *et al.*, 1984) and in cultured PECs treated with MCTP (Wagner *et al.*, 1991; Wagner *et al.*, 1992). Although MCTP-treated cells do not divide, it is possible that they continue to undergo some DNA synthesis either as part of the DNA repair process or in a frustrated attempt to proliferate. Accordingly, a second objective of this study was to determine the effect of MCTP treatment on DNA synthesis and DNA content of cultured PECs.

Materials and Methods

Cell Culture: Primary isolation and subculture of PECs was performed as described previously in chapter II. These studies were performed with subconfluent as well as

confluent cultures of endothelial cells (as indicated) to determine if a stimulus for cell proliferation (ie., subconfluency) further alters cell function after MCTP treatment. PECs were plated at low (40,000 cells/well) or high (80,000 cells/well) density into 12-well tissue culture clusters containing 1.5 ml Opti-MEM with 5% FCS and 1% Ab/Am per well. Plates were incubated under standard conditions of 37°C and 7.5% CO_2/92.5% air until the high-density cultures reached confluence (typically, 48-72 hours). At this time, the medium in each well was replaced with Medium 199 (GIBCO) containing 0.5% FCS for 48-72 hours to growth-arrest the PECs. At the end of this period, the low-serum medium was removed and replaced with 2 ml fresh Opti-MEM (5% FCS, 1% Ab/Am).

Treatment with MCTP: MCTP was synthesized from MCT as described previously in Chapter II. MCTP was dissolved DMF and diluted to the indicated concentrations. Equal volumes of DMF served as vehicle controls in all experiments. At time 0, growth-arrested PECs were placed in fresh Opti-MEM and received a single administration of MCTP at a volume of 5 μl/2 ml medium to achieve the nominal concentrations of 1.5 or 150 μM MCTP. These concentrations, selected on the basis of previous work with MCTP in cultured PECs, typically result in minimal (low concentration) or marked (high concentration) toxic responses in cultured PECs, respectively (Reindel *et al.*, 1991). Treated cells were incubated under standard conditions for the indicated time periods with one change of medium on day 4 posttreatment. No additional MCTP was added with the fresh culture medium.

Experimental Procedures: At the designated times of 0, 3, 6, 12, 24, 36, 48 and 168 hours post-treatment, three wells of PECs per treatment group were analyzed for each of the following: cell number, protein content, DNA content, and radiolabeled thymidine, uridine and leucine incorporation.

Number of cells per well was determined as described previously in Chapter II.

Protein content of the monolayer was determined by the method of Bradford (1976). After washing with HBSS, monolayers were dissolved in 1 ml of 0.1 M NaOH. Two 100 μl aliquots of the cell lysate per well were analyzed, and the results reported as mean protein content per cell.

Total DNA content per well was determined by the spectrofluorometric method of LaBarca and Paigen (1980). Monolayers were washed and cells removed with trypsin/EDTA. Cells were transferred to a 3-ml polypropylene tube, spun in a centrifuge at 1400 x g, and resuspended in 0.65 ml DNA assay buffer [2 M NaCl, 0.05 M Na_2HPO_4 and 2 mM EDTA-2Na in water, pH 7.4]. The suspension was sonicated and refrigerated until analysis. Each cell sonicate was analyzed in duplicate, and the results reported as mean DNA content per cell.

Thymidine, uridine and leucine incorporation were determined by a modification of the method of Madhukar *et al.* (1989). At the post-treatment times indicated, cells were pulse-labeled with 2 μCi/ml [5-methyl-^{3}H] thymidine (40-60 Ci/mmol; Amersham, Arlington Heights, IL), 0.2 μCi/ml [5,6-^{3}H] uridine (35-50 Ci/mmol; NEN/Dupont, Hoffman Estates, IL), or 2 μCi/ml L-[4,5-^{3}H(N)] leucine (40-60 Ci/mmol; NEN/Dupont) under standard incubation conditions for 2 hours. The medium used for all labeling procedures contained 10^{-5}M cold thymidine, as well

as 120 mg cold leucine and 0.3 mg cold uracil/liter, ensuring that the radiolabeled compounds would serve as tracers only and would not significantly impact on the precursor pools. After incubation, the culture plates were placed on ice for 30 minutes, then they were washed three times with cold, phosphate-buffered saline (PBS) to remove all unincorporated tracer. The cells were treated with cold 10% trichloroacetic acid (TCA) on ice for 10 minutes, washed first with 70% ethanol, then with 95% ethanol and air-dried. 0.5 ml 1 N NaOH was added to solubilize the precipitate overnight, then 0.5 ml 1 N HCl was added to neutralize the digest; 200 μl aliquots were mixed with 15 ml Safety-Solve® liquid scintillant (Research Products International Corporation, Mount Prospect, IL), and radioactivity was determined in a scintillation counter. Disintegrations per minute (d.p.m.) in each well were divided by cell number to determine average incorporation per cell.

[^{3}H]Thymidine incorporation was also evaluated with autoradiography by a modification of the method of Wang *et al.* (1989). Cells were grown at high or low density in Lab Tek® two-chamber glass slides (Nunc, Inc., Naperville, IL), then treated with MCTP as described above. At 24, 48 or 168 hours post-treatment, [^{3}H] thymidine was added to each well at a final activity of 2 μCi/ml for a 2 hour incubation, after which the slides were washed with cold PBS (4 x 15 minutes) and fixed with cold 5% TCA for 20 minutes. Slides were dipped briefly in absolute ethanol and air-dried. They were dipped in NTB-2 emulsion (Kodak, Rochester, NY) and air-dried, then sealed in a light-tight can with desiccant at 4°C for 48 hours. Autoradiographs were developed in Kodak D-19 developer, then they were fixed and stained with Giemsa stain. Four chambers were evaluated per treatment group.

Results are reported as the fraction of labeled cells per total cells counted and the number of silver grains per labeled cell.

Statistical Analysis: Data are presented as means of 5 (high density) or 3 (low density) separate experiments, each of which consists of 3 wells per experimental parameter. The standard error of difference (Steel and Torrie, 1980) for each data set is presented. Data were analyzed by a blocked ANOVA. Individual comparisons between treatments (MCTP and DMF vehicle) were made using Tukey's omega as the *post hoc* test ($p < 0.05$).

Results

The effects of MCTP on PEC number are presented in Figure 16. Although PECs plated at high density had formed a confluent monolayer by the time of treatment, cells treated with DMF vehicle or the low concentration of MCTP continued to divide and increase in number throughout the experimental period. This has been a consistent finding with PECs. The cells continue to divide in the confluent monolayer after release from arrest, so that the cell density increases with time. PECs treated with the higher concentration of MCTP were inhibited in their ability to proliferate and did not increase in number with time (Figure 16A). Similar results were obtained when PECs were treated at subconfluent densities (Figure 16B); however, cell numbers in control wells doubled in the high-density cultures but

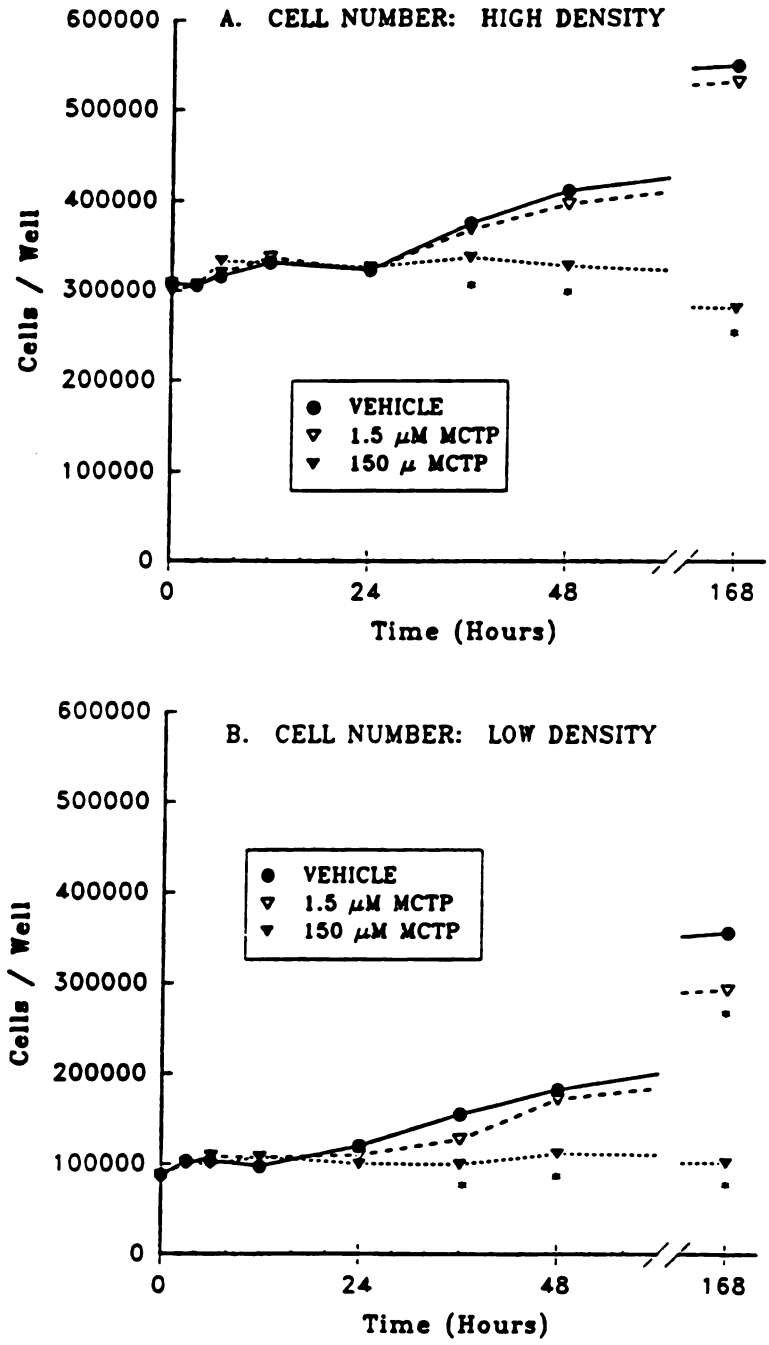


Figure 16: Effects of MCTP on endothelial cell number.

Monolayers of PECs at high (A) or low (B) density received a single administration of MCTP at time 0. Values represent means of replicate studies. (n=5, High Density; n=3 Low Density) [Standard error of difference = 15,900 (HD); 11,190 (LD)] * Significantly different from vehicle control ($p<0.05$)

increased between three- and four-fold in the low density cultures. Because of these changes in cell numbers, remaining data are presented on a "per cell" basis.

Changes in cellular protein reflect changes in cell size. In the high density cultures (Figure 17A), protein content per cell increased very modestly between 12 and 24 hours in cells treated with DMF or with 1.5 μM MCTP and then returned to a basal value of 0.3 ng/cell after 48 hours, presumably once cell division had taken place. Cells treated with the higher concentration of MCTP increased in protein content very slightly over time to 0.4 ng/cell at 7 days post-treatment. The response was similar but exaggerated in the cells plated at low density (Figure 17B): controls increased in number and reached confluence by one week post-treatment, whereas cells treated with the high concentration of MCTP failed to increase in number but instead enlarged and spread out to cover the surface of the culture dish and had twice the protein content of vehicle-treated cells.

DNA content of PECs treated with MCTP is shown is Figure 18. DNA content of cells treated at high density with vehicle or with the low concentration of MCTP remained nearly constant throughout the treatment period (Figure 18A). Cells treated with 150 μM MCTP had an increased DNA content by 7 days post-treatment. PECs treated at low density initially had a greater DNA content compared to cells treated at high density. In cells treated with vehicle or with the low concentration of MCTP, DNA content decreased initially then slowly increased over 24 hours, before ultimately declining to a basal level. PECs treated at low density with the high concentration of MCTP increased dramatically in DNA content late in the post-

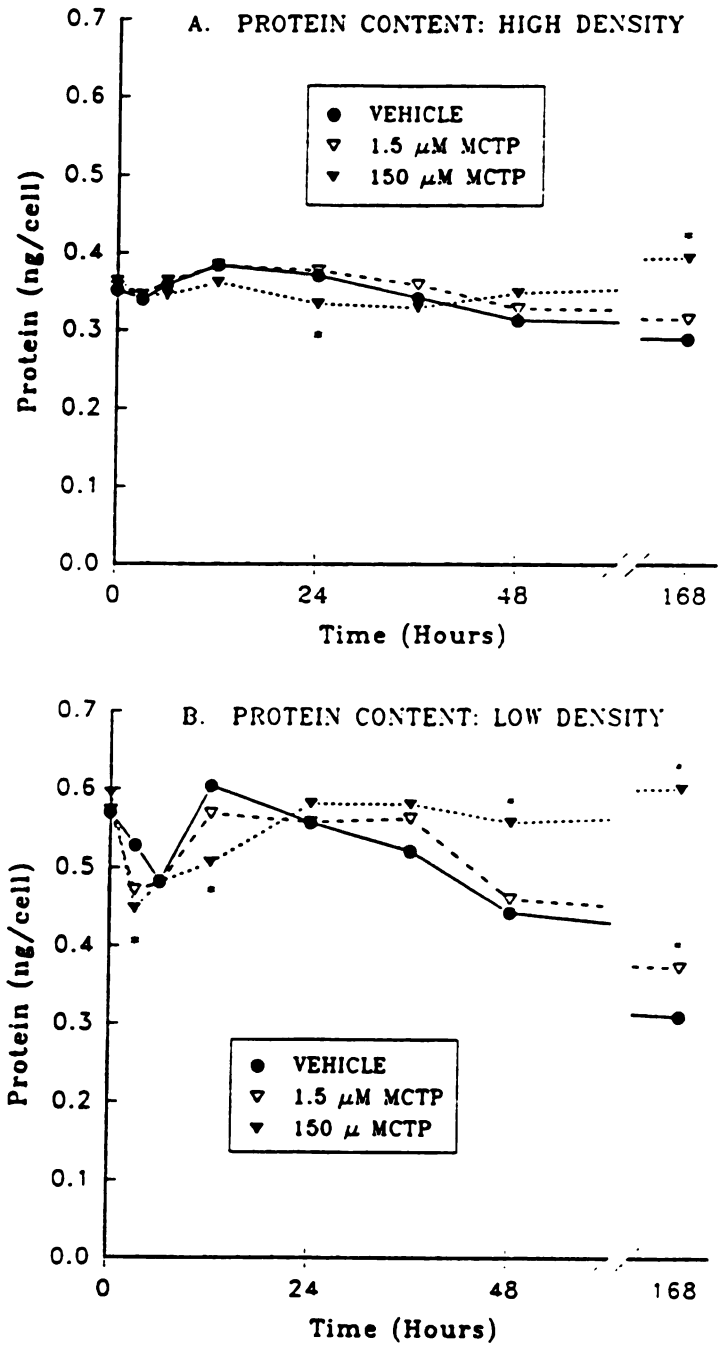


Figure 17: Effects of MCTP on cellular protein content.

PECs at high (A) and low (B) density were exposed to a single administration of MCTP at time 0. Values represent means of replicate studies. (n=5, High Density; n=3, Low Density) [Standard error of difference = 0.028 (HD); 0.053 (LD)]

* Significantly different from vehicle control ($p<0.05$)

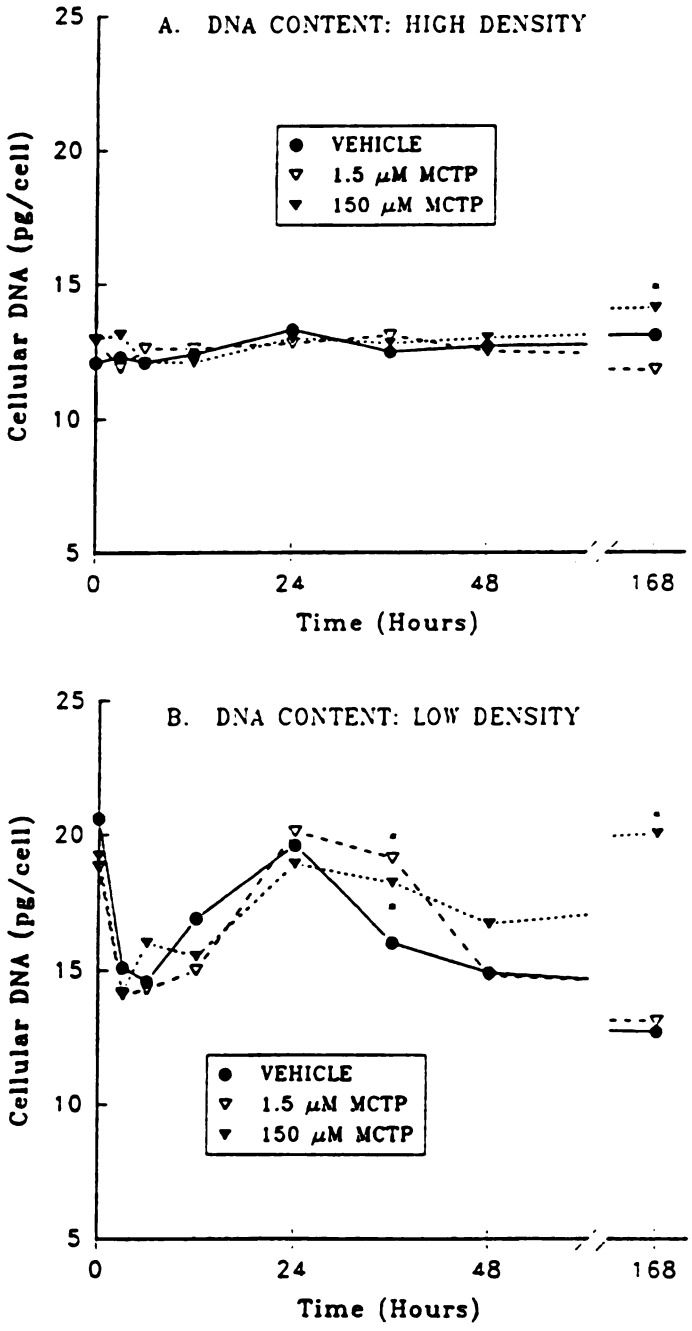


Figure 18: Cellular DNA content of MCTP-treated PECs.

PECs at high (A) or low (B) density were exposed to a single administration of MCTP at time 0. Values represent means of replicate studies.
(n=5, High Density; n=3, Low Density) [Standard error of difference = 0.75 (HD); 1.70 (LD)] * Significantly different from vehicle control ($p<0.05$)

treatment period, so that by 7 days these had a final DNA content nearly double that of controls.

DNA synthesis as determined by incorporation of [^{3}H]thymidine is presented in Figure 19. Incorporation of radiolabel into vehicle-treated cells peaked at 24 hours in both high (Figure 19A) and low density (Figure 19B) cultures, but cells treated at low density incorporated more than twice as much labeled thymidine as cells treated at high density. A similar response was seen in cells treated with the low concentration of MCTP. In contrast, incorporation was consistently less in cells treated with 150 μM MCTP than in controls through 48 hours. However, thymidine incorporation increased gradually in the high concentration group with time, and by 7 days post-treatment incorporation was equal to (high density) or much greater than (low density) incorporation by vehicle-treated cells. In the low-density group, cells treated with the low concentration of MCTP also incorporated significantly more thymidine than controls by day 7.

Autoradiography was performed to analyze DNA synthesis at the level of individual cells and to determine whether the changes seen in thymidine incorporation were due to changes in thymidine pool size, dilution or altered transport of labeled thymidine rather than changes in DNA synthesis (Wang *et al.*, 1989). Table 1 shows the results from these studies. In all treatment groups, cells treated at low density had a higher percentage of labeled nuclei than cells treated at high density. At 24 hours post-treatment, there was little difference between treated and control groups in the percent of cells labeled, but cells treated with 150 μM MCTP were labeled less intensely. At 48 hours post-treatment, the percent of

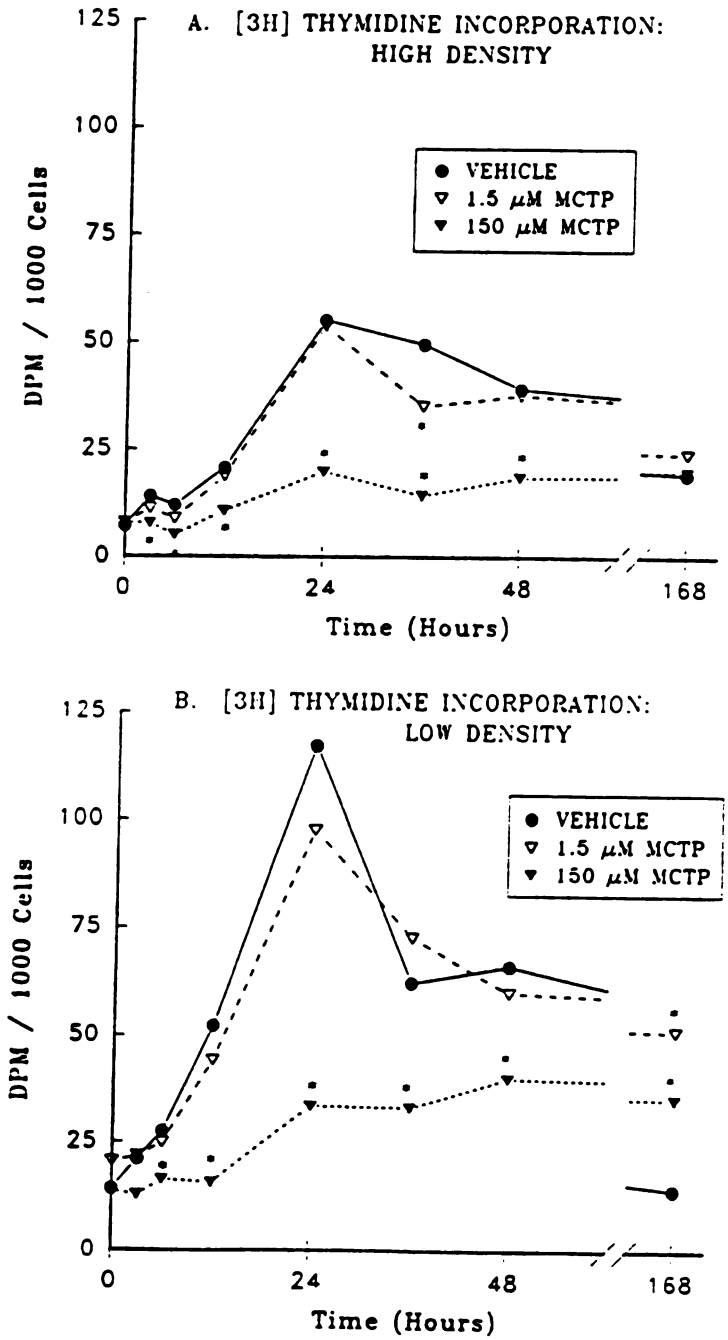


Figure 19: Effects of MCTP on incorporation of [^{3}H]thymidine.

PECs grown at high (A) or low (B) density were exposed to a single administration of MCTP at time 0. Values represent means of replicate studies. (n=5, High Density; n=3, Low Density) [Standard error of difference = 4.86 (HD); 7.00 (LD)]
* Significantly different from vehicle control ($p<0.05$)

TABLE 1: DNA SYNTHESIS BY PECs AS EVALUATED BY AUTORADIOGRAPHY

TIME	CELL DENSITY	TREATMENT	% OF CELLS WITH LABEL (a)	DISTRIBUTION OF GRAINS PER LABELED CELL			NUMBER OF CELLS COUNTED
				< 10	10 - 50	> 50	
24 Hours	High	Vehicle	2.02 +/- 0.64	10%	19%	72%	1031
		1.5 uM MCTP	3.42 +/- 0.55	19%	30%	51%	1065
		150 uM MCTP	1.92 +/- 0.18	60%	30%	10%	1038
	Low	Vehicle	15.74 +/- 0.50	10%	27%	63%	727
		1.5 uM MCTP	18.01 +/- 0.81	8%	20%	72%	637
		150 uM MCTP	22.32 +/- 0.89*	20%	69%	11%	892
48 Hours	High	Vehicle	2.41 +/- 0.30	4%	36%	60%	1031
		1.5 uM MCTP	2.57 +/- 0.39	26%	26%	48%	1030
		150 uM MCTP	4.88 +/- 0.74*	71%	29%	0%	628
	Low	Vehicle	7.51 +/- 0.72	9%	28%	62%	705
		1.5 uM MCTP	7.55 +/- 0.22	10%	31%	59%	647
		150 uM MCTP	17.50 +/- 0.84*	85%	15%	0%	269
7 Days	High	Vehicle	1.86 +/- 0.13	5%	10%	85%	1070
		1.5 uM MCTP	3.66 +/- 0.13	3%	32%	65%	1009
		150 uM MCTP	11.62 +/- 0.66*	88%	12%	0%	656
	Low	Vehicle	3.66 +/- 0.31	0%	29%	71%	1035
		1.5 uM MCTP	8.68 +/- 0.53*	21%	31%	48%	958
		150 uM MCTP	16.89 +/- 0.32*	69%	31%	0%	302

(a) Values represent means +/- SEM of 2 areas counted on each of 2 coverslips per treatment group.
* Significantly different from vehicle control ($p<0.05$).

labeled cells increased in PECs treated with the high concentration of MCTP and most of these cells were labeled much less densely. Although the percentage of labeled cells remained similar to controls in the group treated with 1.5 μM MCTP, these cells were labeled less intensely. By day 7, there was no difference in labeling between low and high density controls. However, cells treated with either concentration of MCTP showed a higher percentage of labeled cells, with a shift from intense incorporation of thymidine (>50 grains/cell) in fewer cells to a lesser incorporation (<10 grains/cell) in a larger population of cells.

Incorporation of radiolabeled leucine and uridine were measured as indicators of protein and RNA synthesis, respectively. In control cells, incorporation of each of these was higher in the low density groups (Figures 20 and 21). Although leucine incorporation was depressed shortly after treatment with 150 μM MCTP as compared to controls, measurable leucine incorporation was maintained. By 7 days post-treatment, leucine incorporation into cells treated with MCTP was significantly greater than that in vehicle-treated controls. The same was true for uridine incorporation: whereas incorporation per cell was significantly lower during the first 36 hours after treatment with high concentrations of MCTP, treated cells continued to synthesize RNA throughout the post-treatment period and by 7 days were incorporating more labeled uridine than controls. Incorporation was generally much greater in cells at low density, but, as control PECs reached confluence (ie., day 7), incorporation decreased to the levels seen in high-density cultures (Figures 20A & 21A).

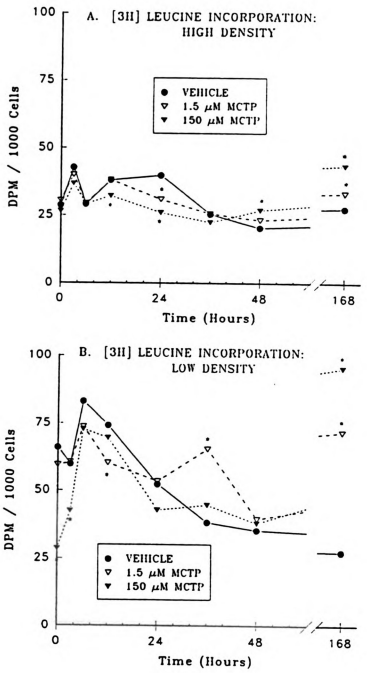


Figure 20: Effects of MCTP on incorporation of [^{3}H]leucine.

PECs grown at high (A) or low (B) density were exposed to a single administration of MCTP at time 0. Values represent means of replicate studies. (n=5, High Density; n=3, Low Density) [Standard error of difference = 4.7 (HD); 10.2 (LD)] * Significantly different from vehicle control ($p<0.05$)

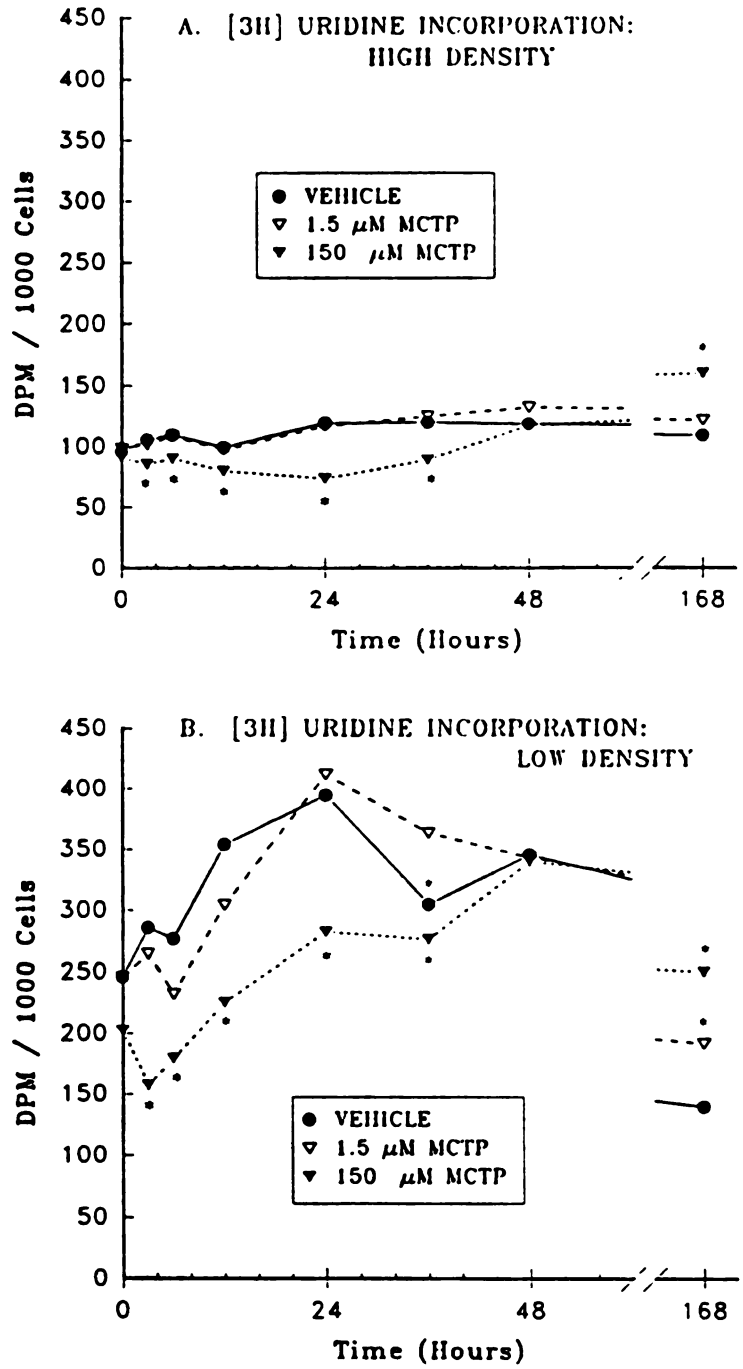


Figure 21: Effects of MCTP on incorporation of [^{3}H]uridine.

PECs grown at high (A) or low (B) density were exposed to a single administration of MCTP at time 0. Values represent means of replicate studies. (n=5, High Density; n=3, Low Density) [Standard error of difference = 13.1 (HD); 41.1 (LD)] * Significantly different from vehicle control ($p<0.05$)

Recognizing that larger cells may synthesize more protein as their cytoplasmic volume increases, protein synthesis was also normalized to protein content (data not shown). Although leucine incorporation into high density, MCTP-treated cells was no longer significantly increased at 7 days, in all other respects the pattern of leucine incorporation was similar whether normalized to protein content or cell number.

Discussion

This study was undertaken to understand how MCTP treatment of cultured porcine pulmonary artery endothelial cells affects DNA synthesis and normal cellular functions as represented by synthesis of RNA and protein. PECs treated with MCTP *in vitro* are unable to divide yet remain attached to the culture dish and continue to exclude trypan blue, suggesting that they remain viable in culture for long periods of time (Reindel *et al.*, 1991). Treated cells also increase in size and maintain an apparently intact monolayer throughout a 7 day treatment period. It has yet to be determined what functional changes occur in cultured endothelial cells after treatment with MCTP and how similar changes *in vivo* might contribute to the progression of lung vascular injury and the development of pulmonary hypertension which are characteristic of MCTP pneumotoxicity (Roth and Ganey, 1988; Roth *et al.*, 1989). Our data indicate that although cells treated with high concentrations of MCTP do not divide, they are not functionally quiescent but continue to synthesize DNA, RNA and protein.

MCTP is a bifunctional alkylating agent, capable of crosslinking DNA. DNA crosslinks have been demonstrated in livers of MCT-treated rats (Petry *et al.*, 1984) as well as in cultured PECs treated with MCTP (Wagner *et al.*, 1991). In PECs, the DNA crosslinking appears to be long-lasting and may be responsible for the arrest of cell proliferation. It is not yet clear whether or not cultured PECs retain the ability to repair these crosslinks, but our results suggest that DNA synthesis and/or repair continues at low levels in these cells after MCTP treatment (Figure 19; Table 1). Although PECs treated with 150 μM/ml MCTP incorporated significantly less labeled thymidine than controls for the first 48 hours after treatment, they nevertheless continued to incorporate thymidine gradually, and by 7 days post-treatment they were synthesizing DNA at levels equal to or greater than controls. This effect was greater in PECs treated at low density, suggesting a larger population of active cells. Results using incorporation of labeled thymidine into TCA-precipitable material were supported by autoradiographic studies: in all cases, altered incorporation of [^{3}H] thymidine reflected a sub-population of cells actively synthesizing DNA.

From previous work with synchronized PECs we know that, following release from arrest, DNA synthesis peaks between 18 and 24 hours, preceding the increase in cellular DNA content by a few hours. Cell division occurs between 36 and 48 hours. After this time, synchronization is lost to some extent and the pattern is much less distinct. As stated above, PECs in an apparently confluent monolayer undergo some increase in cell number after release from arrest. In this study, whereas a majority of cells at low density divided (note 100% increase in vehicle-treated cell

number from 24 to 48 hours, Figure 16B), a relatively low percentage of those at high density did so (vehicle-treated cell numbers increased only 25% during the same time interval, Figure 16A). This was also evidenced by the autoradiographic results in Table 1. While 2% of high density, vehicle-treated cells were labeled with thymidine during a 2 hour period, 16% of low density, vehicle treated cells were labeled. Incorporation of [^{3}H]thymidine per cell was also much lower in the high density controls than in low density controls (Figures 19A and 19B, 24 hours), supporting the suggestion of a smaller population of active cells.

Taken together, the results suggest that changes in thymidine incorporation seen in Figure 4 and Table 1 are consistent with changes in DNA synthesis. While changes in DNA content at 24 hours were subtle in the high density cells, it must be considered that relatively few cells were increasing their DNA content at this time, and these cells were not acting in perfect synchrony. Some cells may have increased their DNA content earlier and gone on to divide sooner (some increase in cell numbers was evident by 36 hours), whereas others may have done so somewhat later.

In cells treated with MCTP, incorporation of thymidine occurred at a much reduced rate per cell. Some of the incorporated thymidine may have been used in repair of damaged DNA. Little is known about the DNA repair mechanisms in this cell type in culture. There may be a basal level of repair which is comparable to that in the endothelium *in vivo,* or there may be an altered capacity for repair, perhaps as a result of increased DNA damage under culture conditions. The extent to which DNA repair was responsible for the increased thymidine incorporation in these cells is unknown, but since DNA crosslinking occurs with exposure to MCTP, repair of

damaged DNA is likely to represent at least part of the incorporation. However, some of the thymidine seems to have been utilized in the synthesis of new DNA despite the fact that cells treated with a high concentration of MCTP did not divide. This is supported by the gradually increasing DNA content of MCTP-treated PECs throughout the 7 day post-treatment period. By 7 days, PECs treated at low density with 150 μM MCTP had nearly twice the DNA content of controls.

Changes in DNA content were also less evident in cells at high density since the percentage of cells actively incorporating [^{3}H]thymidine was apparently very small initially (Table 1). At low density, a higher percentage of treated cells were incorporating labeled thymidine relative to controls, and this may explain why the DNA content increased in parallel in treated and control cells despite a lower rate of incorporation in those treated with 150 μM MCTP. But while control cells divided and reestablished their basal DNA content, the treated cells maintained or increased their DNA content over time. Low-density culture conditions provide a strong stimulus for cell proliferation and may have triggered cells to enter the DNA synthesis phase of the cell cycle, but since they were unable to divide some of the MCTP-treated cells may have remained in a tetraploid configuration.

We examined the synthesis of RNA and protein in MCTP-treated PECs to determine whether these basic cellular functions were maintained after treatment. Whereas synthesis of both RNA and protein was somewhat lower than controls for the first 48 hours after treatment with 150 μM MCTP, their formation did continue above a basal level. Indeed, by 7 days post treatment, MCTP-treated cells were synthesizing significantly more RNA and protein than controls. These responses

were much more pronounced in cells treated at low density. This may reflect a greater percentage of synthetically active cells and/or an increase in the synthetic activity of the general population of cells. Undoubtedly, cells treated at low density with 150 μM MCTP had an increased cellular protein content that reflects increased cell volume; but whereas some of the increased protein synthesis may be attributed to increased cytoplasmic volume, there is a disproportionate increase in the synthetic activity of these cells, suggesting that the cells are quite active. Of primary interest, however, is the fact that both RNA and protein synthesis are ongoing under all conditions, suggesting that MCTP-treated cells are not functionally quiescent.

Endothelial cells are metabolically complex and play a role in the maintenance of vascular integrity and homeostasis. Disruption of normal endothelial cell activity could result in a loss of the delicate balance these cells help to maintain. It has been reported recently that exposure to a variety of cellular stresses, including exposure to endotoxin (Chesney *et al.,* 1974a; Fox and DiCorleto, 1984; Albelda *et al.,* 1989), heat-shock (Kelley and Schlesinger, 1978; Thomas *et al.,* 1982; Williams *et al.,* 1989), hypoxia (King *et al.,* 1989; Kourembanas *et al.,* 1990; Ogawa *et al.,* 1990b) and acetylated low density lipoprotein (Fox and DiCorleto, 1986), may result in altered gene expression in target tissues such as the vascular endothelium. Changes in the amount or type of particular proteins produced by cells may be involved in the expression of injury seen under these conditions. It is possible that MCTP treatment causes a change in the production of proteins by endothelial cells in a similar manner. Many of the changes seen in the pulmonary vasculature after MCTP treatment *in vivo*, such as vascular remodeling (Bruner *et al.,* 1983; Reindel

et al., 1990), thrombosis of the pulmonary vessels (Bruner *et al.*, 1983; Hilliker *et al.*, 1982), accumulation of platelets (Hilliker *et al.*, 1982; White and Roth, 1988) and altered vascular reactivity (Hilliker and Roth, 1985a), could be initiated or exacerbated by changes in the profile of endothelial cell-derived proteins, including growth factors, chemotaxins, coagulation and fibrinolytic factors and vasoactive agents.

These results suggest that marked changes in PEC function occur with MCTP treatment. The increased RNA and protein synthesis observed may reflect overexpression of proteins normally synthesized by cultured PECs, but it is also possible that a somewhat different profile of proteins is expressed in MCTP-treated cells. Changes of this nature might play a role in the delayed and progressive development of MCTP pneumotoxicity.

Chapter IV

MONOCROTALINE PYRROLE TREATMENT DOES NOT ENHANCE THE RELEASE OF MESENCHYMAL CELL GROWTH FACTORS FROM CULTURED ENDOTHELIAL CELLS

Summary

Cultured porcine pulmonary artery endothelial cells (PECs) treated with MCTP are unable to divide but continue to synthesize RNA and protein, ultimately in greater amounts than controls. This increase in protein synthesis may reflect overexpression of normally synthesized proteins, but cell injury could also result in expression of a new profile of endothelial cell gene products. In certain models of vascular injury, endothelial cells demonstrate increased production of mesenchymal cell mitogens which may contribute to the vascular remodeling process. The purpose of this study was to determine whether PECs treated with MCTP also release factors that possess growth-stimulatory activity. MCTP-treated PEC monolayers were co-cultured with growth-arrested Swiss 3T3 fibroblasts in serum-free medium. In separate studies, conditioned, serum-free medium was collected from treated PECs and added to growth-arrested Swiss 3T3 cells. At various times posttreatment, proliferation of Swiss 3T3 cells was determined. Under coculture conditions, growth stimulation of 3T3 cells by untreated PECs was maximal. Medium conditioned by untreated PECs also stimulated 3T3 cell proliferation but to a lesser extent; MCTP treatment did not augment the release of mitogenic activity in this system. Cultured endothelial cells from other species constitutively produce and release high levels of platelet-derived growth factor (PDGF) and other mitogens, and PECs may do this as well. MCTP treatment does not appear to increase the release of growth factors above this basal level in cultured PECs; however, the response of the quiescent endothelium *in situ* to MCTP may be quite different.

Introduction

Pulmonary vascular alterations which occur after MCT(P) treatment *in vivo* include both endothelial cell changes and remodeling of the vessel walls. The vascular remodeling is characterized by extension of smooth muscle into normally nonmuscular arterioles and increased medial thickness in the small pulmonary arteries (Hislop and Reid, 1974; Kay and Heath, 1969; Lalich *et al.,* 1977; Merkow and Kleinerman, 1966b; Meyrick and Reid, 1979; Meyrick *et al.,* 1980; Reindel *et al.,* 1990). Similar lesions are evident in other forms of chronic pulmonary vascular injury, including hypoxia- and irradiation-induced pulmonary hypertension (Abraham *et al.,* 1971; Meyrick and Reid, 1978; Perkett *et al.,* 1986; Reid, 1979). Vascular remodeling may precede the development of pulmonary hypertension or can occur secondarily in response to increased pulmonary vascular pressure; regardless, once established, it contributes to the maintenance of the hypertensive state by decreasing the compliance and reducing the cross-sectional area of the pulmonary vasculature (Reid, 1979; Meyrick and Reid, 1979).

The endothelium exerts a regulatory influence on the growth and proliferation of other vascular cells, and changes in EC function with respect to this capacity may contribute to the vascular remodeling process (Davies, 1986; Reid, 1979; Schwartz and Ross, 1984). ECs modulate smooth muscle cell (SMC) proliferation through composition of the extracellular matrix, direct communication via intercellular junctions, and the release of humoral growth stimulatory and inhibitory factors (Davies, 1986; Carey, 1991; Castellot *et al.,* 1981). Increased synthesis and/or release

of endothelium-derived growth factors (EDGFs) have been demonstrated *in vitro* under conditions which cause EC injury, including exposure to chronic hypoxia, irradiation, endotoxin and mechanical wounding (Vender *et al.,* 1987; Witte *et al.,* 1989; Albelda *et al.,* 1989; Libby *et al.,* 1986; McNeil *et al.,* 1989), and which are also associated with EC alterations and vascular remodeling *in vivo* (Meyrick and Reid, 1978; Perkett *et al.,* 1986; Meyrick and Brigham, 1986; Reidy, 1990; Powell *et al.,* 1990). Similar release of EDGFs has not been established *in situ* but, if present, could help to define a role for the endothelium in the development of chronic pulmonary vascular disease.

Cultured PECs treated with MCTP, a putative pneumotoxic metabolite of MCT, demonstrate limited cytolysis but are unable to divide (Reindel *et al.,* 1991). Treated cells become spindle-shaped in appearance or enlarge if monolayer integrity is disturbed, but they remain viable in culture for long periods of time (Reindel *et al.,* 1991; Hoorn *et al.,* 1990). Basic cellular function, as illustrated by the ability to synthesize RNA and protein, is maintained in treated PECs; indeed, by 7 days after MCTP treatment, cells demonstrate higher synthetic activity than vehicle-treated controls (Hoorn and Roth, 1992). The profile of proteins synthesized by MCTP-treated cells is not known. They may continue to synthesize the normal spectrum of EC proteins but in larger amounts; alternatively, they might express a new set of gene products in response to injury, including EDGFs. These studies were conducted to determine if MCTP-treated ECs release increased amounts of mesenchymal cell growth factors.

Materials and Methods

Cell Culture: Primary isolation and subculture of PECs was performed as described previously in Chapter II. PECs were maintained in Opti-MEM supplemented with 5% FCS and 1% Ab/Am under standard conditions of 37°C and 7.5% CO_2 and 92.5% air. Swiss 3T3 fibroblasts (CCL 92; American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% FCS and 1% Ab/Am under similar conditions. Medium was changed three times per week, unless otherwise indicated.

Preparation of MCTP: MCTP was prepared from MCT as described previously in Chapter II. MCTP was dissolved in DMF at a concentration of 20 mg/ml. All dilutions were performed in DMF, and equal volumes of DMF served as the vehicle control in all studies. Untreated cells and cells stimulated with 2-5 units/ml bovine thrombin were also utilized as indicated.

Experimental Conditions:

a.) *Coculture Experiments:* PECs were plated on 22 mm polycarbonate or collagen culture plate inserts (Costar). When the PECs reached confluence (48-72 hr), 1.5 ml of fresh medium was added to each insert and the monolayers received a single administration of MCTP or DMF vehicle (5 μl vol). Meanwhile, 3T3 cells were plated at 25,000 cells per 35 mm well. 24 hours later, the medium on the 3T3 cells was replaced with DMEM containing 0.25% bovine serum albumin (BSA) and

1% Ab/Am [serum-free medium; SFM] for 72 hours to growth-arrest the cells. At the end of this time, the PEC-containing inserts (72 hours posttreatment with MCTP) were rinsed twice with CMF-HBSS and added to the growth-arrested 3T3 cells. Fresh SFM was added to both chambers, and the cocultures were maintained for 72 hours. A second set of growth-arrested 3T3 cells was prepared for coculture with PECs 8 days posttreatment with MCTP, and these were also maintained for 72 hours.

In addition, several wells of growth-arrested 3T3 cells were cocultured with untreated PEC monolayers or monolayers that had been stimulated with thrombin 24 hours previously. These were handled in the same way as those cocultured with MCTP or vehicle. Additional wells of 3T3 cells were incubated with cell-free inserts in the presence either of SFM or of DMEM containing 10% FCS to define minimum and maximum proliferation for the system, respectively.

b.) *Conditioned Medium Experiments:* PECs were plated in T-75 tissue culture flasks (Costar) and were incubated under standard conditions for 7-10 days prior to treatment to establish quiescent monolayers. At this time, monolayers were rinsed twice with CMF-HBSS and 15 ml fresh Opti-MEM was added. PECs received a single administration of MCTP or DMF vehicle (37.5 μl) to achieve the nominal concentrations utilized in these studies. At 48 hours posttreatment, the monolayers were rinsed twice with CMF-HBSS, and 15 ml of SFM was added to each flask. The PEC-conditioned medium (CM) was collected after 72 hours (5 days posttreatment with MCTP), and 15 ml of fresh SFM was again added to each flask; this was collected 48 hours later (7 days posttreatment with MCTP).

The CM was spun in a centrifuge at 800 x g for 10 minutes, and a portion was diluted 1:1 with fresh SFM. 3-ml aliquots of 100% or diluted CM were added to wells containing growth-arrested 3T3 cells (prepared as above), and the plates were incubated under standard conditions for 72 hours.

As described above, CM from untreated PECs or PECs stimulated with thrombin for 24 hr was also used on separate wells of growth-arrested 3T3 cells. Additional wells maintained with unconditioned SFM or with DMEM containing 10% FCS were used to determine minimum and maximum proliferation for the system, respectively.

Enumeration of PECs: At the end of the coculture period, or subsequent to the collection of CM from the PEC monolayers, PECs were removed from the inserts or flasks and counted as described previously in Chapter II.

Quantitation of 3T3 Cell Proliferation:

a.) *Enumeration of 3T3 cells:* At the end of the 72 hour period of coculture or incubation with CM, adherent 3T3 cells were removed from the wells and counted as described previously in Chapter II.

b.) *Incorporation of [^{3}H]thymidine:* In some studies, cell proliferation was also quantified by the incorporation of radiolabeled thymidine over the 8-24 hr and 24-48 hr intervals of the coculture period or during the 18-36 hr interval after the addition of CM. [5-methyl-3H] thymidine (40-60 Ci/mmol; Amersham) was added to the culture medium at a concentration of 1 μCi/ml for the period indicated. After

incubation, incorporation of thymidine into TCA-precipitable material was quantified as described in Chapter III.

Statistical Analysis: Data are presented as means +/- SEM of 3 separate experiments, each consisting of 3 wells per experimental parameter (CM studies: 2 T-75 flasks). Percentage data were transformed by the $\arcsin^{-1/2}$ transformation before statistical analysis. Data were analyzed using a random ANOVA, and individual comparisons were made using Tukey's omega as the *post hoc* test. The criterion for significance was $P < 0.05$.

Results

Cellularity of MCTP-treated PEC monolayers on inserts or in flasks followed a pattern typical for this cell type. Vehicle-treated monolayers and monolayers treated with a low concentration of MCTP contained similar numbers of cells to untreated PEC monolayers, whereas those treated with higher concentrations of MCTP contained fewer cells. This effect was no more pronounced at 10 days posttreatment than at 5 days. [Figures 22 & 23]

The effects of coculture with MCTP-treated PECs on the proliferation of growth-arrested 3T3 cells is shown in Figure 24. 3T3 cell number is presented as a percent of the response achieved by medium containing 10% FCS (i.e., maximum

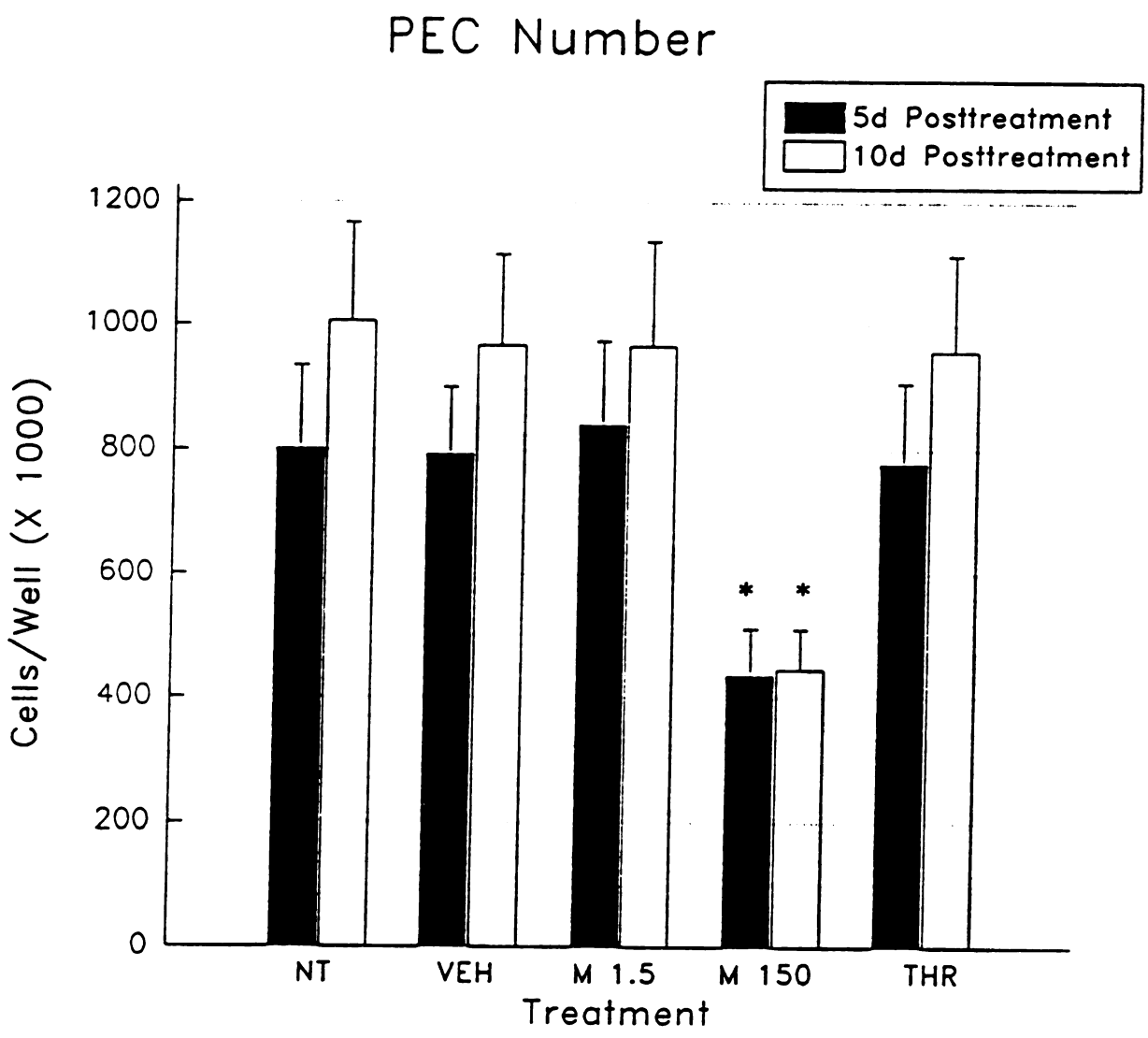


Figure 22: Effects of MCTP on cellularity of monolayers grown on inserts.

Confluent monolayers of PECs grown on culture plate inserts were treated with MCTP at time 0. At the times indicated posttreatment, cells were removed from the inserts and counted. Values represent means +/- SEM of 3 replicate experiments, each treatment group consisting of 3 individual wells. * Significantly different from vehicle control ($p<0.05$).

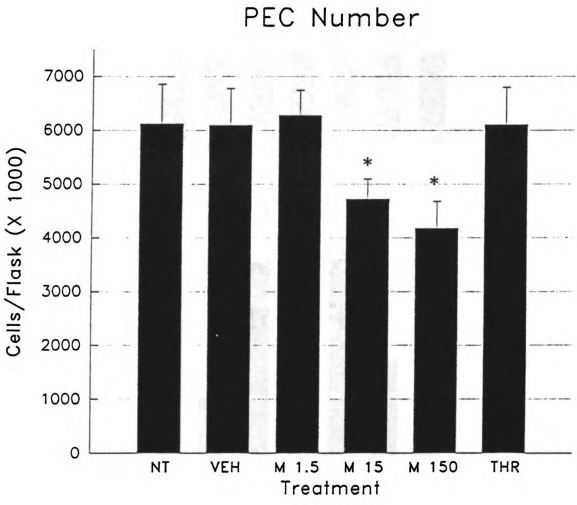


Figure 23: Effects of MCTP on cellularity of monolayers grown in flasks.

Post-confluent monolayers of PECs received a single administration of MCTP at time 0. After collection of conditioned medium at 7 days posttreatment, cells were removed from the flasks and counted. Values represent means +/- SEM of 4 replicate studies, each treatment group consisting of 2 flasks. * Significantly different from vehicle control ($p < 0.05$).

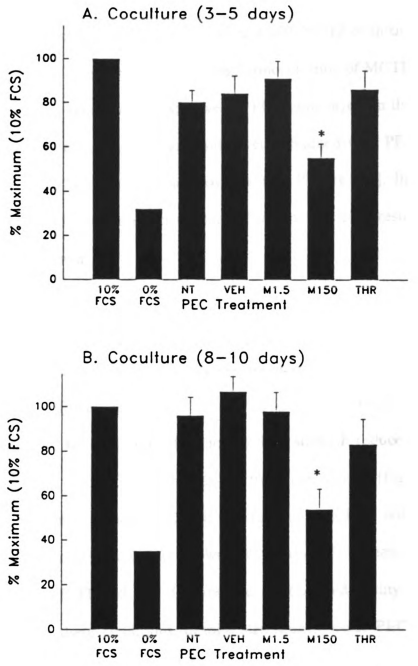


Figure 24: Effects of co-culture with MCTP-treated PECs on Swiss 3T3 cell number.

Growth-arrested 3T3 cells were co-cultured with MCTP-treated PECs during the indicated intervals posttreatment. Values represent means +/- SEM of 3 replicate experiments, each treatment group consisting of 3 wells. Results are expressed as a percent of the proliferation stimulated by exposure to 10% FCS. * Significantly different from vehicle control ($p<0.05$)

response). Untreated PECs stimulated Swiss 3T3 cell proliferation at a level 80-100% of that stimulated by 10% FCS, and treatment with MCTP or thrombin did not augment this response. Cells treated with a high concentration of MCTP stimulated less proliferation than vehicle controls; however, PEC monolayers in this treatment group were also less cellular. Similar results were obtained with PECs 3-5 days posttreatment [Figure 24A] and 8-10 days posttreatment [Figure 24B]. Incorporation of [^{3}H]thymidine paralleled these increases in 3T3 cell number, but results were less uniform [data not shown].

The effects of CM from MCTP-treated monolayers on 3T3 cell number are shown in Figure 25. CM from untreated PECs stimulated proliferation of growth-arrested 3T3 cells at approximately 80% of maximum, and CM from MCTP-treated cells at 2-5 days [Figure 25A] or 5-7 days [Figure 25B] posttreatment did not significantly increase this response. Dilution to 50% strength reduced the growth stimulation capacity of the CM slightly in all treatment groups [Figure 25], and dilution to 25% strength reduced 3T3 cell proliferation to a level not significantly different from that in SFM [data not shown]. CM from PECs treated with higher concentrations of MCTP did not demonstrate a decreased ability to stimulate proliferation under these conditions, despite significantly lower PEC numbers in these treatment groups. Again, results of [^{3}H]thymidine incorporation reflected comparable effects on cell proliferation [data not shown].

CM from thrombin-stimulated PECs did increase 3T3 cell proliferation under these conditions, but this response was quite variable among experiments. 50%

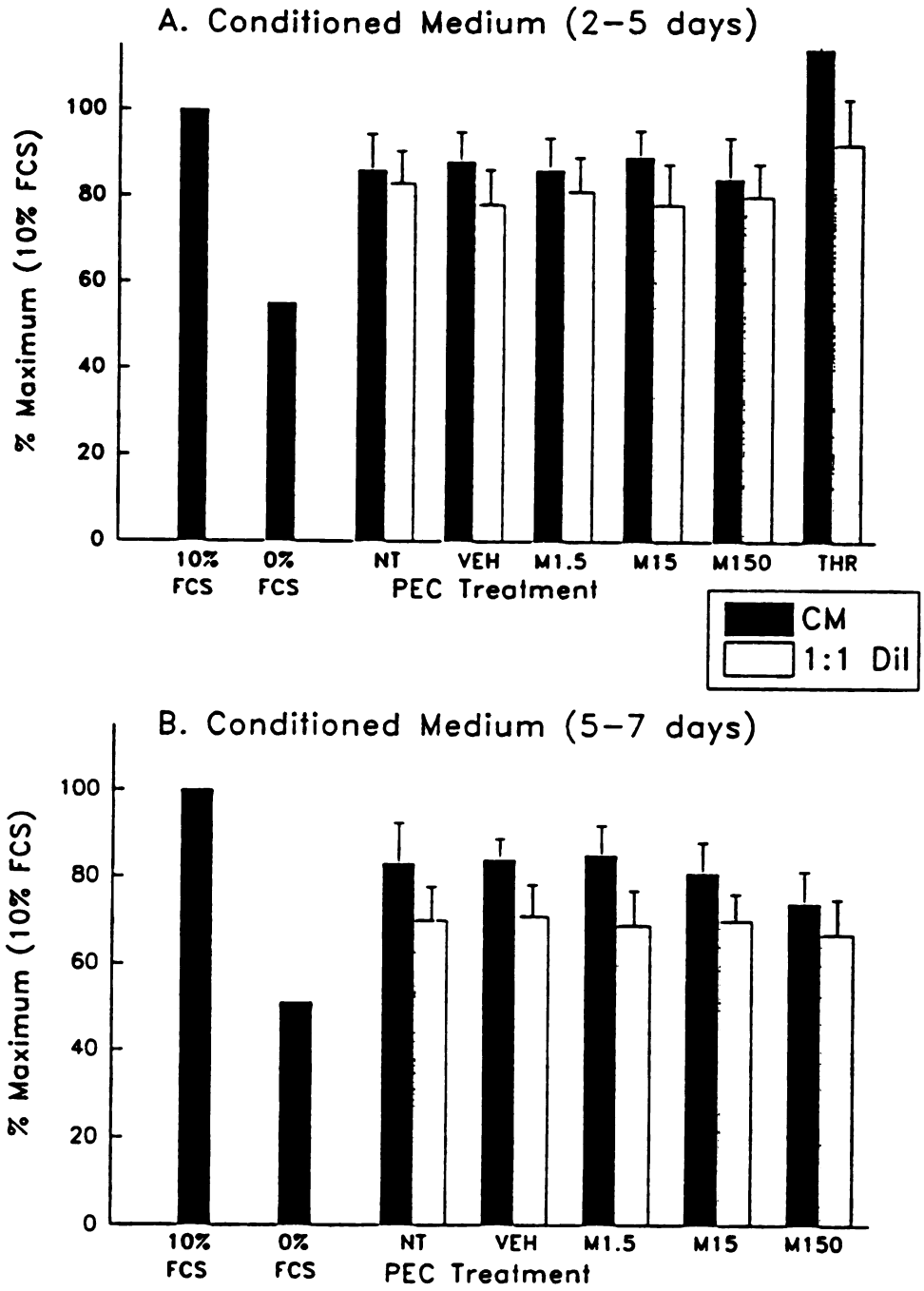


Figure 25: Effects of medium conditioned by MCTP-treated PECs on Swiss 3T3 cell number.

Growth-arrested 3T3 cells were incubated for 72 hr with conditioned medium collected during the indicated intervals posttreatment. Medium was applied at full strength or diluted 1:1 with fresh, serum-free medium as indicated. Values represent means +/- SEM of four replicate experiments, each treatment group consisting of 3 individual wells. No significant difference from controls.

dilution of this CM decreased its ability to stimulate 3T3 cell proliferation to a greater extent than dilution of CM from untreated or MCTP-treated cells.

Discussion

The endothelium plays an important role in the regulation of SMC growth and proliferation in the vessel wall, and several models of lung injury which are characterized by structural remodeling of the vasculature also demonstrate evidence of relatively early EC alterations (Meyrick and Reid, 1978; Perkett *et al.*, 1986; Crapo *et al.*, 1980). Cultured ECs from a variety of species synthesize and release factors which are mitogenic and chemotactic for SMCs and other mesenchymal cells in response to stimulation with thrombin or after toxic or physical injury (Harlan *et al.*, 1986; Schini *et al.*, 1989; Hsieh *et al.*, 1991; Vender *et al.*, 1987; Witte *et al.*, 1989). MCTP-treated PECs remain active synthetically, and it is not unreasonable to suggest that they may produce altered amounts of normally expressed proteins or, alternatively, that they may express a new complement of proteins in response to exposure to MCTP. Therefore, it is plausible that similar alterations in EDGF production and/or release could occur in MCTP-treated PECs, and the studies presented here were designed to determine the effects of MCTP treatment on the release of soluble mediators from PECs that influence mesenchymal cell proliferation.

A number of preliminary experiments were performed in order to optimize the experimental conditions and allow appropriate controls to be incorporated into the design. The use of a transformed cell line of 3T3 fibroblasts eliminated the difficulties of maintaining two primary cell lines (i.e., PECs and SMCs) at optimal levels simultaneously. 3T3 cells are frequently used to bioassay for growth factor production and respond with cell division to FCS and SMC mitogens like PDGF, bFGF and ET (Vogel *et al.*, 1978; Wang *et al.*, 1989; Csonka and Jellinek, 1986; Wang *et al.*, 1981). Preliminary work with the growth-arrested 3T3 cells used in these studies demonstrated that they were able to respond appropriately to these factors (at concentrations of 1-10 ng/ml) in our hands as well [data not shown]. In addition, serum-free conditions were established that could sustain PECs and permit a "normal" response to MCTP yet would not mask the production of growth factors by these cells. Despite these and other precautions, the results of the studies did not provide evidence that MCTP altered release of growth factor(s) by PECs.

The 3T3 cell bioassay system used in these studies is a very sensitive one and is frequently used to detect the production or release of low levels of SMC mitogens; concentrations of growth factor which are undetectable by Western blotting or radioimmunoassay can stimulate cell proliferation under these conditions (Vogel *et al.*, 1978; Wang *et al.*, 1989). However, bioassay is of limited usefulness in the presence of high levels of growth factor because once 100% proliferation has been achieved there is no way to detect further increases in growth factor concentration.

Under coculture conditions, stimulation of 3T3 cell proliferation by untreated PECs was maximal: virtually 100% of 3T3 cells divided. Thus, MCTP treatment and

thrombin stimulation could not augment this response. PECs treated with 150 μM MCTP stimulated less proliferation of 3T3 cells, but there were also 40-50% fewer cells on the inserts in this treatment group. Apparently, cellular production of mitogenic activity is maintained after MCTP treatment and is not increased by treatment with high concentrations of MCTP; however, enhanced release of mitogenic activity in other treatment groups cannot be detected in this system.

The effects of basement membrane composition can influence EC shape and behavior and may affect the release of mitogens (Huber and Weiss, 1989; Madri and Pratt, 1986; Gospodarowicz and Lui, 1981; Gospodarowicz and Ill, 1980). In an effort to decrease basal EDGF production, both collagen and polycarbonate culture plate inserts were utilized, but this variable had minimal impact on the outcome of the studies. PEC monolayers were also maintained for longer periods of time in culture to establish a more quiescent monolayer prior to treatment, but this interval was limited by the tendency for older monolayers to detach from the insert during the repeated manipulations required for feeding, treatment, etc.

Because of the limitations of the coculture system, studies with PEC CM were initiated. Monolayers grown in flasks could be maintained in culture for longer periods of time, and the conditioned medium generated in this manner could be diluted as necessary to modify the 3T3 cell proliferative response. CM from untreated cells varied in its ability to stimulate 3T3 cell proliferation but generally elicited a response 70-80% of maximum. Dilution of the CM resulted in a further decrease in this proliferative response. Maintenance of the monolayers for 21 days in collagen-coated flasks prior to treatment reduced the mitogenic potential of the

CM to 50-60% of maximum, but still did not result in completely quiescent monolayers. However, CM from thrombin-stimulated PECs was able to increase proliferation to a variable extent, suggesting that the bioassay system was able to detect an augmentation in the release of mitogenic activity under these conditions.

CM from MCTP-treated PECs did not increase or decrease the proliferation of 3T3 cells as compared to CM from untreated or vehicle-treated controls, and dilution decreased the growth-promoting activity of the CM to a similar extent in all treatment groups. These results suggest that MCTP does not alter the production of mesenchymal cell growth factors by cultured PECs. Decreases in 3T3 cell proliferation which were seen in the coculture system when PECs were treated with high concentrations of MCTP are not evident in this system despite similar decreases in monolayer cellularity. It is possible that the cells in the flasks treated with 150 μM MCTP generate more mitogenic activity per cell than those in the other treatment groups, but this would be of questionable significance in a vessel as the release per unit area is not apparently different from unstimulated cells.

It is possible that the time intervals examined in these studies were inappropriate. Using information from previous studies, intervals for coculture and collection of CM were selected to bracket the onset of increased RNA and protein synthesis in MCTP-treated PECs (Chapter III). It is possible that a spike of mitogen release would be missed using this experimental protocol; however, a transient release of growth factor would be of questionable significance in the development of vascular remodeling in this model.

There are certain difficulties inherent in a cultured cell system, particularly in dealing with the regulation of proliferation. Although the endothelium *in situ* is relatively quiescent in terms of growth factor production (Shepherd and Katusic, 1991), the act of taking ECs out of their normal environment and growing them in culture changes them in such a way that their unstimulated production of mitogens (predominantly PDGF) is increased greatly (Vlodavsky *et al.*, 1987; DiCorleto and Bowen-Pope, 1983; Barrett *et al.*, 1984). Growth under low-serum conditions appears to enhance this effect in human ECs. Despite this high level of basal activity, EC injury *in vitro* has been associated with increased release of EDGFs (Madri *et al.*, 1991; Vender *et al.*, 1987; Albelda *et al.*, 1989). Thus, our results would appear to indicate that increased production and/or release of mitogens is not a feature of MCTP-induced EC injury.

Vascular remodeling is a characteristic feature of MCT(P)-induced pneumotoxicity. Whether this is due to hyperplasia, hypertrophy and/or increased chemotaxis of SMCs has not been definitively established in this model, but a change in the regulation of SMC growth and behavior is evident (Meyrick and Reid, 1982; Orlinska *et al.*, 1988; Olson *et al.*, 1985). The elaboration of EDGFs is only one way in which ECs may influence activity of SMCs. Changes in intercellular communication between ECs and SMCs or in the composition of the extracellular matrix can affect the growth and phenotype of SMCs (Sheridan and Atkinson, 1985; Herman, 1990; Carey, 1991; Davies, 1986). SMC chemotactic factors and mitogens may be derived from platelets, which accumulate in the lung during MCTP-induced pneumotoxicity, perhaps as a result of EC alterations (Ross *et al.*, 1986; Assoian and

Sporn, 1986; Oka and Orth, 1983; White and Roth, 1988). Alternatively, there may be decreased production of growth-inhibitory substances (i.e., heparan sulfate) by ECs (Castellot *et al.*, 1981). These alterations would not be evident in the systems utilized to evaluate cell proliferation in this study.

Despite our findings, the contribution to vascular remodeling of EDGFs produced by pulmonary endothelium exposed to MCT(P) *in vivo* should not be ruled out. Although a valuable tool in many ways, the cultured cell system is a limited one for studies of this nature. Cultured ECs exist in a perpetually activated state with respect to growth factor production. Quiescent endothelium *in vivo* may respond to MCT(P)-induced injury (and other forms of injury) quite differently in this respect. Little work has been done *in vivo* on the role of endothelium-derived growth factors in this and other models of vascular injury, perhaps because the technology has not been available until recently. Preliminary studies with PDGF-neutralizing antibody suggest that this mediator is not involved in MCTP pneumotoxicity (Ganey *et al.*, 1988); however, the potential for paracrine modulation within the microenvironment of the vessel wall cannot be ruled out by those studies. *In situ* hybridization or immunohistochemical techniques with cDNA probes and/or antibodies for the mediators of interest (as they become available) may be able to determine more conclusively whether changes in growth factor production by the endothelium play a part in this model of lung vascular injury.

Chapter V

EFFECTS OF MONOCROTALINE PYRROLE ON CULTURED RAT PULMONARY ENDOTHELIUM

Summary

Monocrotaline pyrrole (MCTP) causes direct toxicity to cultured bovine and porcine pulmonary artery endothelial cells (BECs and PECs, respectively), but there exist species differences both in whole-animal response to the parent alkaloid and in cellular response to direct application of MCTP. In this study, the changes in cultured rat pulmonary vascular endothelial cells (RECs) after a single administration of MCTP were characterized in order to compare these with changes previously identified in this species *in vivo*. MCTP caused a delayed and progressive release of lactate dehydrogenase from REC monolayers which consisted primarily of isozymes 4 and 5. Progressive cell detachment was evident and remaining cells became enlarged, with morphologic changes comparable to those reported previously in BECs, including cytoplasmic vacuolization and nuclear enlargement. MCTP also caused an inhibition of cell proliferation at concentrations of 0.15 μM MCTP or greater, and DNA crosslinking was evident at 24 and 48 hours posttreatment. These results suggest that MCTP is directly toxic to cultured RECs, and the development of changes is reminiscent of that seen in the rat *in vivo*. The cytostatic nature of the compound, in combination with its cytolytic effect on RECs, could contribute to the development of pulmonary edema and other lung vascular changes seen in rats treated with MCT or MCTP.

Introduction

MCTP, a putative toxic metabolite of MCT, is unstable in aqueous solution (Bruner *et al.*, 1986), and it is likely that rapid binding of this electrophile to cellular constituents of the pulmonary vasculature is the initiating event in the pneumotoxicity seen with this compound. DNA crosslinking has been reported as a relatively early phenomenon following MCT treatment in vivo (Petry *et al.*, 1984), as well as in cells treated with MCTP in vitro (Wagner *et al.*, 1991; Wagner *et al.*, 1992). Whereas the pattern and progression of the pulmonary injury caused by MCTP *in vivo* have been well characterized in the rat, the link between binding of this short-lived electrophile and the delayed vascular changes has not been clearly identified.

The endothelium has been suggested as a target for binding of MCTP in the lung, and changes in pulmonary vascular endothelial cell structure and function have been reported as relatively early events in MCT and MCTP pneumotoxicity (Reindel *et al.*, 1990; Bruner *et al.*, 1983; Hilliker *et al.*, 1982; Molteni *et al.*, 1984; Rosenberg and Rabinovitch, 1988). We have shown that a single administration of MCTP to cultured bovine and porcine pulmonary artery endothelial cells (BECs and PECs, respectively) causes delayed and progressive injury which is reminiscent of that which occurs *in vivo* (Reindel and Roth, 1991; Reindel *et al.*, 1991). However, the response of endothelial cells of these two species to MCTP is quite different. Both BECs and PECs are sensitive to the antiproliferative effects of MCTP, and both respond with a release of prostacyclin (Reindel *et al.*, 1991). However, whereas BECs are sensitive to the cytolytic and hypertrophic effects of MCTP, PECs are relatively insensitive,

showing minimal cell detachment and release of LDH and more subtle morphologic changes.

The manifestation of toxicity of the parent alkaloid, MCT, in cattle and pigs is also quite different *in vivo*. In cattle, MCT causes a hepatic venoocclusive disease and pneumotoxicity is not evident (Sanders *et al.*, 1936; Sippel, 1964). Clinical reports indicate that ingestion of MCT by pigs results in delayed and progressive lung injury which is similar to that described in the rat. These differences may be due in part to differences in metabolism or in distribution of the toxic metabolite(s) (Shull *et al.*, 1976; Cheeke and Pierson-Goeger, 1983). However, differences in sensitivity of endothelial cells to reactive pyrroles might also influence the organ pathology observed in animals intoxicated with monocrotaline or other pyrrolizidine alkaloids (Reindel *et al.*, 1991).

Because most attention has centered on the rat in the MCT model of pulmonary vascular injury and in light of the species differences in response to MCT and MCTP both *in vivo* and *in vitro*, this study was conducted to determine how cultured rat endothelial cells respond to direct administration of MCTP.

Materials and Methods

Preparation of endothelial cells: Rat pulmonary vascular endothelial cells were generously provided by Dr. James Varani (Dept. of Pathology, University of Michigan, Ann Arbor, Michigan). These cells were isolated by the method of Ryan

and White (1986) and demonstrate characteristic cobblestone morphology. RECs were further characterized as described in Chapter II.

RECs were maintained in DMEM (GIBCO) containing 10% FCS and 1% Ab/Am and were incubated under standard conditions of 37°C and 6% CO_2/94% air. Cells were passed at a ratio of 1:10 to 1:20 following enzymic (0.025% trypsin-0.27 mM EDTA) or mechanical dissociation. Cells used for these studies were between passages 6 and 10. Unless otherwise noted, culture medium was replaced with fresh DMEM containing 10% FCS and 1% Ab/Am every other day.

Preparation of MCTP: MCTP was prepared from MCT as described previously in Chapter II. MCTP was dissolved in DMF at a concentration of 20 μg MCTP/ml, and all dilutions of MCTP were made with DMF. A 2.5 μl volume of MCTP solutions or DMF vehicle (0 μM MCTP) per milliliter of medium was used in all studies to achieve the nominal concentrations of MCTP (0.015-150 μM) used in these studies.

Effects of MCTP on monolayer cellularity and morphology: After exposure of confluent REC monolayers in 12-well tissue culture clusters to a single administration of MCTP on day 0, cells remaining in the monolayers were photographed and counted (as described previously in Chapter II) on days 1-5 posttreatment.

Cellular release of lactate dehydrogenase activity: RECs were plated into 12-well tissue culture clusters and allowed to proliferate until monolayers reached confluence. At this time (day 0), fresh medium was added and monolayers were exposed to a single administration of 0, 1.5, 15 or 150 μM MCTP in the culture medium. On each of the subsequent 5 days, one 12-well plate of cells was used for determination of LDH activity by the method of Bergmeyer and Bernt (1974). Medium from above monolayers was removed for LDH analysis, and monolayers were washed twice with CMF-HBSS. Two milliliters of fresh CMF-HBSS were added, and cells were lysed with 15 μl of a 10% solution of Triton X-100 (Sigma).

Percent LDH release was determined by the following formula:

$$\% \text{ release} = \frac{\text{LDH in medium above monolayer}}{\text{LDH from lysed cells} + \text{LDH in medium above monolayer}} \times 100$$

Determination of LDH isozymes: Aliquots of culture medium and cell lysate collected each day for analysis of total LDH release as described above were also submitted for LDH isozyme analysis. Prior to electrophoresis, samples were diluted with phosphate-buffered saline to a total activity of 400-600 IU/L. Isozymes were separated in agarose gels (Paragon Lactate Dehydrogenase Isoenzyme Electrophoresis Kit; Beckman Instruments, Inc., Fullerton, CA). A commercially prepared control serum (ID Zone Abnormal LDH Control; Beckman Instruments, Inc.) containing all five isozymes was run with each gel. Gels were dried and

scanned on a densitometer (Appraise™ Densitometer, Beckman Instruments, Inc.). The LDH isozyme profile of a blank (DMEM containing 10% FCS, which was not exposed to cells) was also evaluated, and these values were subtracted from the values for cell-conditioned medium to arrive at the LDH isozyme profile of LDH released from RECs.

Colony-forming efficiency: RECs were plated at low density (500 cells per plate) in 100-mm tissue culture plates. Cells were incubated for 3-4 hours to allow for cell attachment and then were exposed to a single administration of 0, 0.015, 0.15, 1.5, 15 or 150 μM MCTP. Fresh medium (without MCTP) was added on day 7 posttreatment; on day 14, cell colonies on plates were fixed with 10% neutral buffered formalin and stained with crystal violet (0.5%). Colonies of >50 cells were counted. Results are expressed as a percentage of the number of colonies in plates exposed to vehicle.

DNA Crosslinking: Two days prior to MCTP treatment, confluent monolayers of RECs were harvested by trypsin digestion and plated (approx. 100,000 cells/T-75 flask) in medium containing 0.1 μCi [^{3}H]thymidine (40-60 Ci/mmol; Amersham, Arlington Heights, IL)/ml to allow for incorporation into cellular DNA. Labeling medium was replaced with fresh medium containing no radioactivity after 24 hours. Cells were treated one day later (day 0) with 0, 1.5, 15 or 150 μM MCTP and incubated for 24 or 48 hours.

At this time, cells were harvested by scraping, resuspended, and assayed for DNA crosslinking by the alkaline elution technique of Kohn *et al.* (1981). Briefly, suspensions of cells in phosphate buffered saline (pH 7.2) were X-irradiated (100 rads) using a Variable Flux Gamma Irradiator (Model E-0117-M-1; U.S. Nuclear Corp., Burbank, CA) to induce DNA single strand breaks. Aliquots of cells were then loaded onto polyvinyl chloride filters (2.0 μm pore size; Omega Specialty Instruments, Chelmsford, MA) which were mounted in Swinnex filter assemblies (Millipore, Bedford MA) affixed with smokestack funnels (Millipore). Cells were lysed with 10 ml of lysing buffer (2% sodium dodecyl sulfate [SDS] and 25 mM Na_2EDTA; pH 9.7) and rinsed with 10 ml of a solution containing 0.1% SDS and 20 mM H_4EDTA (pH 10). Outflow needles were affixed via polyethylene tubing to a peristaltic pump, and 30 ml elution buffer (0.1% SDS and 20 mM H_4EDTA; pH 12.3) was added to the funnels. This buffer was drawn through the filters at a rate of approximately 0.04 ml/min. The alkaline eluting buffer denatures DNA into single strands, leaving covalently bonded crosslinks intact. Crosslinked DNA complexes are excluded or retarded from passing through the filter as compared with noncrosslinked, single-stranded DNA. Fractions were collected at 90 min. intervals, and incorporated [^{3}H]thymidine was quantified using a liquid scintillation counter (Beckman, Model LS-315OP). A crosslinking factor was calculated as described by Kohn and coworkers (1981):

$$X_r = (1\text{-}R_c \,/\, 1\text{-}R_t)^{1/2} - 1$$

where X_t is the crosslinking factor induced by treatment t, R_c is the fraction of DNA from control cells remaining on the filter at 18 ml of elution, and R_t is the fraction of DNA from MCTP-treated cells remaining on the filter at the same elution volume. Hence, the crosslink factor normalizes the elution rate of DNA from treated cells to that of a corresponding vehicle-treated control. An increase in crosslinking factor indicates an increase in total DNA crosslinking.

Statistical analysis: Data are presented as means +/- SEM or as means with standard error of differences (SED) presented for the data set (Steel and Torrie, 1980). Percentage data were transformed by the $\arcsin^{-1/2}$ transformation before statistical analysis. The data from the time-course assessment of monolayer cellularity were analyzed by a completely blocked analysis of variance (ANOVA), and individual comparisons were made using Tukey's omega as the *post hoc* test. DNA crosslinking data were analyzed using Student's t-test for single comparisons between treated cells and their vehicle control. Data for the remaining studies were analyzed using a random ANOVA, and individual comparisons were made using Tukey's omega as the *post hoc* test. The criterion for significance was $P<0.05$.

Results

Morphology of REC monolayers. RECs showed dose-dependent injury first evident as increased cell detachment which was noticeable between 1 and 2 days

posttreatment. Cell detachment became more pronounced with time, and cells which remained attached to the dish progressively enlarged. These enlarged cells became increasingly abnormal with time, developing large cytoplasmic vacuoles and stress fibers and demonstrating prominent nuclear changes [Figure 26]. However, they remained viable and were able to exclude trypan blue. By 4 days posttreatment, small gaps were evident in the monolayers treated with the highest concentration of MCTP (150 μM). Monolayer integrity appeared to be maintained by cellular hypertrophy throughout the posttreatment period in the other groups treated with MCTP.

Monolayer cell density was quantified by counting the cells remaining attached to the wells after MCTP treatment [Figure 27]. Whereas cell numbers remained relatively constant or increased somewhat in monolayers treated with 0 or 1.5 μM MCTP, they progressively decreased in monolayers treated with the higher concentrations of MCTP, resulting in a large disparity in monolayer cellularity between treatment groups by day 5.

Cellular release of LDH activity expressed as a percentage of total releasable activity is shown in Figure 28. RECs treated with 150 μM MCTP released significantly more LDH activity than vehicle controls by 2 days posttreatment, and this persisted throughout the 5 day period. In none of the other treatment groups was released LDH activity significantly elevated.

The LDH isozyme profile was examined in cells treated with 0 or 150 μM MCTP. Cultured RECs primarily contained isozymes 4 and 5 (39% and 58%, respectively), with small amounts of isozyme 3 present as well (Figure 29A).

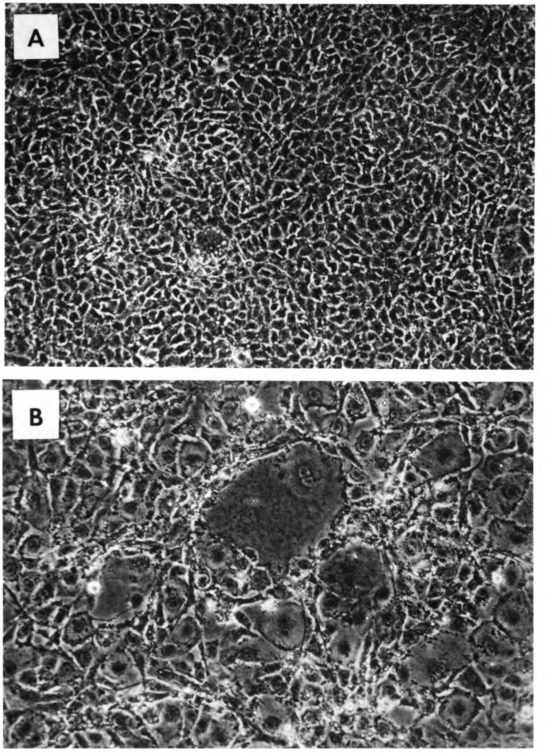


Figure 26: Photomicrographs of REC monolayers 4 days after a single administration of vehicle (A) or 150 μM MCTP (B). [Phase contrast. 180X Magnification.]

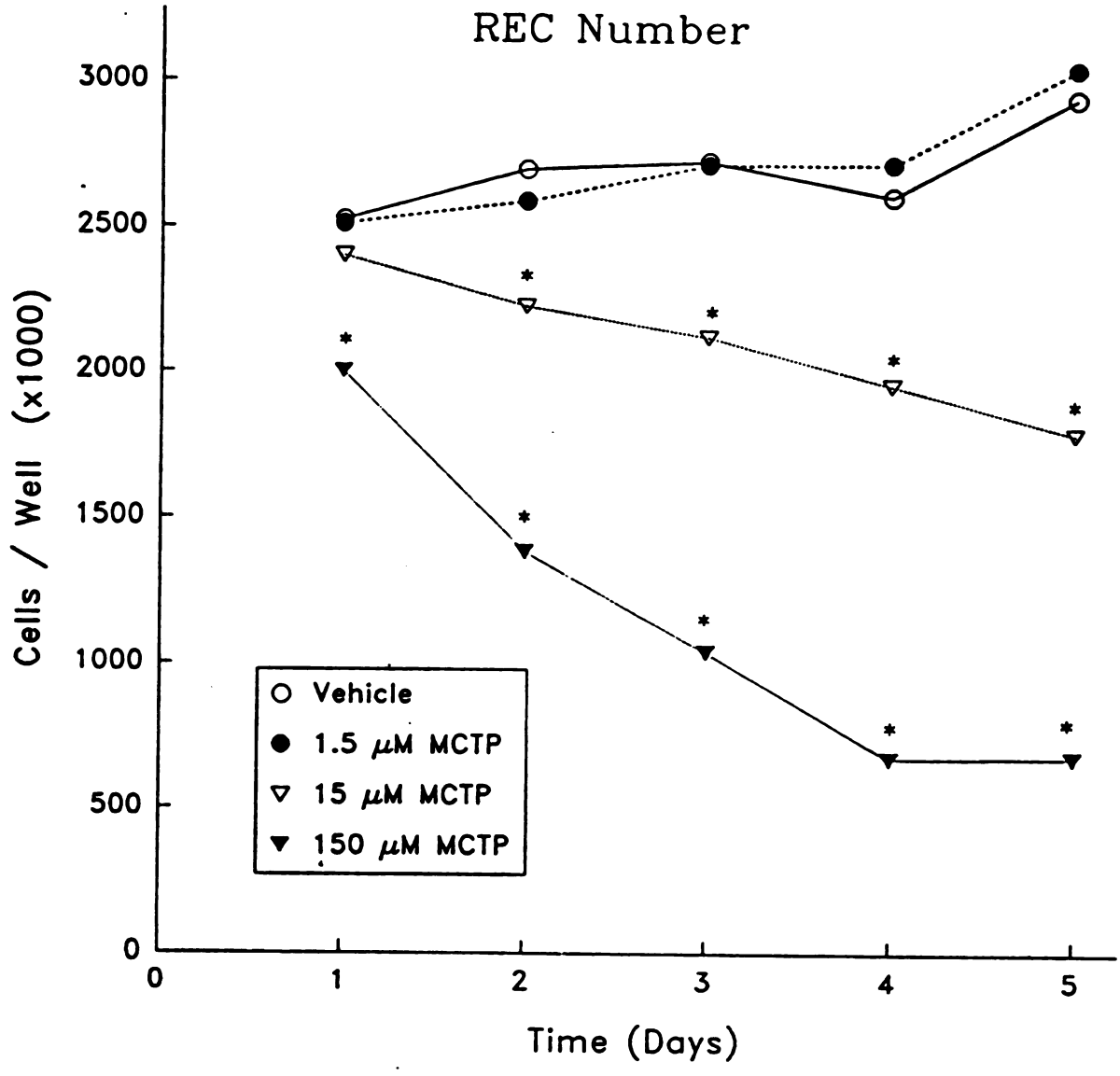


Figure 27: Effects of MCTP on REC monolayer cellularity.

Confluent monolayers of RECs received a single administration of MCTP on day 0. Values represent means of four replicate studies. [Standard error of difference = 158] * Significantly different from vehicle control ($p < 0.05$)

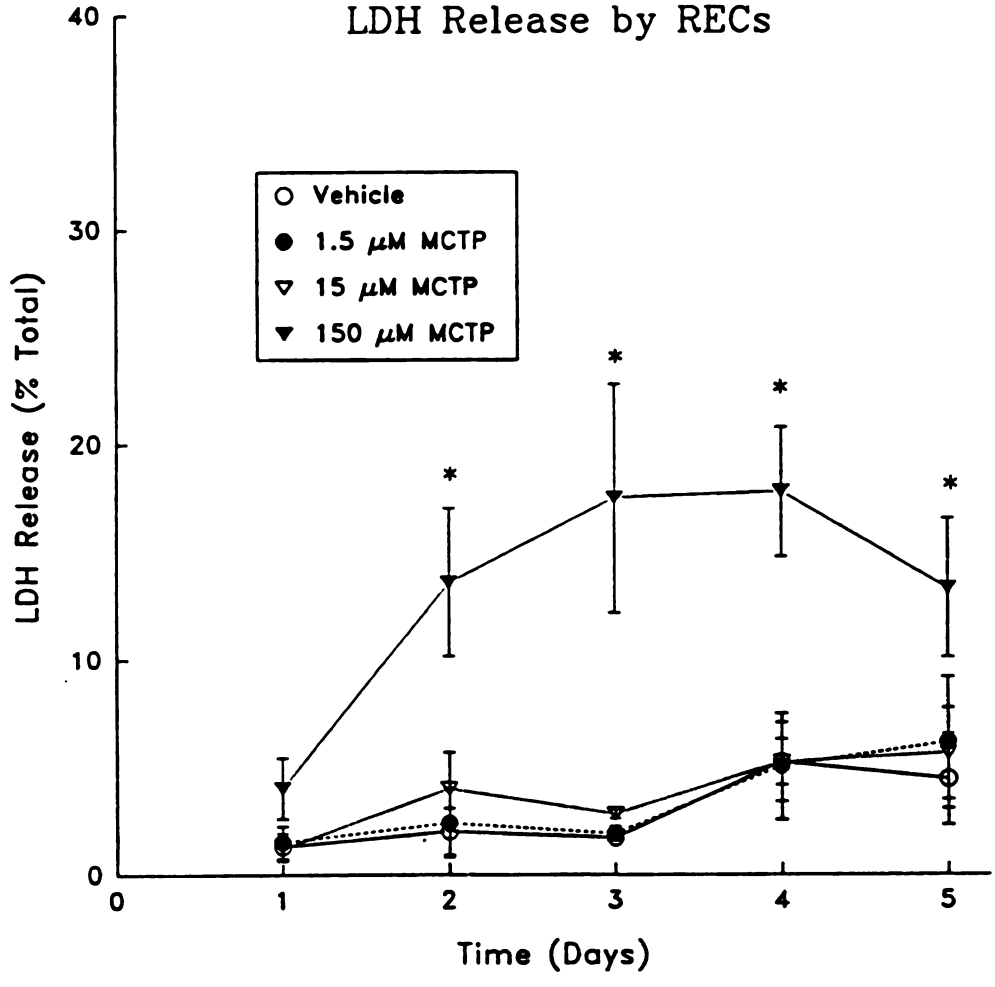
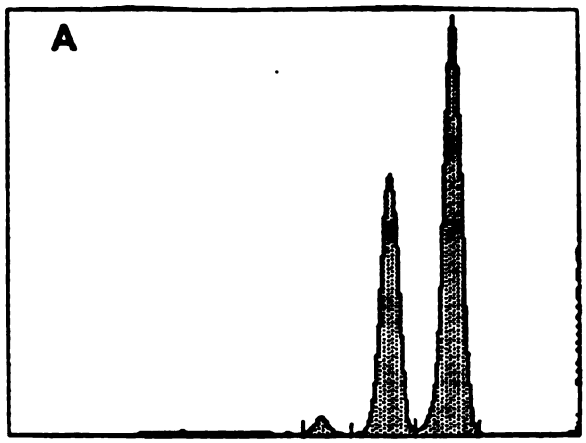


Figure 28: Release of lactate dehydrogenase activity from REC monolayers.

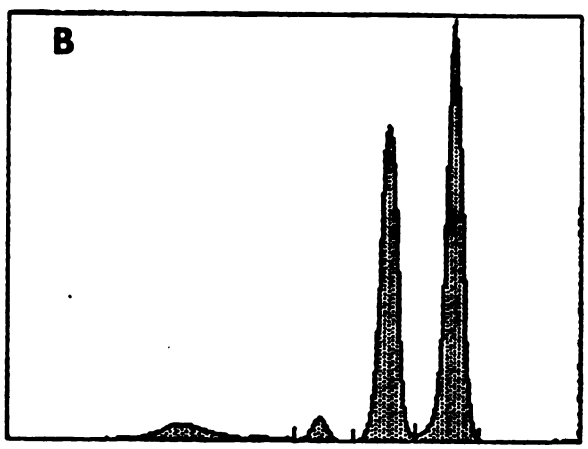
MCTP was administered once at time 0 at the concentrations indicated. Data are presented as means +/- SEM of four replicate studies. * Significantly different from vehicle control ($p<0.05$)



Lactate Dehydrogenase

Fraction	Relative %	IU/L
3	3	33
4	37	408
5	60	658

Total IU/L = 1099



Lactate Dehydrogenase

Fraction	Relative %	IU/L
3	3	8
4	43	106
5	54	134

Total IU/L = 248

Figure 29: Representative electrophoretic patterns of LDH isozymes in RECs.

Cells were treated with vehicle (A) or 150 μM MCTP at time 0 and were lysed 5 days later with 15 μl of 10% Triton X-100 for analysis.

Isozymes 1 and 2 were not detected. This pattern did not change qualitatively with MCTP treatment. Figure 29B shows the electrophoresis pattern of LDH isozymes in RECs 5 days after treatment with MCTP. Isozymes 4 and 5 still represent 43% and 55%, respectively, of the total LDH activity. Table 2 shows the pattern of LDH isozymes found in culture medium over the 5 days posttreatment. Although the percent of total LDH released from RECs increased with time for the first 3 days after MCTP treatment, the profile of LDH isozymes released from the treated cells into the culture medium was essentially the same as that of the cell lysates. The conditioned medium contained isozymes 3, 4 and 5, in similar proportions to the cell lysates.

Colony-forming efficiency of RECs treated with MCTP is shown in Figure 30. Concentrations of 0.15 or 1.5 μM MCTP partially inhibited REC proliferation, whereas higher concentrations resulted in a complete inhibition of cell division. In plates exposed to concentrations of 15 μM MCTP or greater, individual cells were attached to the plate surface, but there was no colony formation. These cells were greatly enlarged and vacuolated, but they remained viable as determined by their ability to exclude trypan blue.

The ability of MCTP to cause DNA crosslink formation in RECs is demonstrated in Figure 31. There was a concentration-dependent increase in DNA crosslinking at 24 hours posttreatment in RECs exposed to MCTP as compared to vehicle controls. After 48 hours of continuous exposure to the MCTP-containing medium, there was a further increase in DNA crosslinking in all treatment groups.

Table 2: Lactate Dehydrogenase (LDH) Isozyme Profile of Conditioned Medium from Rat Endothelial Cells

		Isozyme Fraction (% of total)[a]			
Treatment[b]	Day	3	4	5	% Total LDH Released[c]
Vehicle	1	0	0	0	1
	2	11	56	0	1
	3	10	42	4	1
	4	4	28	66	11
	5	2	36	62	9
MCTP	1	9	43	14	6
	2	5	44	49	23
	3	4	41	55	31
	4	6	43	42	13
	5	6	39	47	20

[a] Isozymes were separated by electrophoresis in agarose gels and were quantified by densitomitry. Values were calculated from a single analysis of a representative sample submitted on the day indicated. The profile of isozymes 3, 4 and 5 found in the supernatant is presented as the percent of total LDH units (isozymes 1-5) each represents.

[b] Cells were treated once at time 0 with 150 μM monocrotaline pyrrole (MCTP) or vehicle.

[c] This value represents the quantity of LDH released into supernatant expressed as a percent of the sum of supernatant and cell lysate LDH activity.

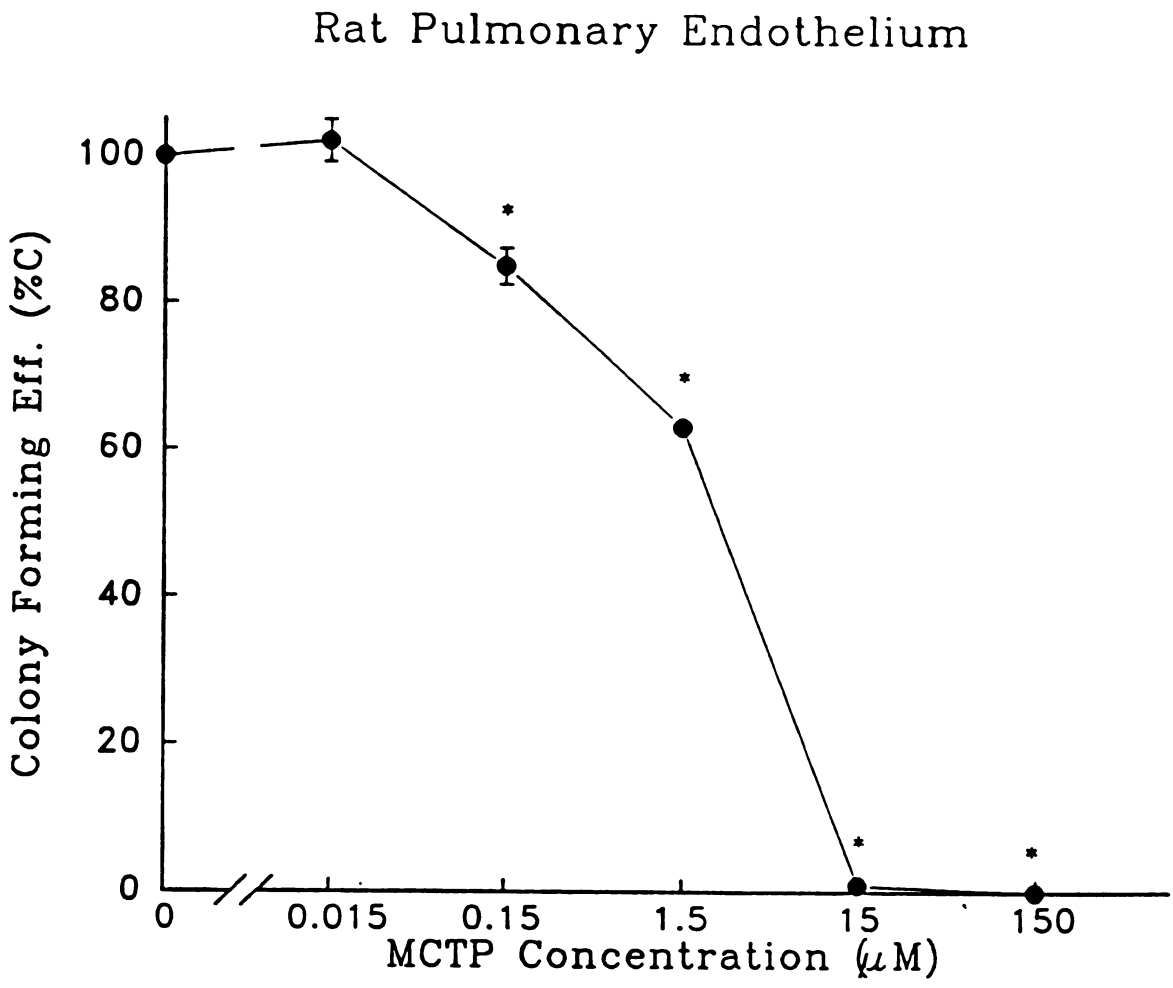


Figure 30: Effects of MCTP on colony-forming efficiency of RECs.

RECs plated at very low density received a single administration of MCTP on day 0. Colonies were fixed, stained and counted 14 days later. Values represent means +/- SEM of four replicate studies. * Significantly different from vehicle control ($p<0.05$)

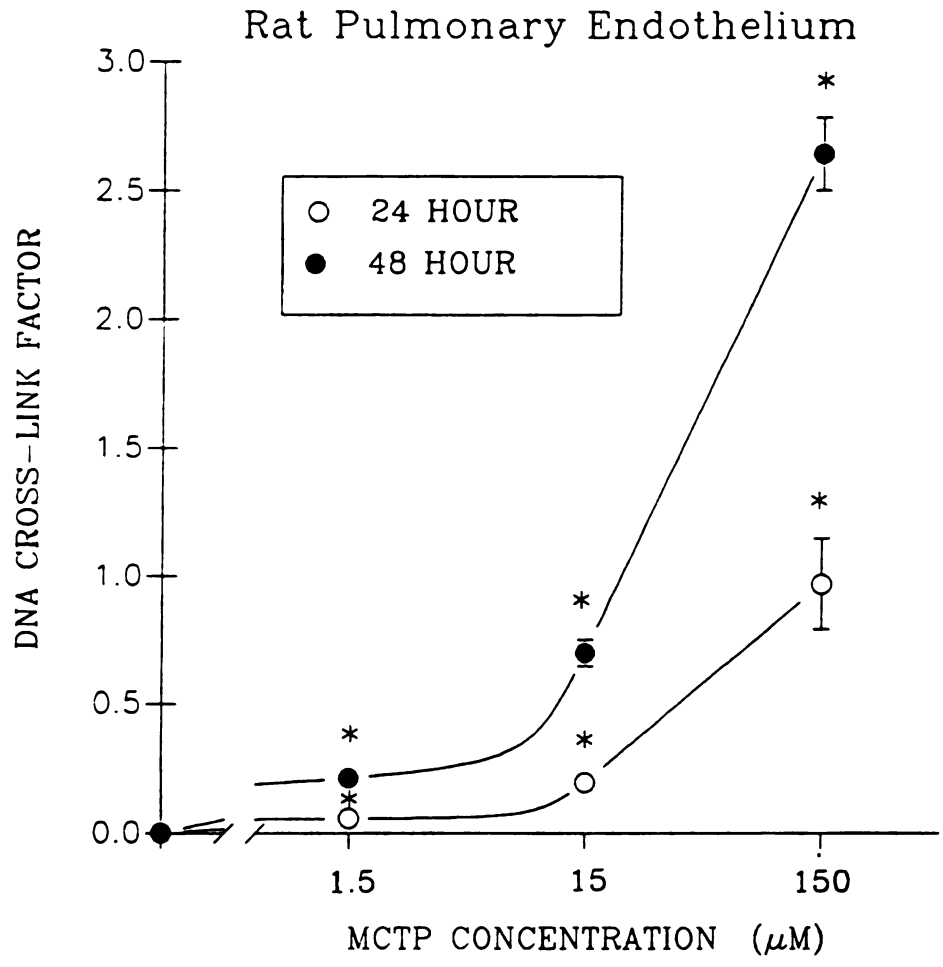


Figure 31: Total DNA crosslinking in RECs.

RECs were treated once with MCTP at time 0. Values represent means +/- SEM of replicate studies (n=3-4 per group). * Significantly different from vehicle control ($p<0.05$)

Discussion

The pulmonary vascular endothelium, by virtue of its proximity to the flowing blood and its large surface area, has been proposed as a likely site for binding of much of the reactive metabolite(s) of MCT (Roth and Reindel, 1990). The consequences of this binding may be very important, both in terms of overt endothelial cell structural injury and more subtle changes in endothelial function. Changes in endothelial structural and functional integrity have been noted as relatively early features of the delayed and progressive pulmonary vascular injury caused by the administration of MCT or MCTP to rats *in vivo*. Although many of the reported endothelial cell changes are subtle initially, their onset precedes the onset of vascular remodeling and increases in pulmonary pressure, suggesting a role for the altered endothelium in the development and progression of the pneumotoxicity caused by these compounds (Plestina and Stoner, 1972; Rosenberg and Rabinovitch, 1988; Allen and Carstens, 1970).

The present studies in cultured rat endothelium demonstrated that MCTP was directly cytotoxic to these cells. The injury we observed in RECs was delayed and progressive, reminiscent of that seen in the rat *in vivo*. Injury to monolayers was not apparent for at least 24 hours after a single administration of MCTP. From this time forward, there were concentration-dependent cell detachment and release of LDH which were persistent. The cells which remained in the culture plates became markedly enlarged, with cytoplasmic vacuolization, prominent stress fibers, and enlarged nuclei. These cytolytic and hypertrophic changes closely resemble those

previously reported in cultured BECs treated with MCTP (Reindel and Roth, 1991; Reindel *et al.,* 1991) but are more pronounced than those which occur in PECs.

The electrophoretic pattern of LDH isozymes in these cells was investigated in an effort to identify endothelium as a potential source of LDH detected in the bronchoalveolar lavage fluid (BALF) and serum of rats treated with MCTP *in vivo*. The profile of LDH isozymes in RECs did not change with MCTP treatment. The predominant isozymes detected in lysates of either MCTP- or vehicle-treated cells were 4 and 5, and these isozymes were released into the medium in the same relative proportions from injured RECs. Not surprisingly, it appears that inasmuch as LDH isozymes 4 and 5 make up a substantial part of the LDH released into bronchoalveolar lavage fluid *in vivo*, other isozymes are detected as well (Schultze *et al.*, unpublished observations). Thus, the endothelium may be a source of some, but not all, of the activity noted in BALF and serum of MCTP-treated rats.

In addition to these cytolytic consequences of MCTP treatment, RECs were also found to be very susceptible to the antiproliferative effects of MCTP, perhaps more so than are either BECs or PECs (Reindel *et al.,* 1991). Concentrations of as little as 0.15 μM MCTP caused a significant decrease in REC colony formation, and concentrations >1.5 μM MCTP completely inhibited cell division. We have found MCTP to be a potent antiproliferative agent in all of the cultured cell types we have tested, including BECs, PECs, bovine and porcine smooth muscle cells, Madin-Darby canine kidney cells and Crandel-Rous feline kidney cells (Reindel *et al.,* 1988; Reindel and Roth, 1991; Reindel *et al.,* 1991) ;unpublished observations).

DNA:DNA and DNA:protein crosslinking has been demonstrated in the livers of rats treated with MCT, suggesting that the reactive metabolite generated by the liver is a potent, bifunctional alkylating agent (Petry *et al.*, 1984). Studies in our laboratory revealed that MCTP treatment results in persistent DNA crosslinking in cultured PECs (Wagner *et al.*, 1991; Wagner *et al.*, 1992), and this has also been demonstrated in RECs in the present study. Alkylation leading to DNA crosslinking may represent one of the earliest events in the toxicity. It is likely that the DNA crosslinking which results shortly after MCTP treatment interferes with cell division. However, the DNA crosslinking does not appear to inhibit DNA synthesis completely, since MCTP-treated PECs continue to synthesize DNA and increase their DNA content although they are unable to divide (Hoorn and Roth, 1992).

The cytostatic effects of MCTP appear to be long-lived and may interfere with the ability of the endothelial monolayer to effect repair in a normal fashion. In the face of endothelial cell loss *in vivo*, due either to intimal injury or normal cell turnover, a persistent defect in proliferative repair could result in a gradual loss of vascular integrity. The progressive vascular leak seen in the lungs of MCTP-treated rats reflects such a deterioration of the endothelial barrier (Sugita *et al.*, 1983b; Bruner *et al.*, 1983; Reindel *et al.*, 1990).

In summary, MCTP causes direct injury to rat endothelium *in vitro* which is delayed and progressive, supporting the contention that rat pulmonary endothelium is a likely target for MCTP binding and injury *in vivo*. MCTP-induced injury in cultured RECs is both cytostatic and cytolytic and suggests several ways in which endothelial impairment or altered function could contribute to the pulmonary

vascular injury in this species when treated *in vivo*. Impaired cell proliferation, perhaps as a consequence of DNA crosslinking, would leave the pulmonary endothelium vulnerable to any action resulting in injury or stimuli for increased cell turnover. This aspect of MCTP cytotoxicity, combined with the cytolytic nature of the injury, could contribute to the vascular alterations which occur in the rat treated with MCT *in vivo*.

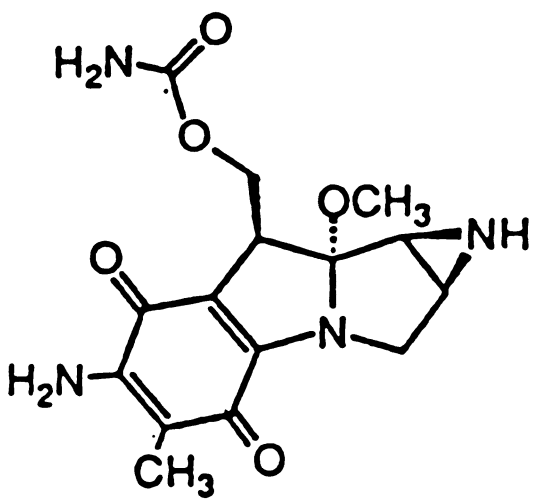
Chapter VI

THE EFFECTS OF MITOMYCIN C ON CULTURED ENDOTHELIUM: COMPARISON WITH MONOCROTALINE PYRROLE-INDUCED CYTOTOXICITY

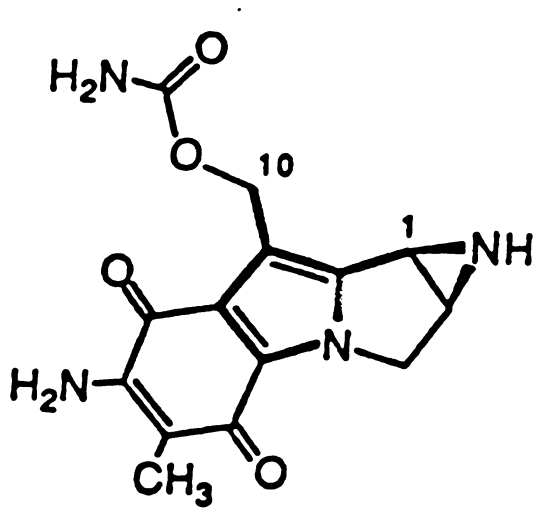
Summary

EC alterations are proposed to underlie the delayed and progressive development of pneumotoxicity which is seen after exposure to MCT(P), but the mechanism by which this compound initiates injury remains unknown. MCTP injures ECs treated *in vitro*, resulting in an inhibition of proliferation at low concentrations and marked cytolytic and morphologic alterations at higher concentrations. MCTP is a bifunctional alkylating agent, and DNA crosslinking has been observed in cultured ECs treated *in vitro* with concentrations of MCTP that are cytostatic but which cause little cytolysis. DNA crosslinking, by inhibiting EC proliferation and limiting monolayer repair capability, may engender the delayed and progressive cytotoxic response of ECs monolayers to MCTP administration. Mitomycin C (MMC) is a bifunctional alkylating agent used in cancer chemotherapy. Like MCTP, reactive metabolites of MMC can also crosslink DNA, and the cytotoxicity of this compound has been attributed to this capacity as well as to other capacities such as redox cycling. Treatment of cultured EC monolayers with MMC resulted in a pattern of injury reminiscent of MCTP-induced EC injury, including both cytolytic and cytostatic effects. MMC treatment also resulted in DNA crosslinking at concentrations which inhibited proliferation but caused limited overt cytotoxicity, supporting an association between DNA crosslinking and cytostasis. MMC was much more potent than MCTP in producing EC effects. Comparable morphologic alterations were evident at 1 μM MMC and 150 μM MCTP, and 0.01 μM MMC and 1.5 μM MCTP inhibited proliferation to a similar extent. Nevertheless, the similarity of the EC reaction to

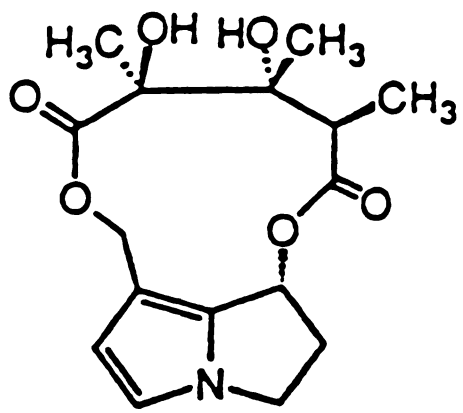
bifunctional alkylating agents of different classes suggests that DNA crosslinking in ECs results in a characteristic response of this cell type to a loss of monolayer integrity.



Mitomycin C



7-Amino-1,2-aziridinomitosene



Dehydromonocrotaline

Figure 32: Structures of mitomycin C, the reactive metabolite, 7-amino-1,2-aziridinomitosene, and dehydromonocrotaline (MCTP).

Introduction

Many of the pyrrolic metabolites of PZAs, as well as a number of potent cancer chemotherapeutic agents, are bifunctional electrophiles. The antiproliferative and cytotoxic effects of these compounds have generally been attributed to their common ability to crosslink DNA and other cellular macromolecules, as related monofunctional alkylating agents, which do not form crosslinks, are generally much less cytotoxic (Bull *et al.,* 1968; Culvenor *et al.,* 1969; Zwelling *et al.,* 1979; Wheeler, 1975; Bodell *et al.,* 1985; Mattocks and Legg, 1980).

DNA crosslinking occurs as a relatively early event in the course of MCT(P) toxicity, both in the liver after MCT treatment *in vivo* and in cultured endothelial cells exposed to MCTP *in vitro* (Petry *et al.,* 1984; Wagner *et al.,* 1992). Cell proliferation is inhibited in both systems, and surviving cells become markedly enlarged, with pronounced nuclear alterations (Culvenor *et al.,* 1969; Jago, 1969; Reindel *et al.,* 1991). It is hypothesized that similar binding to DNA in the endothelium of the lung vasculature may initiate a cascade of cellular alterations that contribute to the development of MCT(P) pneumotoxicity *in vivo* (Roth *et al.,* 1989).

Mitomycin C (MMC) is an antitumor antibiotic used in the treatment of neoplasia of the gastrointestinal tract or breast (Crooke and Bradner, 1976). Like MCT, MMC is bioactivated to a potent, bifunctional alkylating species that is capable of crosslinking DNA, and much of the cytotoxic potential of this agent has been attributed to this capacity (Dulhanty *et al.,* 1989; Iyer and Szybalski, 1963). Reductively activated MMC bears a structural similarity to the PZAs, but has an

added quinone function (Weidner *et al.*, 1990) [Figure 32]. Interestingly, intravenous administration of MMC occasionally has been reported to cause delayed-onset, progressive pulmonary vascular injury which is characterized by EC enlargement, vascular leak, and the development of pulmonary hypertension (Jolivet *et al.*, 1983; Waldhorn *et al.*, 1984; Cantrell *et al.*, 1985; Chang *et al.*, 1986; McCarthy and Staats, 1986). Structural analogues of MMC have also been shown to cause a delayed pneumotoxicity in rats, with vascular alterations which are reminiscent of those caused by MCT(P). These alterations include necrotizing arteritis and medial hypertrophy, with the development of pulmonary edema and right ventricular dilatiation and hypertrophy. Endothelial changes were not mentioned in this study, and the pulmonary effects were considered, perhaps erroneously, to be a consequence of cardiotoxicity (Bregman *et al.*, 1989).

To begin to understand the importance of DNA crosslinking to the inhibition of proliferation and the progressive development of cytotoxic alterations which occur in ECs treated with MCTP, the effects of MMC on cultured EC structure and function were examined. Although MMC has been used as an experimental tool to inhibit EC proliferation, the antimitotic effects of this compound have not been directly correlated with DNA crosslinking in ECs, and the overall response of the endothelium to MMC treatment has not been well characterized. The identification of similarities in the response of ECs to bifunctional alkylating agents of different classes may help to define a role for DNA crosslinking in the early cellular alterations that lead to the delayed development of chronic pulmonary vascular injury.

Materials and Methods

Preparation of endothelial cells: Porcine pulmonary artery endothelial cells (PECs) were isolated and characterized as described previously in Chapter II. Rat pulmonary endothelial cells (RECs) were generously provided by Dr. James Varani (Dept. of Pathology, University of Michigan, Ann Arbor, Michigan). PECs and RECs were maintained and subcultured as described previously in Chapters II and IV, respectively. PECs used in these studies were between passages 4 and 10; RECs were at passages 27 and 28. Unless otherwise noted, culture medium was replaced with fresh Opti-MEM (5% FCS, 1% Ab/Am) or DMEM (10% FCS, 1% Ab/Am) every other day.

Preparation of MMC: MMC (Sigma) was dissolved in sterile water at a concentration of 100 μM, and all dilutions of MMC were made with sterile water. A 5 μl volume of MMC solution or vehicle (0 μM MMC) per milliliter of medium was used in all studies to achieve the nominal concentrations of MMC (0.001-10 μM) used in these studies.

Effects of MMC on monolayer cellularity and morphology: PECs or RECs were plated into 12-well tissue culture clusters and allowed to proliferate until monolayers reached confluence. At this time (day 0), fresh medium was added and monolayers were exposed to a single administration of 0, 0.1, 1 or 10 μM MMC. At 4 hrs and

on days 1, 2, 3, 5, 7 and 10 (PECs only) posttreatment, cells remaining in the monolayers were examined and counted as described previously in Chapter II.

Cellular release of LDH activity: PECs and RECs were plated and treated as described above. At the indicated times posttreatment, one 12-well plate of each cell type was used for determination of LDH activity as described in Chapter V.

Colony-forming efficiency: PECs and RECs were plated at low density (1000 cells/plate) in 100 mm tissue culture plates. Cells were incubated for 3-4 hr to allow for cell attachment and then were exposed to a single administration of 0, 0.001, 0.01, 0.1, 1 or 10 μM MMC. Fresh medium (without MMC) was added on day 7 posttreatment. On day 14, cell colonies were fixed, stained and counted as described in Chapter V.

DNA crosslinking: These studies were carried out (on PECs only) as described previously in Chapter V. PECs were treated with 0, 0.01, 0.1 or 1 μM MMC, and the alkaline elution assay was performed at 24 and 48 hr posttreatment.

Statistical analysis: Data are presented as means +/- SEM. Percentage data were transformed by the $\arcsin^{-1/2}$ transformation before statistical analysis. DNA crosslinking data were analyzed using Student's t-test for single comparisons between treated cells and their vehicle controls. Data for the remaining studies were analyzed

using a random ANOVA, and individual comparisons were made using Tukey's omega as the *post hoc* test. The criterion for significance was $P<0.05$.

Results

Morphology of cell monolayers. Both PECs and RECs showed concentration-dependent injury first evident as increased cell detachment by 24 hr posttreatment. Cell detachment was more pronounced in RECs but progressed with time in both cell types. As monolayers became less cellular, the remaining cells progressively enlarged and became irregular in shape. The concentration-dependent effects of MMC on PEC and REC monolayers at 5 days posttreatment are shown in Figures 33 and 34. By this time, few cells remained in the monolayers treated with 10 μM MMC, and gaps were evident in the monolayers treated with 1 μM MMC. Remaining PECs became spindle-shaped, whereas RECs were markedly enlarged and abnormal in appearance with cytoplasmic vacuoles and enlarged nuclei. All adherent cells in these dose groups remained viable and were able to exclude trypan blue. PEC and REC monolayers treated with 0.1 μM MMC showed only minor morphologic alterations, but they appeared less dense than controls at this time; there was little cell detachment evident in this treatment group.

Monolayer cell density was quantified by counting the cells remaining attached to the wells after MMC treatment [PEC: Figure 35; RECs: Figure 36]. PEC number increased slightly over the 10 day period of observation in the control group. Cell

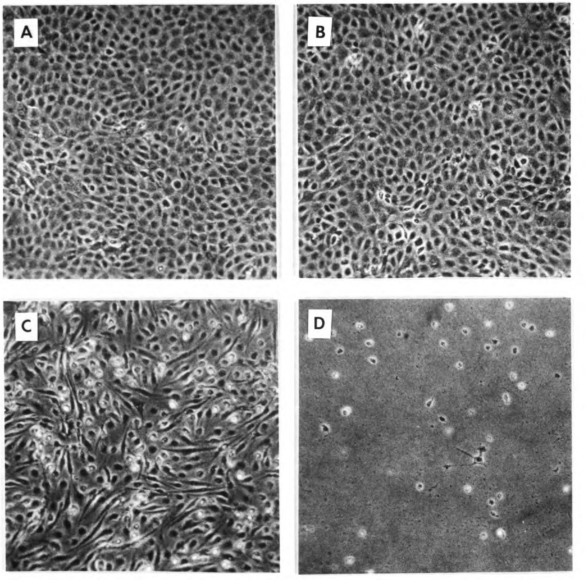


Figure 33: Photomicrographs of PEC monolayers 5 days after a single administration of 0 (A), 0.1 (B), 1 (C) or 10 (D) μM MMC. [Phase contrast].

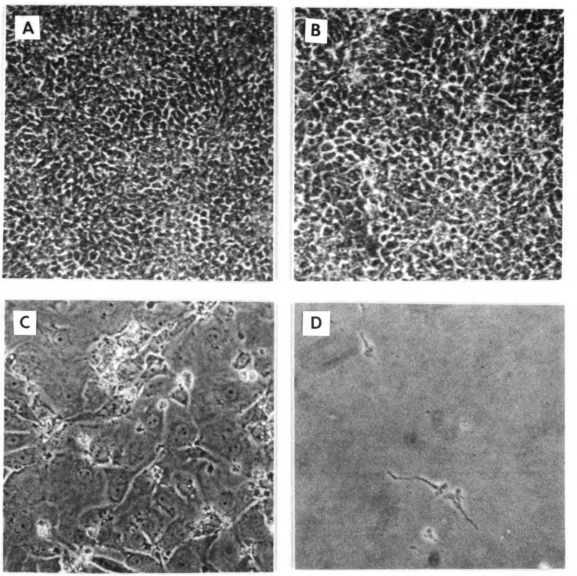


Figure 34: Photomicrographs of REC monolayers 5 days after a single administration of 0 (A), 0.1 (B), 1 (C) or 10 (D) μM MMC. [Phase contrast].

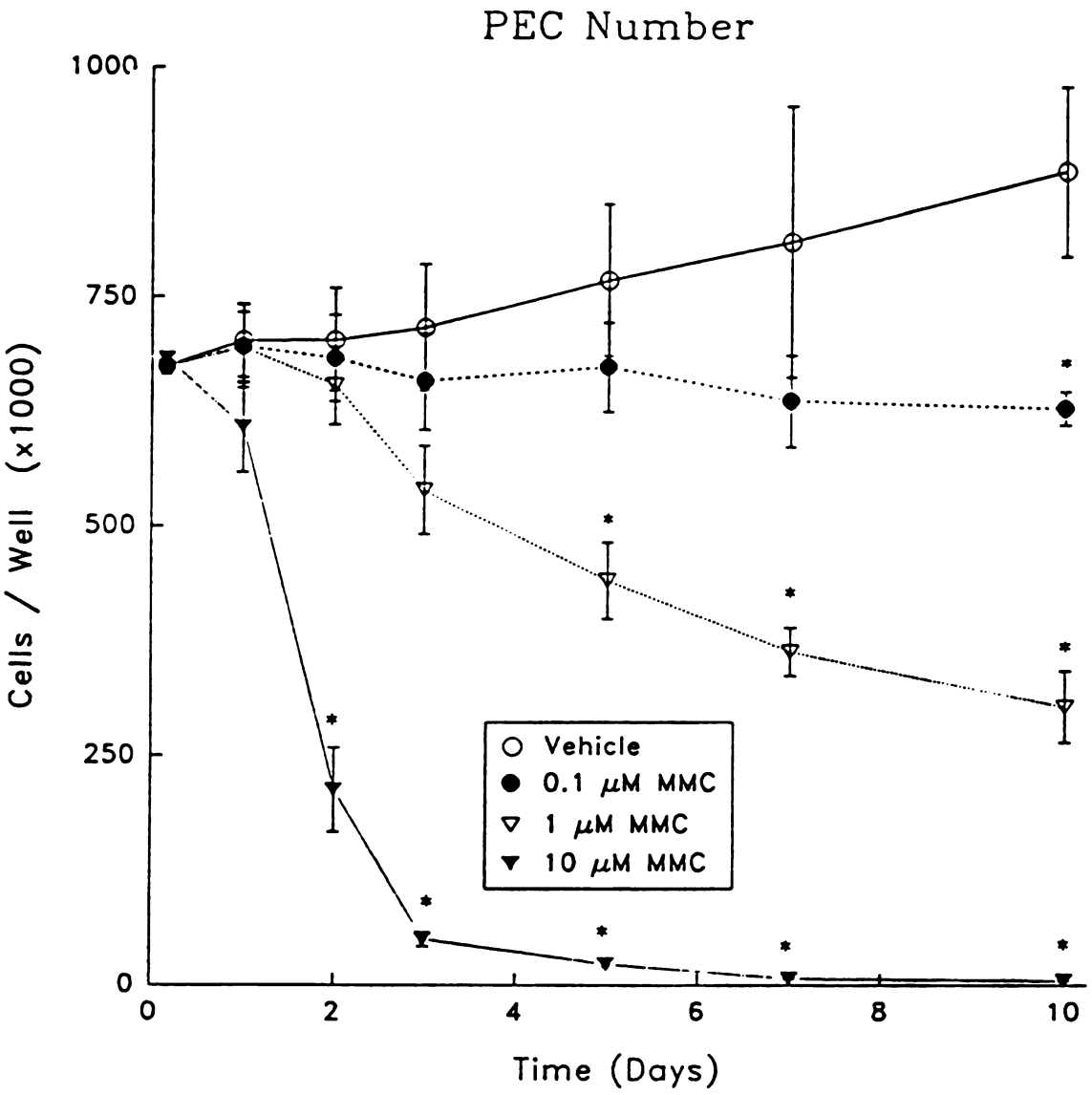


Figure 35: Effects of MMC on PEC monolayer cellularity.

PEC monolayers received a single administration of MMC at time 0. Values represent means +/- SEM of 3 replicate studies. * Significantly different from vehicle control ($p<0.05$)

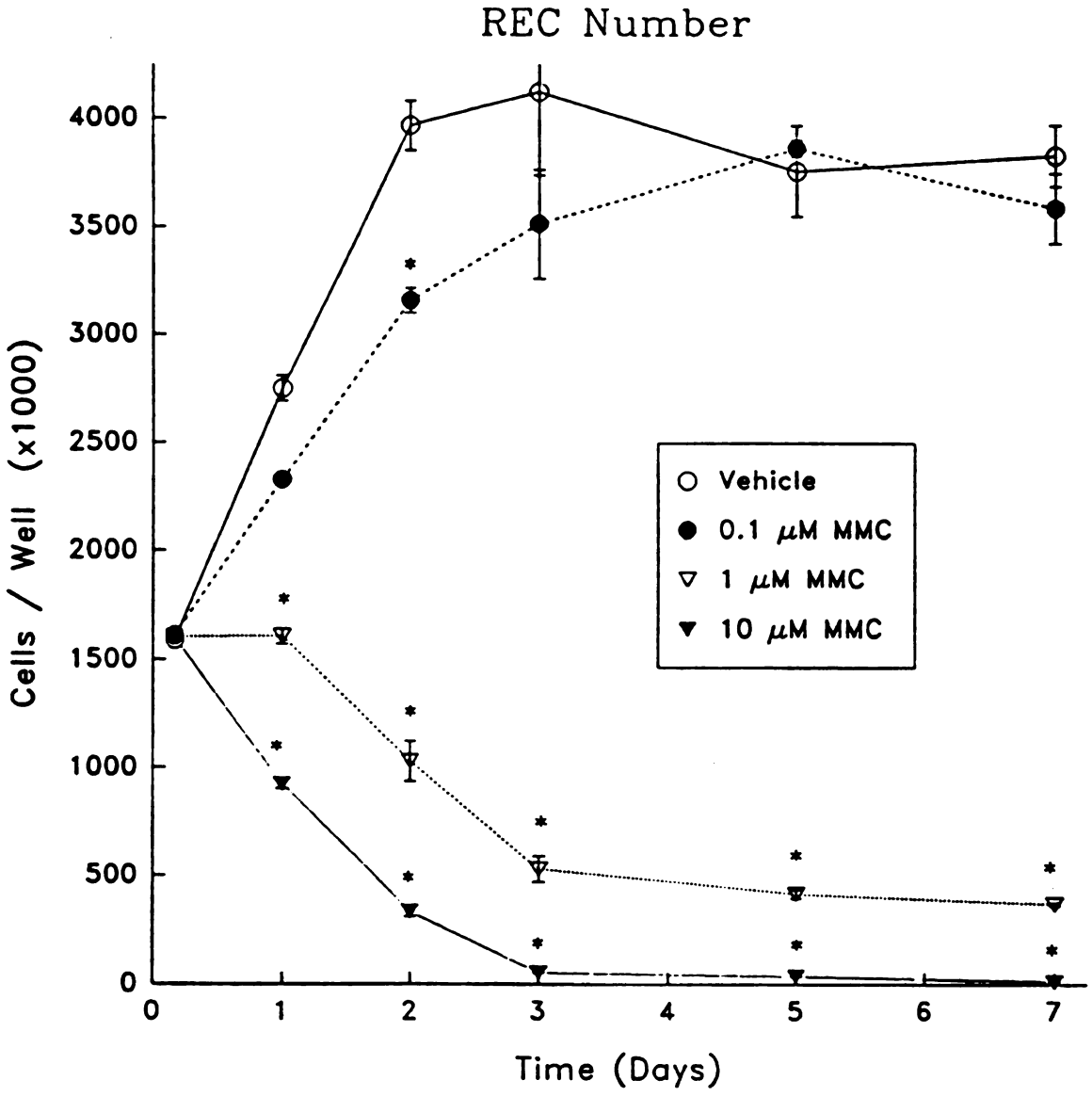


Figure 36: Effects of MMC on REC monolayer cellularity.

REC monolayers received a single administration of MMC at time 0. Values represent means +/- SEM of 3 replicate studies. * Significantly different from vehicle control ($p < 0.05$)

number remained relatively constant in PECs treated with 0.1 μM MMC and declined gradually in monolayers treated with 1 μM MMC. PEC monolayers treated with 10 μM MMC rapidly decreased in cellularity, with the number of adherent cells less than 5% of controls by 5 to 7 days posttreatment.

REC monolayer cellularity increased dramatically in both the control group and the lowest MMC treatment group (0.1 μM) during the first two days of the experiment but remained constant thereafter. The rate at which cell number increased was less in the cells treated with 0.1 μM MMC. By 7 days posttreatment, these dense REC monolayers began to detach spontaneously from the culture plates in sheets irrespective of treatment, making evaluation at 10 days posttreatment impossible. In monolayers treated with higher concentrations of MMC, REC number decreased progressively with time. REC monolayers treated with 10 μM MMC had few adherent cells remaining after 3 days posttreatment.

Release of LDH activity expressed as a percentage of total releasable activity is illustrated in Figures 37 and 38 (PECs and RECs, respectively). PEC LDH release was minimal for the first 24 hours posttreatment. After this time, LDH activity in the medium from PEC monolayers treated with 10 μM MMC increased rapidly until 3 days posttreatment, then began to decline by day 5 when few cells remained in the monolayers. LDH release was minimal in other treatment groups; monolayers treated with 1 μM MMC showed a slight increase in release of LDH activity, but only at 7 days posttreatment.

LDH release from REC monolayers was minimal at 4 hr posttreatment in all groups but increased dramatically during the next three days in cells treated with 10

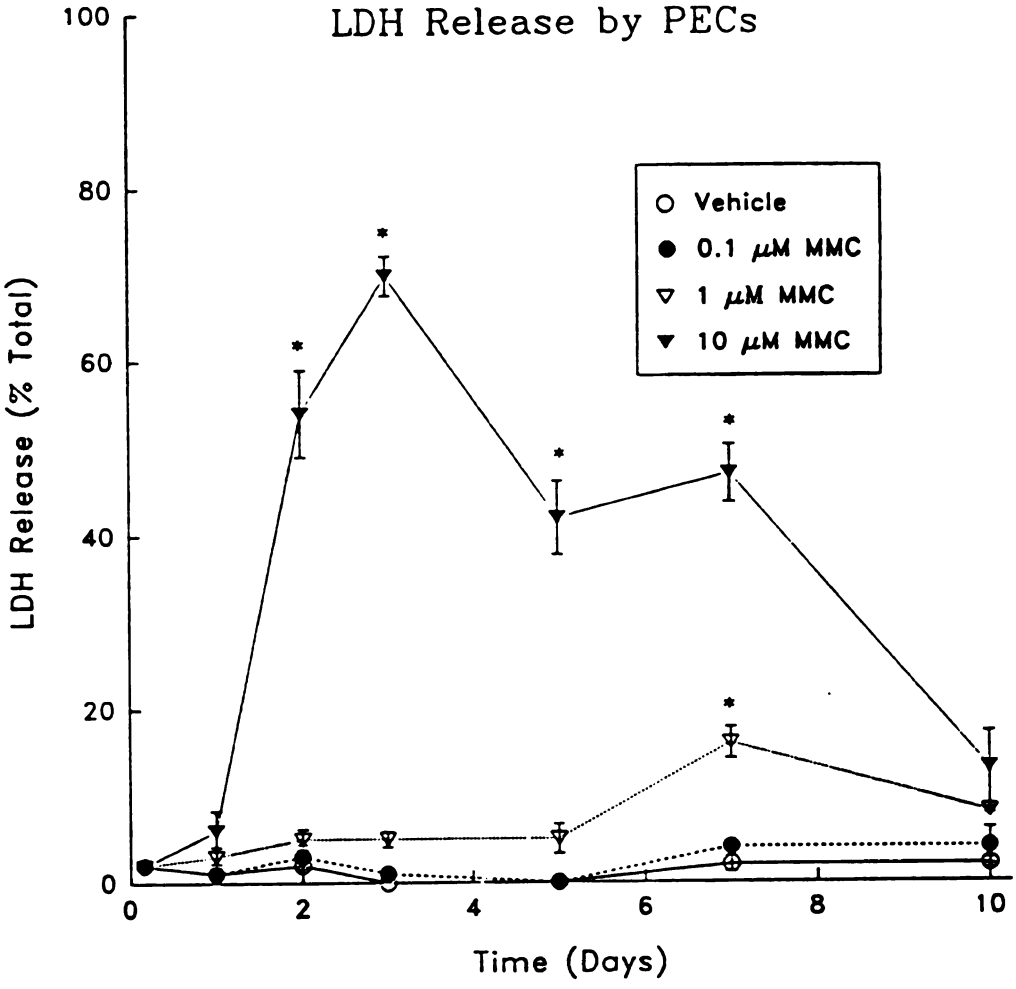


Figure 37: Release of LDH activity from PEC monolayers.

MMC was administered once at time 0 at the concentrations indicated. Data are presented as means +/- SEM of three replicate studies. * Significantly different from vehicle controls $p < 0.05$)

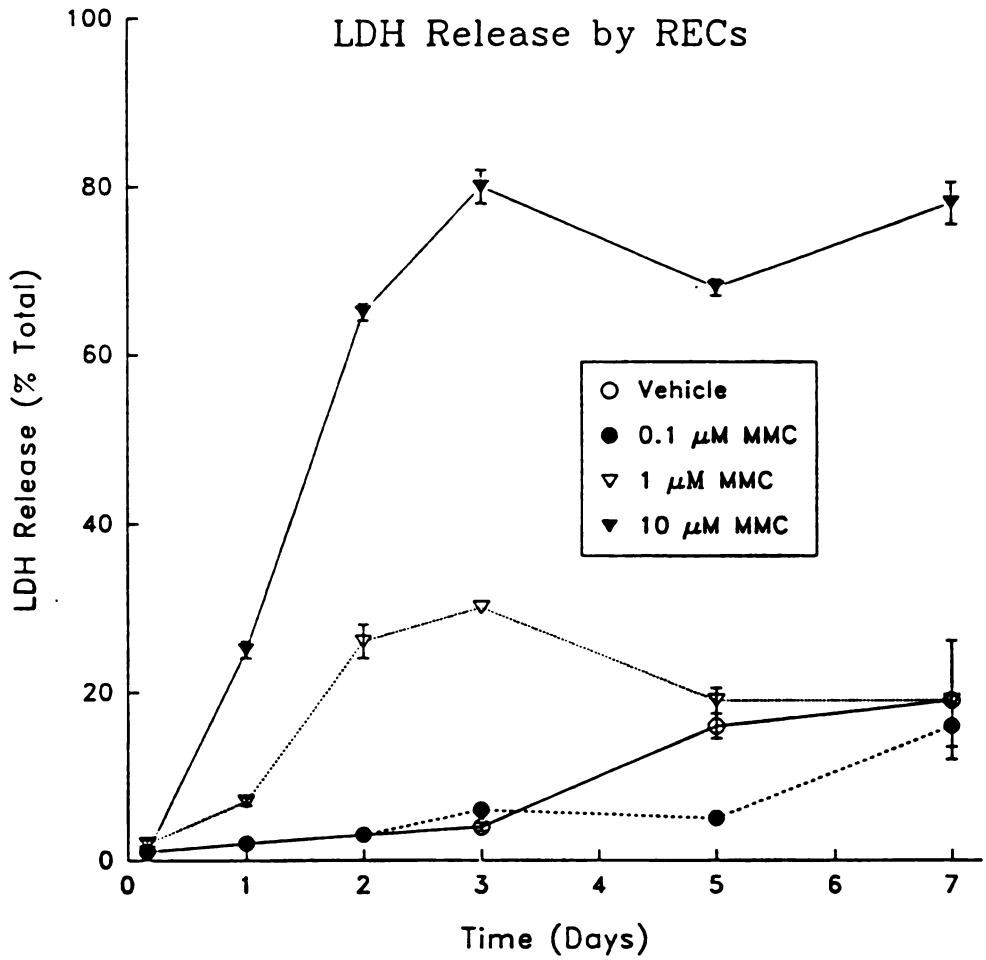


Figure 38: Release of LDH activity from REC monolayers.

MMC was administered once at time 0 at the concentrations indicated. Data are presented as means +/- SEM of three replicate studies. * Significantly different from vehicle controls $p<0.05$)

μM MMC. The increase in LDH release was more gradual and progressive from cells treated with 1 μM MMC, peaking at about 25% by 3 days posttreatment. LDH release was minimal from REC monolayers treated with 0.1 μM MMC until 7 days posttreatment; by this time, LDH release was increased from control monolayers as well.

Colony-forming efficiency of PECs treated with MMC is shown in Figure 39. MMC at a concentration of 0.01 μM inhibited PEC proliferation by 50%, whereas higher concentrations resulted in a complete inhibition of cell proliferation. At 0.1 μM MMC, enlarged, abnormal cells could be seen as individual cells or small groups attached to the plate surface. Very few cells remained on the plates treated with 1 μM MMC, and no cells were evident in the highest treatment group.

MMC treatment inhibited REC proliferation in a similar manner [Figure 40]. Concentrations of 0.01 μM decreased colony formation by 33%, and higher concentrations completely inhibited cell proliferation.

DNA crosslinking in PECs in response to MMC treatment is shown in Figure 41. A concentration-dependent increase in total DNA crosslinking was evident at 24 hr posttreatment as compared to controls, and this crosslinking persisted through 48 hours.

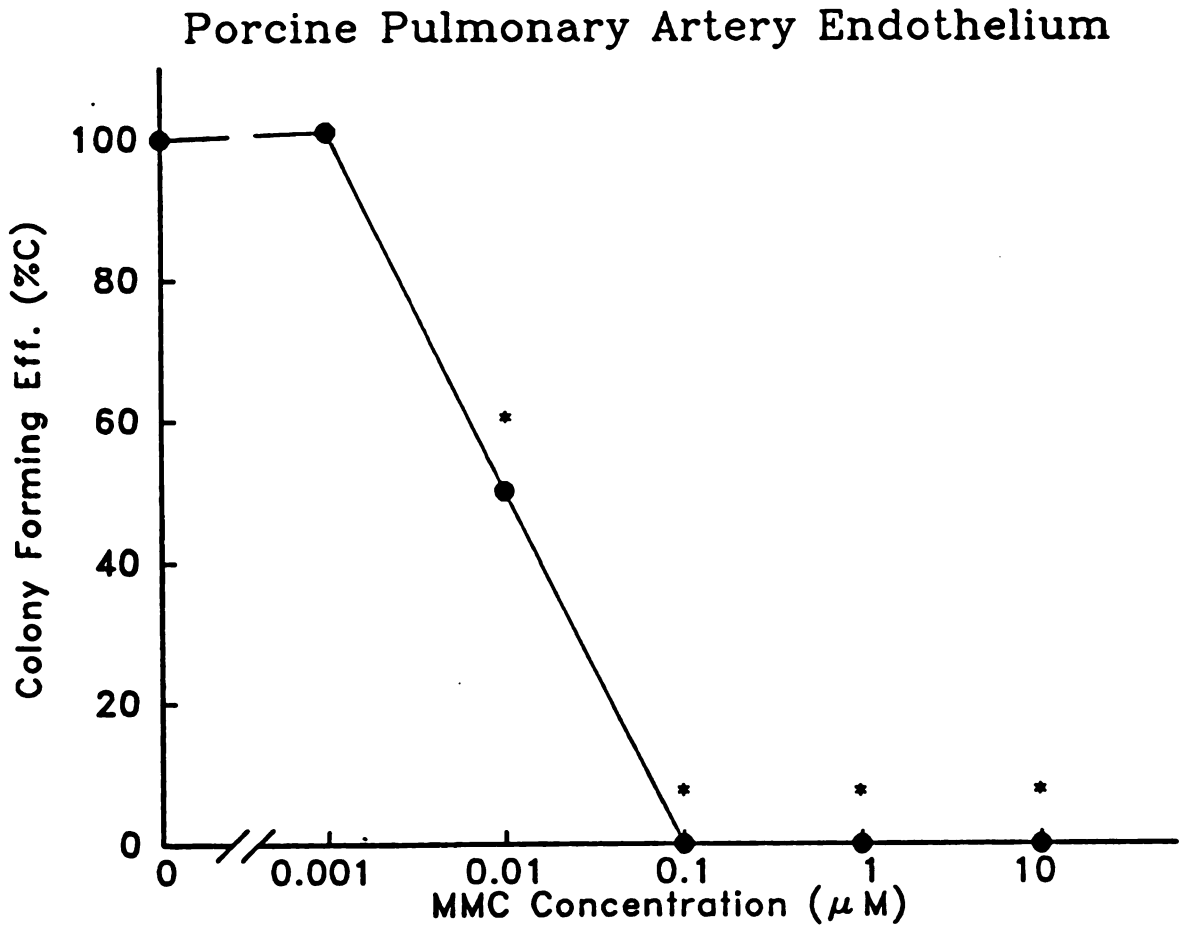


Figure 39: Effects of MMC on colony-forming efficiency of PECs.

PECs plated at very low density received a single administration of MMC on day 0. Colonies were fixed, stained and counted 14 days later. Values represent means +/- SEM of three replicate studies. * Significantly different from vehicle control ($p<0.05$)

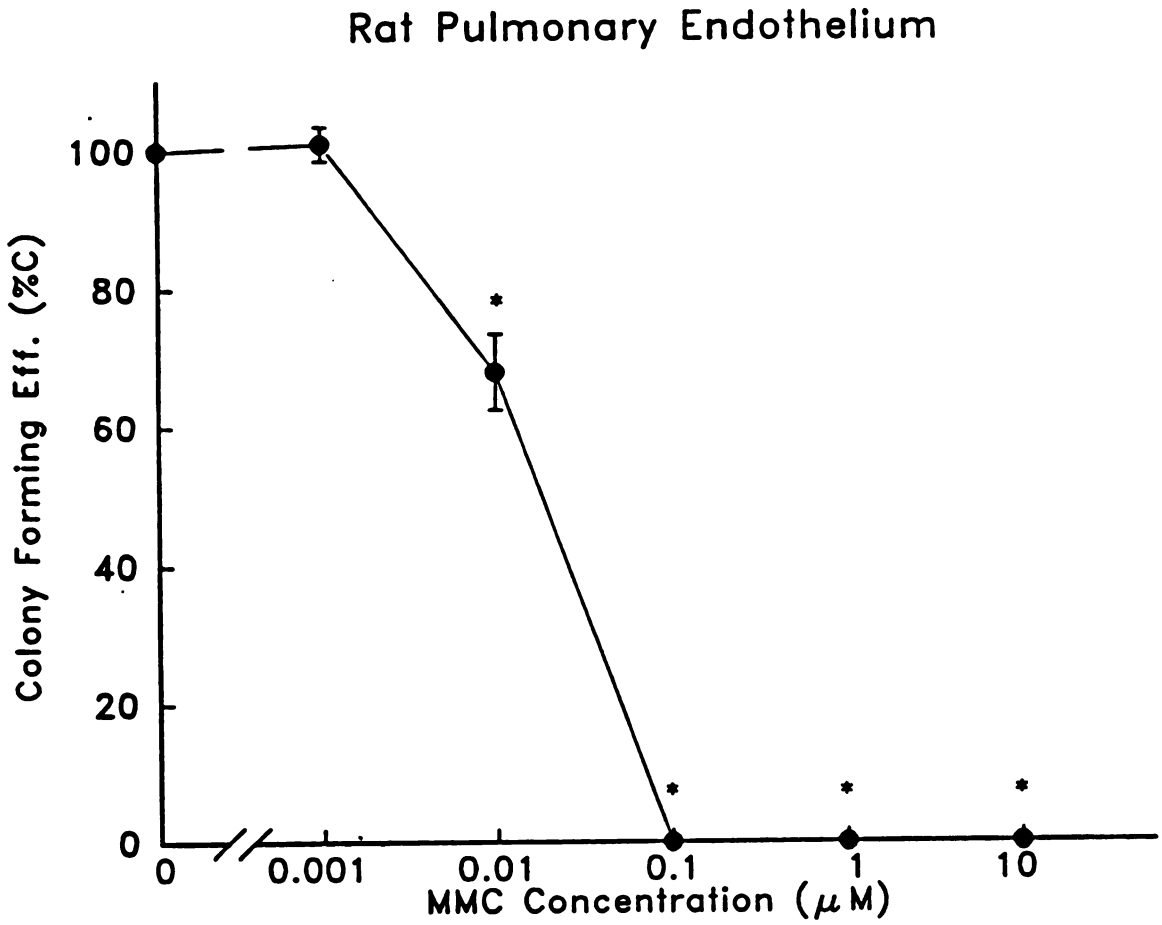


Figure 40: Effects of MMC on colony-forming efficiency of RECs.

RECs plated at very low density received a single administration of MMC on day 0. Colonies were fixed, stained and counted 14 days later. Values represent means +/- SEM of three replicate studies. * Significantly different from vehicle control ($p<0.05$)

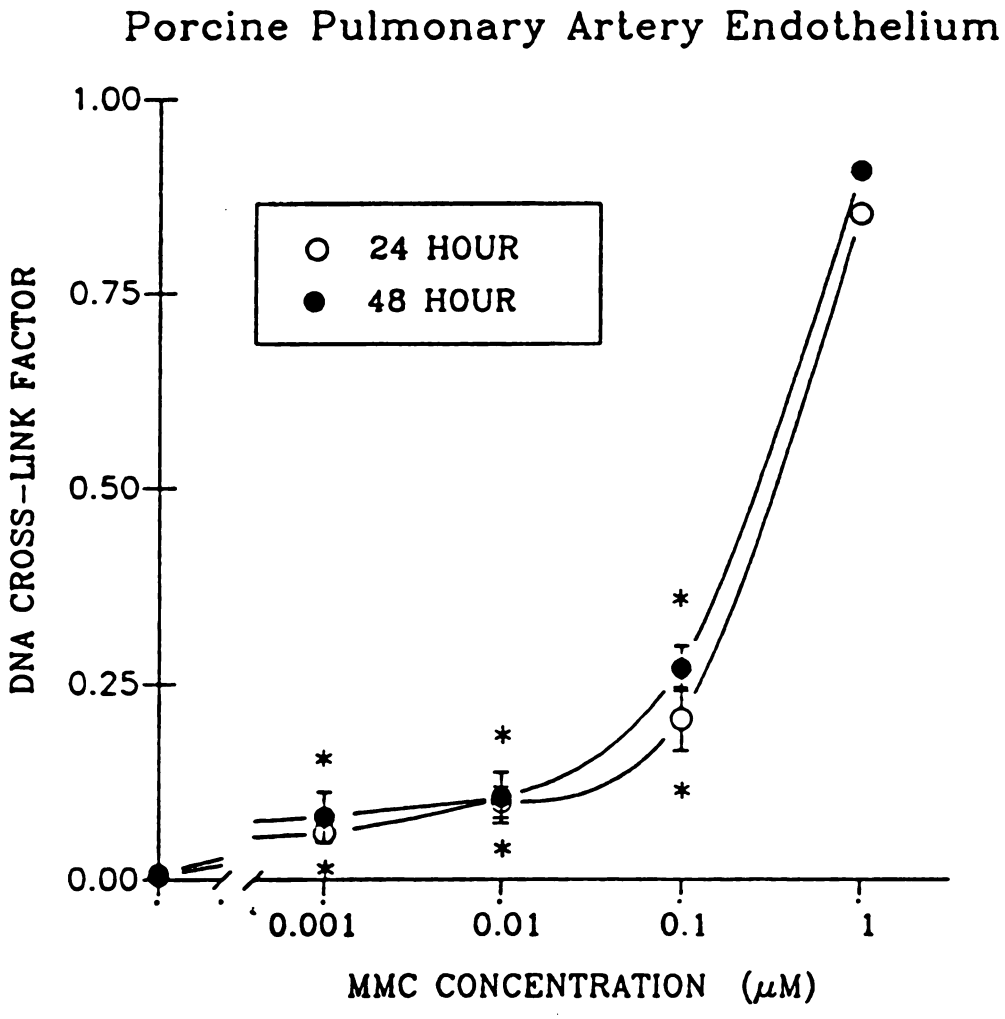


Figure 41: Total DNA crosslinking in PECs.

PECs were treated once with MMC at time O. Values represent means +/- SEM of replicate studies (n = 4-5 per group). * Significantly different from vehicle control ($p<0.05$)

Discussion

Treatment with MCTP causes injury to cultured ECs derived from a number of species (Reindel *et al.*, 1991) , Chapter V). PECs demonstrate subtle morphologic alterations in response to treatment with high concentrations of MCTP; they become spindle-shaped in appearance and demonstrate some nuclear enlargement. However,they are relatively insensitive to the cytolytic effects of this compound, and demonstrate only limited cell detachment and LDH release (Reindel *et al.*, 1991). RECs are much more sensitive to the cytolytic effects of MCTP and respond with marked increases in cell detachment, increased LDH release and dramatic morphologic changes in the surviving cells (Chapter V). However, MCTP causes a similar inhibition of proliferation in both PECs and RECs at concentrations which cause little or no other evidence of cytotoxicity.

MCTP treatment also results in persistent DNA crosslinking in cultured PECs and RECs. Significant crosslinking is evident after treatment with low concentrations of MCTP that cause limited cytolytic injury but significant cytostasis (Wagner *et al.*, 1992; Chapter V). These responses suggest that DNA:DNA or DNA:protein crosslinking may be responsible for the antimitotic effects of MCTP but is probably not directly responsible for its cytolytic effects.

As with MCTP, treatment of cultured PECs and RECs with the bifunctional alkylating agent, MMC, resulted in delayed and progressive injury that was concentration-dependent. MMC caused cytostasis at lower concentrations, and increased cell detachment and release of LDH were evident at higher concentrations.

DNA crosslinking occurred in PECs at concentrations that caused marked inhibition of proliferation but only modest cytolysis. Accordingly, MMC causes an EC response that is quite similar to that caused by MCTP.

The cytolytic alterations in ECs developed somewhat more rapidly in response to MMC than to MCTP, and both PECs and RECs were sensitive to these effects. This may be a consequence of the concentrations of MMC used in this study. Nevertheless, the endothelial response (i.e., cell spreading, enlargement and shape change) to a loss of monolayer integrity was very consistent within each species to both MCTP and MMC.

Although the effects of MMC on cultured endothelium are similar to those reported for MCTP-treated endothelium, the concentrations required to cause these effects are quite different. Concentrations of 1 μM MMC caused changes in monolayer cellularity and morphology comparable to concentrations of 150 μM MCTP or greater, and the DNA crosslink factor associated with MMC-induced cytostasis was much lower than that associated with an inhibition of proliferation by MCTP {Reindel *et al.*, 1991; Chapter V). The amount of bioactive MMC metabolite may, in fact, be much lower than the nominal concentrations indicated, thus MMC appears to be a much more potent EC toxicant than MCTP.

MMC, like MCT, is metabolically activated to one or more bifunctional alkylating species; covalent binding to DNA and other cellular macromolecules occurs only after reduction. Under aerobic conditions, NADPH:cytochrome P_{450} reductase or xanthine oxidase are thought to be responsible for bioactivation (Keyes *et al.*, 1984; Pan *et al.*, 1986). However, under aerobic conditions, reduction by DT-

diaphorase is thought to predominate, and inhibition of this enzyme by dicumerol increases resistance to the toxicity of MMC (Ernster, 1967; Keyes *et al.*, 1985; Dulhanty *et al.*, 1989). Endothelial DT-diaphorase activity has not been identified per se in the literature; however, the results of these studies and those of others (Duperray *et al.*, 1988) in cultured ECs would suggest that these cells are able to bioactivate MMC to a cytotoxic metabolite that crosslinks DNA.

MMC is thought to exert its cytotoxic effects by covalent binding of a reductively activated metabolite to DNA or other cellular macromolecules and/or by the formation of reactive oxygen species as a result of redox cycling (Dulhanty *et al.*, 1989; Monks *et al.*, 1992; Iyer and Szybalski, 1963). DNA:DNA or DNA:protein crosslink formation by the quinone methide or aziridinomitosene metabolites of MMC, or by reactive MCTP, may be responsible for their common antiproliferative effects, resulting in an endothelial monolayer that is defective in its capacity to effect repair. MMC and MCTP demonstrate a similar sequence preference for DNA binding, resulting in crosslinking between deoxyguanosine residues on complementary strands at 5'-CG duplex sequences (Niwa *et al.*, 1991; Weidner *et al.*, 1990). MMC and MCTP also have a similar distance between electrophilic centers, and these features may result in crosslinking at similar sites in EC DNA and consequently similar effects.

Cell death may ensue as a delayed response to the DNA damage, but there may be other, dissimilar toxic consequences of exposure to MMC or MCTP which are responsible for the cytolytic effects of these compounds, such as alteration of calcium homeostasis, alkylation of cellular proteins or redox cycling and resultant

oxidative stress. For example, the quinone function of MMC, which MCTP lacks, may contribute to the direct cytotoxic effects of this compound (Monks *et al.*, 1992).

Cell detachment or lysis results in the formation of gaps in the EC monolayer, providing a stimulus for cell migration, spreading and proliferation. Although MMC and MCTP may cause a loss of monolayer integrity by similar or dissimilar mechanisms, they similarly impair the ability of ECs to respond to a stimulus for proliferation. Thus, a critical consequence of persistent DNA crosslinking in the endothelium may be an altered capacity for monolayer repair, which leaves the vascular wall vulnerable to endothelial disruption of any sort, including cell death in response to disease, toxic insult or aging.

Lung vascular injury is not a common sequel to chemotherapy with MMC *in vivo*, but a number of cases have been reported after intravenous administration of this compound. EC alterations, vascular leak, arterial medial hypertrophy, and the development of pulmonary hypertension and right ventricular enlargement are characteristic of the pulmonary vascular damage which can result from treatment with MMC. Similar changes have been reported in the lungs of animals exposed to MCT(P). The pneumotoxic effects of both MMC and MCTP typically are delayed in onset and progressive in nature (McCarthy and Staats, 1986; Chang *et al.*, 1986; Jolivet *et al.*, 1983; White and Roth, 1989). The formation of fibrin thrombi in capillaries, increased vascular permeability and other evidence of endothelial dysfunction have been reported after administration of both compounds, and the development of hemolytic anemia after MMC treatment has been attributed to changes in vascular EC function (McCarthy and Staats, 1986; Jolivet *et al.*, 1990;

Valdivia *et al.*, 1967a,b; Merkow and Kleinerman, 1966b). Thus, not only are the effects of MMC and MCTP on cultured endothelium very similar, but the consequences of exposure of the pulmonary vasculature to these agents *in vivo* are similar as well.

In conclusion, the nature of the endothelial response to MMC demonstrated by these studies *in vitro* and the spectrum of reported pulmonary vascular alterations as a result of treatment with this agent *in vivo* bear a marked similarity to those reported previously for MCTP. These compounds are derived from different sources, are activated by different mechanisms, and possess some distinct chemical properties; nevertheless, they share a common pyrrolizidine nucleus and both are bioactivated to bifunctional alkylating species. Their shared ability to crosslink DNA (and other macromolecules) may underlie the inhibition of proliferation seen after administration of both of these agents and may contribute to the unusual, delayed and progressive endothelial response to MMC and MCTP treatment *in vitro*. Similar effects on the capacity for proliferation and repair in the endothelium of pulmonary vessels *in vivo* may contribute to the evolution of chronic lung vascular disease caused by these, and perhaps other, bifunctional alkylating agents.

SUMMARY AND CONCLUSIONS

I began these studies hoping to determine whether there existed changes in EC function in response to MCTP treatment that were consistent with an endothelial contribution to the development of MCTP-induced pulmonary vascular alterations. MCTP treatment does affect basic cellular function of ECs, increasing their synthesis of DNA, RNA and protein in a delayed and progressive manner. This is an important point in understanding the endothelial response to injury. In the study of cancer chemotherapeutic agents, inhibition of proliferation is typically considered to be synonymous with cell death (Rosenblum *et al.*, 1978). Our work with MCTP suggested that these conditions should not be viewed as equivalent as they apply to chronic lung injury; cells may be inhibited in their ability to divide yet remain viable and continue to function, though perhaps in an altered manner (Hoorn and Roth, 1992). Although the specific profile of proteins produced by MCTP-treated cells remains unknown, the fact that these cells continue to function and, in fact, synthesize increased amounts of RNA and protein after treatment suggests that ECs could contribute to the vascular alterations seen after MCT(P) treatment.

My efforts to define some of the specific EC functional alterations in response to MCTP met with limited success. ACE activity decreased in response to MCTP treatment, but only at high concentrations. In addition, the delayed nature of the decrease and its correlation with the extent of overt cytotoxicity suggested that changes in ACE activity occur as part of the EC response to injury, not as a direct effect of MCTP on the enzyme itself. Although this tells us a little more about the EC response to injury, surface ACE activity of ECs does not appear to be directly affected by low concentrations of MCTP. These findings suggest that changes in

ACE activity *in vivo*, if present, likely occur as secondary changes in response to an altered physical or chemical environment for the enzyme.

The cultured EC system did not lend itself well to investigation of the role of MCTP-induced EC alterations in regulation of SMC growth and proliferation. I was unable to demonstrate increased release of mesenchymal cell growth factors from MCTP-treated ECs. It is possible that the system was incapable of upregulating the production and release of mitogens. This was difficult to determine definitively, as the basal release of EDGFs was quite high and the response to thrombin stimulation was inconsistent. However, after many modifications of the system and exhaustive repetition of these studies, it seems evident that MCTP treatment does not increase markedly the release of growth factors from constitutively active cells.

There is still a case to be made for a contribution of EC-derived mitogens in the vascular remodeling process associated with MCT(P) treatment *in vivo*. Quiescent endothelium *in vivo* could respond to injury quite differently with regard to growth factor synthesis than the model *in vitro* used in these studies. The relevant question, "Does MCTP treatment increase the expression, synthesis or release of growth factors from quiescent endothelium *in vivo*?" may be quite different from the question we actually asked, "Does MCTP augment the release of growth factors from cells *in vitro* that constitutively express these factors at high levels?". In retrospect, the cultured EC system at its present state of development is an inadequate model to address the former question. Examination of gene expression in the lung vasculature *in situ* may be a more appropriate way to investigate this hypothesis.

From these investigations with cultured endothelium treated with MCTP, we have learned a great deal about the endothelial response to injury *in vitro*. Damage to endothelial monolayers as a consequence of treatment with certain other EC toxicants (e.g., endotoxin, cyclosporin A) is typically more rapid in onset and is less persistent than the damage that results from exposure to MCTP. The limited proliferative repair capacity and the dramatic hypertrophic response seen with MCTP treatment does not occur with these more acutely toxic compounds, suggesting that delayed and progressive EC injury may represent a response by endothelium to toxic damage that is unique to certain toxicants.

The EC alterations observed after the administration of MCTP *in vitro* appear to represent a summation of responses to a number of distinct events which occur as a result of this treatment. A relatively early event is the binding of MCTP to cellular nucleophiles such as DNA. The highly reactive nature of MCTP and its instability in aqueous solution suggest that initial binding to ECs occurs shortly after administration of the compound. Since DNA crosslinking increases with time during the 48 hr period after a single administration of MCTP, this initial binding may be transient, with MCTP shifting from nucleophile to nucleophile within the cell. Bifunctional alkylating agents generally bind at one reactive center initially; if conditions are favorable and the initial site of alkylation is not repaired, the other nucleophilic center also binds, forming a crosslink. Thus, stable DNA crosslinks may take some time to form in MCTP-treated ECs. By 48 hr posttreatment with MCTP, crosslinking appears to be maximal; this suggests that either 1) final formation of

stable crosslinks has occurred by this time, or 2) the rate of crosslink formation is in equilibrium with the rate of crosslink removal.

Although DNA crosslinking is frequently associated with an inhibition of cell proliferation, it is difficult to determine conclusively whether a cause/effect relationship exists. Linking of complementary strands of DNA should hinder strand separation and progression of the replication fork, thus physically impeding the mitotic process. In support of this theory, most crosslinking agents are cycle active and arrest cells late in M phase, during G_1 phase, or early in S phase; cells are least sensitive during late S phase (Meyn and Murray, 1984; Murray and Meyn, 1986). Bifunctional alkylating agents are typically more cytostatic than their monofunctional counterparts (Mattocks and Driver, 1983; Mattocks and Legg, 1980; Tokuda and Bodell, 1987; Bodell, 1990), suggesting that a bifunctional event is critical; however, crosslinking of other vital cellular proteins may occur coincident with DNA crosslinking, and these may have antiproliferative effects, too. Nevertheless, DNA crosslinking remains a plausible mechanism underlying the antimitotic effects of such agents.

The ability of ECs in a blood vessel to respond to a loss of monolayer integrity by spreading, migrating into the defect and proliferating is probably critical to the repair capacity of this tissue. Unrepaired disruption of the intimal surface of the blood vessel or capillary wall can result in increased vascular permeability, increased thrombogenicity of the intimal surface, and altered vasoactivity, perhaps with severe consequences to the surrounding tissues. Migration and spreading of ECs may be sufficient to maintain vascular integrity temporarily in the face of limited,

discrete monolayer disruption; however, proliferation is vital in the repair of larger defects or more diffuse intimal damage.

MCTP-treated ECs are able to migrate and spread but are persistently impaired in their ability to divide. DNA crosslinking has frequently been associated with cytostasis. This primary defect in ECs treated with MCTP (e.g., crosslinking of DNA or other critical macromolecules), with the consequent inhibition of proliferation, may account for the characteristic pattern of injury seen *in vitro*. PECs treated with a single administration of MCTP exhibit little cell detachment or lysis but are inhibited in their ability to proliferate. Monolayers of treated PECs demonstrate only subtle morphologic alterations, and synthesize DNA, RNA and protein at slightly elevated levels. However, if the monolayer is artificially disrupted, or if the cells are treated before they reach confluence, they enlarge dramatically, become vacuolated, have marked nuclear changes, and synthesize substantially larger amounts of DNA, RNA and protein. Thus, a stimulus for proliferation appears to unmask the effects of persistent crosslinking in these cells.

There are differences in the response of ECs derived from different species and sources to MCTP treatment. In the case of BECs or RECs, treatment with MCTP causes significant cell detachment and cytolysis. This may occur as a delayed response to the DNA damage or may be due to other alterations which occur as a result of MCTP treatment. In any case, the cytolytic response results in a loss of monolayer integrity and provides a stimulus for cell spreading and proliferation. Surviving cells are unable to divide, but they do spread and enlarge greatly. Thus,

although MCTP causes cytolytic damage in these cells which is not evident in PECs, the basic response to injury is the same in all three cell types.

Our studies with MMC suggest that DNA crosslinking and the resultant inhibition of proliferation may be the common denominator in the atypical EC response to injury. MMC and MCTP may cause overt cytolytic changes by very different mechanisms, but they share a common ability to crosslink DNA and the response of MMC- or MCTP-treated endothelial monolayers to a loss of monolayer integrity is very similar. Further investigation with this comparative model should provide many answers with respect to the role of DNA crosslinking in the inhibition of proliferation and in the development of other functional alterations in ECs, such as changes in DNA, RNA and protein synthesis.

In addition to their ability to inhibit cell proliferation, DNA alkylating agents have the potential to influence gene expression. Gene amplification may occur as a result of endoreduplication of the genome when there is an arrest of proliferation but continued DNA synthesis. MCTP-treated PECs continue to synthesize DNA and demonstrate an increased cellular DNA content (Chapter III). Some of the increased RNA and protein synthesis which is evident in MCTP-treated ECs may reflect the expression of genes amplified in this manner, and this possibility requires further investigation. In addition, the act of binding to DNA necessitates repair, and increased rates of repair may lead to defective repair and alterations in DNA sequence, folding and transcription. Furthermore, the very nature of interstrand DNA crosslinks may increase the likelihood of inaccurate repair. Future studies which identify specific alterations in EC function in response to MCTP and MMC

may point to areas of the genome on which to focus a search for critical DNA alterations.

In order to demonstrate a cause-and-effect relationship for DNA crosslinking and various EC responses, it will be necessary to identify crosslink formation after administration of relevant concentrations of MCTP or MMC. Crosslinking should occur at critical posttreatment intervals which precede or coincide with the effects of interest, and it should persist . Conditions which enhance crosslinking should enhance the cytotoxic effects. Ideally, we should also be able to inhibit crosslink formation and prevent occurrence of the EC effects. Although several of these criteria have been met by MCTP and MMC, there is still much work to be done before DNA crosslinking is can be established as a mechanism for the chronic pulmonary vascular injury caused by these compounds. This work may be confounded by the fact that we still know very little about the extent of crosslinking required for cytotoxic injury; and we have yet to confirm that the relevant crosslink formation is that which occurs in DNA. However, comparison of the EC response to MCTP treatment with the response to MMC and other, unrelated, bifunctional agents will allow us to begin to investigate these relationships.

Eventually, the results of studies performed in cultured cells will need to be confirmed in studies performed *in vivo*. DNA crosslinking in the pulmonary endothelium *in situ* has not been demonstrated; it may be difficult to detect small but relevant levels of crosslink formation in this tissue *in vivo*. Parallel studies *in vivo* utilizing MCTP and MMC (and perhaps other bifunctional agents of different

classes) may provide information about the relevance of DNA crosslinking to pulmonary vascular alterations *in vivo*.

A final area addressed by the work presented in this dissertation involves the importance of species differences in the endothelial response to MCTP. In a previous study, the responses of BECs and PECs to MCTP treatment were compared (Reindel *et al.*, 1991). We found that BECs, like RECs, were quite sensitive to the cytolytic effects of MCTP. PECs appeared to be more resistent to these effects, evidencing less cell detachment and release of LDH and only subtle changes in cell size and morphology. However, PECs and BECs were equally sensitive to the cytostatic effects of MCTP. From these studies, we concluded that species differences in sensitivity to reactive pyrrolic metabolites exist and might in part explain the species differences observed in organ pathology after exposure to MCT *in vivo*.

Similar studies utilizing cultured endothelium from a highly relevant species such as the rat have not been performed previously. The results presented here raise new questions with respect to species differences in endothelial sensitivity to MCTP and how these differences may relate to the patterns of toxicity observed *in vivo*. For example, cattle typically develop venoocclusive lesions of the liver shortly after exposure to MCT (Sanders *et al.*, 1936) rather than the chronic, progressive pulmonary vascular lesions which are more typically reported in the rat (Chesney and Allen, 1973d; Meyrick and Reid, 1979; Reindel *et al.*, 1990) or the pig (Peckham *et al.*, 1974). The former lesions might be attributable to a rapid deterioration of the

hepatic vasculature after interaction of the reactive metabolite(s) of MCT with sensitive bovine endothelial cells. On the other hand, in a species with endothelium that is less susceptible to cytolytic injury (e.g., porcine), changes in the hepatic vasculature might be more subtle, allowing time for the consequences of extensive but delayed, progressive pulmonary vascular cell injury to be expressed (Reindel *et al.*, 1991). Assuming this scenario, one might predict that the endothelium of the rat would resemble that of the pig in its sensitivity to MCT. However, this does not appear to be the case; in fact, RECs more closely resemble BECs in their response to injury by MCTP *in vitro*.

Presumably, species differences in ability to generate the necessary toxic metabolite(s) in sufficient quantity from the parent compound and differences in distribution of the reactive metabolites once they are formed may influence the pattern of organ toxicity seen after exposure to MCT (Chesney *et al.*, 1974a; Cheeke and Pierson-Goeger, 1983; Mattocks, 1968). In large animals, susceptibility of the endothelium to injury by MCTP may be quite important in determining the ultimate effects of a broadly distributed dose of toxicant. Bovine endothelium is very sensitive to the cytolytic effects of MCTP, and the deterioration of the liver in this species after MCT ingestion may reflect endothelial injury at the site of metabolite generation. Delayed pneumotoxic injury is more commonly seen in the pig, perhaps because endothelial injury in both the liver and the lung is more subtle, allowing time for pulmonary consequences of inhibited EC proliferation to develop. In addition, the relatively longer time required for an unstable hepatic metabolite to reach the lungs via the circulation in larger animals may reduce pulmonary susceptibility.

Given the short distance from the liver to the lung and the extremely rapid blood circulation time in a small animal like the rat, a larger proportion of metabolite may reach the lung vasculature. In this case, the sensitivity of the endothelium may be of secondary importance compared to the relative concentration of metabolite reaching the pulmonary vascular bed.

Finally, it is important to consider that the species differences observed in response to this compound *in vitro* may be due to inherent differences which are expressed when endothelial cells of different species are maintained in culture. These may include differences in matrix production, glycocalyx composition, protein production or a number of other factors. Although these species differences have not been investigated in detail, a number of differences have been reported between endothelial cells of large and small vessel origin (Kumar *et al.*, 1987; Gumkowski *et al.*, 1987; Stolz and Jacobson, 1991). Some of these may be important in interpreting the cellular response to injury, as the BECs and PECs used in many of these studies were of pulmonary artery origin, whereas the RECs used in these investigations were isolated from smaller, intrapulmonary vessels.

Clearly, much work remains to be done, both in identifying specific, relevant functional alterations in ECs in response to MCTP treatment, and in determining the role of DNA crosslinking in the development of these alterations. These studies in cultured endothelium have established that changes occur *in vitro* which appear to be consistent with the development of chronic vascular alterations *in vivo*, but eventually these observations must be confirmed in the pulmonary vasculature of

treated animals *in situ*. Although one must bear in mind the limitations of the cultured cell system, this model of EC injury should continue to provide important information on mechanisms of MCTP pneumotoxicity, as well as on the role of this pluripotent tissue in the development of vascular disease.

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