TUMOR PROMOTING EFFECTS, MORPHOLOGICAL EVALUATION AND IMMUNOHISTOCHEMICAL DETECTION OF RAS P21 PROTEIN IN PRENEOPLASTIC AND NEOPLASTIC LESIONS OF INITIATED RATS TREATED WITH PHENOBARBITAL AND/OR 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

By

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ABSTRACT

TUMOR PROMOTING EFFECTS, MORPHOLOGICAL EVALUATION AND IMMUNOHISTOCHEMICAL DETECTION OF RAS P21 PROTEIN IN PRENEOPLASTIC AND NEOPLASTIC LESIONS OF INITIATED RATS TREATED WITH PHENOBARBITAL AND/OR 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN

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In an initiation-promotion protocol, Sprague-Dawley rats were initiated with 10 mg/kg nitrosodiethylamine (NDEA) and promoted with either 500 ppm of phenobarbital (PB) until day 170 or 150 ppt of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) until days 170, 240 or 450. Rats were killed at 170, 240 and 450 days. The objectives of the first study were to determine if TCDD would inhibit the regression of altered hepatocellular foci (AHF) induced by PB and to assess the promoting effects of a low dose of TCDD. TCDD had a strong promoting effect even when administration was delayed after initiation. Mean volume of AHF was significantly larger when TCDD was given continuously. Phenobarbital increased tumor incidence at 450 days however, a significant increase in the number of AHF/cm^3 of liver was not found. Thus, whether or not TCDD inhibited the regression of PB-induced foci could not be determined. The objectives of the second study were to characterize AHF in hematoxylin and eosin stained paraffin sections. AHF consisted of clear cell, eosinophilic, vacuolated, mixed and basophilic foci. At day 170, initiated rats promoted with

TCDD had a higher incidence of basophilic AHF compared to initiated rats promoted with PB. At day 240, initiated rats promoted with TCDD had eosinophilic AHF with spongiosis hepatis and basophilic AHF including diffuse, atypical and tigroid, whereas initiated rats promoted with PB and subsequently TCDD had a higher incidence of atypical and diffuse basophilic AHF and eosinophilic foci with peliosis hepatis. The objectives of the third study were to determine if the oncogene product ras p21 protein was present in preneoplastic and neoplastic lesions. This protein was present in AHF (104/124), hepatic nodules (11/13) and hepatocellular carcinomas (9/9). Cytoplasmic and plasma membrane staining were the patterns of reactivity observed. Since neoplastic transformation is associated with membrane staining, it is proposed that foci with this pattern of staining may have a greater propensity for developing into neoplastic lesions. This protein expression was an early event and may be associated with the proliferative process during carcinogenesis.

Dedicated with lots of love to
my wife, Nancy

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KEY TO ABBREVIATIONS

TCDD....................2,3,7,8-tetrachlorodibenzo-p-dioxin
DAB.....................3,3-diaminobenzidine
ATPase..................Adenosinetriphosphatase
AHF.....................Altered hepatocellular foci
F344....................Fischer 344
GGT.....................Gamma glutamyltranspeptidase
G6Pase..................Glucose-6-phosphatase
GST-P...................Glutathione-S-transferase
HBB.....................Hexabromobiphenyl
NDEA....................Nitrosodiethylamine
PB......................Phenobarbital
PBS.....................Phosphate buffered saline
PBB.....................Polybrominated biphenyl
PCB.....................Polychlorinated biphenyl
PHAHs...................Polyhalogenated aromatic hydrocarbons

INTRODUCTION

INTRODUCTION

Several approaches have been used to identify environmental agents which are potentially carcinogenic to humans. Chemicals in the environment are assessed on the basis of epidemiologic evidence from exposed human populations, with supportive evidence derived from short-term tests that correlate with carcinogenicity. These chemicals are classified as potentially hazardous to humans on the basis of medium- and long-term carcinogenesis studies in rodents.

Most short- and mid-term bioassays in rats use altered hepatocellular foci (AHF) as an endpoint in evaluating chemicals for tumor promotion and carcinogenic potential. Tumor promoters and hepatocarcinogens cause an increase in the number and size of AHF prior to the appearance of hepatic nodules and hepatocellular carcinomas.

Many AHF but only a few tumors develop as a result of carcinogen treatment, thus it is important to determine morphologically which foci are most likely to develop into tumors. In previous research, quantitation and characterization of AHF was limited to the evaluation of histochemical staining. Recently emphasis has been placed on evaluating AHF in hematoxylin and eosin (H&E) stained paraffin embedded sections. In H&E stained sections, cells in AHF may have a clear, eosinophilic, vacuolated, mixed or basophilic appearance (Squire, and Levitt, 1975; Maronpot et al. 1986; Bannasch, 1988). It is proposed that the sequence of cellular changes during hepatocarcinogenesis progresses from clear and eosinophilic cell foci through mixed cell foci and nodules to basophilic cell populations prevailing in hepatocellular carcinomas (Bannasch et al. 1985).

In addition to determining which AHF are most likely to develop into tumors, it is also critical to determine at the molecular level which genes are important in the carcinogenesis process. Two major categories of genes appear to play essential roles in mechanisms of carcinogenesis. These genes include proto-oncogenes and tumor suppressor genes. This study will focus on proto-oncogenes.

Proto-oncogenes (c-onc) are normal cellular genes present in a cell which play key roles in growth control (Pimentel, 1986a; Seemayer and Cavenee, 1989). They influence cell proliferation and differentiation (Travali et al. 1990). When proto-oncogenes are overexpressed, mutated, or deregulated they can be associated with transformation of cells (Bishop, 1987; Seemayer and Cavenee, 1989; Vorce and Goodman, 1990).

Of the many proto-oncogenes associated with cancer, the ras family of genes frequently shows increased or altered expression in human and animal tumors (Bos, 1988, Fiorucci and Hall, 1988). The ras genes consist of three functional genes, Ha-ras, Ki-ras, and N-ras which encode similar proteins (p21) with molecular weights of 21,000 (Barbacid, 1987). Mutations in specific amino acids and overexpression of normal proteins have been linked to altered proliferation and/or differentiation and, particularly to the neoplastic process.

Previous studies in our laboratory have shown that a number of polyhalogenated aromatic hydrocarbons (PHAHs) are potent hepatic tumor promoters in initiation-promotion models in rats (Jensen et al. 1982,1984; Rezabek et al. 1987; Dixon et al. 1988; Evans, 1989). These PHAHs include polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB) and congeners 2,2',4,4',5,5'-hexabromobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl and 3,4,3'4'-tetrabromobiphenyl. Included in this group of PHAHs is the environmental contaminant 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD).

The following studies were designed to further define the tumor promoting effects of TCDD in rats, characterize altered hepatocellular foci in H&E stained sections and determine which lesions are important in the carcinogenesis process, and determine if the ras p21 protein is consistently present throughout multistage carcinogenesis or is present only in specific stages of the process.

LITERATURE REVIEW

LITERATURE REVIEW

Multistage concepts of carcinogenesis

Carcinogenesis is defined as the natural history of neoplastic disease (Pitot et al. 1989b). It occurs in two or more sequential stages in a number of *in vivo* (Scribner and Suss, 1978; Pitot and Sirica, 1980b) and *in vitro* (Mondal et al. 1976; Barrett, 1980; Yuspa et al. 1981) histogenetic systems. Chemical carcinogenesis was first recognized as a multistage process by the work of Yamagiwa and Ichikawa (1918) who induced skin tumors in rabbits by using coal tar followed by scarlet oil. In studies undertaken by Rous and Kidd (1941), Mottram (1944) and Berenblum and Shubik (1947) the concept of carcinogenesis occurring in two discrete phases termed initiation and promotion became clearer. Since the early experiments on the mouse skin, numerous investigators have developed initiation-promotion assays in organ systems such as the liver, mammary gland, lung, stomach, colon, thyroid gland and pancreas (Berenblum, 1979; Pitot et al. 1988b).

Current evidence suggests that multistage carcinogenesis involves at least three stages defined as initiation, promotion and progression (Weinstein et al. 1984; Pitot et al.

1988c, Pitot, 1988a). The stages have been most successfully identified in skin and liver models of carcinogenesis (Farber and Sarma, 1987; Fischer et al. 1988).

Initiation

Initiation as the name implies, is viewed as the first event in the carcinogenesis process, occurring either spontaneously or from the damage incurred by a carcinogen (Scribner and Suss, 1978; Peraino et al. 1983). Generally only a small fraction of cells in an exposed organ are targeted by initiating agents (Scherer, 1984a). The process of initiation seems to involve an irreversible genetic event in a single cell which has the capacity to proliferate (i.e., a stem or progenitor cell) (Pitot, 1988a; Trosko et al. 1990a). It is postulated that the process of initiation prevents a stem cell from terminally differentiating (Potter, 1978; Trosko et al. 1990a). Initiation has a minimum of two steps which include the genesis of a biochemical or molecular lesion and fixation of one or more biochemical changes by a round of cell proliferation (Scherer and Emmelot, 1975b; Ishikawa et al. 1980; Farber and Sarma, 1987).

Most chemicals that are carcinogenic in the rat liver are metabolized to their ultimate forms before they can initiate hepatocytes (Pitot, 1988a; Guengerich, 1988). Metabolic activation of chemicals is primarily by the cytochrome P-450 dependent monooxygenase system located mainly in microsomes but also in nuclei (Miller and Miller, 1969; Farber and Sarma, 1987; Pitot, 1988a). The exact nature of the biochemical lesion or lesions is unknown. There is considerable evidence that alterations in DNA, including the formation of DNA adducts by carcinogens, are probably linked to the initiation process (Farber and Sarma, 1987). For example, the alkylating agent and initiator nitrosodiethylamine (NDEA) forms adducts with guanine and adenine bases in DNA (Hemminki, 1983), while the aromatic amine N-2-acetylaminofluorine, following metabolic activation, binds to the C-8 position of guanine residues. The result is a major distortion of the DNA helix which is termed base-displacement (Weinstein, 1981; Grunberger et al. 1985; Weinstein, 1988).

Initiating carcinogens may cause mutations in normal cellular genes (proto-oncogenes) to produce activated proto-oncogenes (oncogenes) which may lead to abnormalities in growth control and differentiation (Weinstein, 1988). There is evidence accumulating that several types of tumors induced in rodents by chemical carcinogens and certain tumors in humans are associated with base pair substitution at specific sites in ras proto-oncogenes (Bishop, 1985; Barbacid, 1987; Weinstein, 1987). Some mouse skin tumor initiating agents appear to cause a specific mutation in cellular oncogenes. For example, many tumors induced in the mouse initiation-promotion protocol using 7,12-dimethylbenz(a)anthracene (DMBA) as an initiator and 12-O-tetradecanoylphorbol-13 acetate (TPA) as a promoter contained an activated c-Ha-ras gene at the 61st codon (Quintanilla et al. 1986).

In addition to proto-oncogenes, DNA sequences that are normally present in the mammalian genome and transcriptional regulatory sequences may also be critical targets during initiation (Weinstein, 1987, 1988). Other biochemical alterations in DNA that may represent relevant changes of the cellular genetic material which could lead to initiation and neoplasia include deletions, chromosomal translocations, amplifications and transpositions within the genome (Bishop, 1985; Weinstein, 1987, 1988).

A critical step in the initiation process is the requirement of cell proliferation for "fixation" of DNA alterations (Columbano et al. 1981; Ying et al. 1981; Farber and Sarma, 1987). Such proliferation can be induced by partial hepatectomy, toxic necrosis produced by a carcinogen or non-specific agent and by physiological growth in neonates (Pitot, 1988a).

Initiated cells are resistant to a variety of cytotoxic chemicals and therefore have a selective proliferative advantage when compared to normal cells (Schulte-Hermann et al. 1981; Farber and Sarma, 1987). The relative effect of initiating agents depends on quantitation of focal lesions following a defined period of promotion (Pitot et al. 1988b,c).

Promotion

Promotion is the process whereby an initiated cell in an organ or tissue develops focal proliferations such as altered hepatocellular foci in the liver, papillomas in the skin and polyps in the colon (Hicks, 1983; Slaga, 1983; Bannasch, 1986b; Cerutti, 1988). One or more of these lesions act as precursors for subsequent steps in the carcinogenic process (Emmelot and Scherer, 1980; Farber and Sarma, 1987). The sequence of initiation followed by promotion is critical to the development of cancer. Omission of either stage results in no or a significantly lower yield of hepatocellular tumors (Peraino et al. 1983; Pitot et al. 1988b).

The most distinctive characteristic of tumor promotion which distinguishes it from the stages of initiation and progression is reversibility (Takahashi et al. 1982; Tatematsu et al. 1983; Moore et al. 1983; Glauert et al. 1986). Boutwell et al. (1964) were the first to demonstrate that reducing the frequency of application of a promoting agent decreased or eliminated the induction of neoplasms. In some model systems of hepatocarcinogenesis the number of altered hepatocellular foci decrease when the promoting stimulus is removed (Takahashi et al. 1982; Tatematsu et al. 1983; Moore et al. 1983; Pitot et al. 1988c; Pitot, 1988a). Tatematsu et al. (1983) using the resistant hepatocyte model stated that the "disappearance" of enzyme altered foci was the result of "remodelling" from their altered form to normal hepatocytes.

Alternatively, Bursch et al. (1984) and others (Columbamo et al. 1984; Garcea et al. 1989) indicated that the disappearance of focal lesions was due to apoptosis (individual cell death). Promotion is therefore dependent on chronic administration of an agent which among other things, causes clonal expansion of initiated cells (Boutwell, 1974; Barrett, 1980; Trosko and Chang, 1989b).

Modulation of tumor promotion by physiological and environmental factors such as aging, diet and hormones is well known (Van Duuren et al. 1975; Sivak, 1979). In animals fed a semisynthetic diet, promotion was less effective than in animals fed a crude cereal-based diet (Glauert et al. 1986). Adrenalectomy and thyroidectomy inhibited hepatocarcinogenesis when aromatic amines were continuously administered (Solt and Farber, 1976).

Conversion of initiated cells to tumor cells require two or more discrete changes, "hits", which represent genomic mutations (Emmelot and Scherer, 1977; Moolgavkar, 1986). Efficient clonal expansion during promotion increases the probability that initiated cells will undergo a second specific mutation (Stout and Becker, 1982). The resultant new cell will again develop into a cell clone which eventually will give rise to a cell with a third specific mutation. In this way initiated cells may progress and develop into tumors (Scherer, 1984).

Progression

The stage of progression is irreversible (Pitot, 1989a). It is the stage at which focal lesions develop into benign or malignant neoplasms (Schulte-Hermann, 1985; Pitot, 1986). A critical event in the progression process may involve a mutation-like event (Hennings et al. 1983; Nowell, 1986). Papillomas progressed to carcinomas when mice were treated with direct acting carcinogens ethylnitrosurea (ENU), N-methyl-N'-nitro-N-nitroguanidine (MNNG) or urethane (Hennings et al. 1983; O'Connell et al. 1986). Progression was not enhanced by treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, a non-genotoxic carcinogen.

Schere (1984) were the first to develop a progression model (initiation-promotion-initiation protocol) in the rat liver. By this regimen, a relatively large number of focal lesions developed within preexisting altered hepatocellular foci, termed foci-in-foci. Foci-in-foci were felt to arise by a second genetic event; the first genetic event being that of initiation. Pitot and his associates (1988c,1989c) recently modified the procedure of Scherer (1984). A two to four fold increase in the number of foci-in-foci were induced in animals subjected to the initiation-promotion-initiation protocol. By quantitating the number of foci-in-foci it was possible to determine the progression potential of a chemical. The entire process of initiation-promotion-initiation was similar to the multihit concept first developed by Knudson

(1971).

Current evidence derived from the retinoblastoma (Friend et al. 1988; Weinberg, 1988; Seemayer and Cavenee, 1989) and Wilms' tumor models (Seemayer and Cavenee, 1989) also indicates that at least two mutational events are needed to trigger the cancer phenotype; the second event, deletion of tumor suppressor genes being important in progression.

A characteristic cellular alteration which occurs during the stage of progression is karyotypic instability (Pitot, 1989a). Cells isolated from livers of animals subjected to an initiation-promotion-initiation protocol had a marked degree of aneuploidy (Pitot et al. 1989c). These findings strongly support the concept that foci-in-foci reflect the earliest beginnings of the stage of progression in rat hepatocarcinogenesis.

Specific proto-oncogenes may be important in progression. The proto-oncogene c-raf-1 was expressed at higher levels in hepatic nodules and hepatocellular carcinomas (Pitot et al. 1988c). The actual role of c-raf-1 in these tumors was not certain. Mutational activation of the c-raf gene was detected in NIH 3T3 cells transfected with DNA from a rat hepatocellular carcinoma (Ishikawa et al. 1985b) and a human gastric tumor (Shimizu et al. 1985). These findings support the hypothesis that the expression of raf-1 may be important in the later stages of carcinogenesis. Transcriptional activation of c-raf-1 may be used as an indicator of preneoplastic lesions with a potential to develop into hepatic neoplasms.

Mechanisms of tumor promotion

The mechanisms of hepatic tumor promotion are not completely known. Current evidence supports the theory that tumor promoters act through nongenotoxic mechanisms, that is, their primary target of action does not involve direct alterations of DNA (Butterworth, 1987; Lutz and Maier, 1988). Proposed mechanisms of tumor promotion include receptor binding and alteration of gene expression, inhibition of gap junctional intercellular communication, oxidant injury and suppression of immune surveillance.

Receptor binding and gene expression

One of several working hypotheses suggest that protein kinase C (PKC) plays a central role in tumor promotion (Chouroulinkov et al. 1989). PKC is the major cellular receptor for the potent skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Jeng et al. 1985; Mills and Smart, 1989). PKC is also a major component in the signal transduction system which exerts normal control over growth and differentiation (Nishizuka, 1984).

When TPA binds to PKC, it stimulates the activation of Ca^{2+} and the phospholipid-dependent enzyme protein kinase C (Ashendel, 1985). TPA-stimulated PKC is thought to phosphorylate serine and threonine residues of critical target proteins which may directly or indirectly regulate the expression of specific genes associated with tumor promotion. TPA may phosphorylate proteins which are specific transcription factors (Nishizuka, 1984). Induction of ornithine decarboxylase (ODC), DNA synthesis and epidermal hyperplasia are biochemical and cellular events closely associated with TPA tumor promotion (Mills and Smart, 1989; Gilmour and O'Brien, 1989). ODC induction by TPA is one of the earliest changes in gene expression during tumor promotion (Mufson, 1984; Ashendel, 1985). Other effects of PKC include arachidonic acid release and inhibition of gap junctional communication (Boreiko et al. 1989; Klann et al. 1989).

A wide variety of hepatic tumor promoting chlorinated hydrocarbons stimulate PKC activity in vitro (Moser and Smart, 1989). Whether stimulation of PKC activity by chlorinated hydrocarbons could increase hepatic DNA synthesis, cell proliferation and promote hepatic tumors is unknown (Brooks et al. 1989). A number of structurally unrelated hepatic, colonic and skin tumor promoters including mezerene (Arcoleo and Weinstein, 1985), chloroform (Roghani et al. 1987), teleocidin, aplysiatoxin (Roghani et al. 1987) and diacylglycerol (Kishimoto et al. 1980) stimulated PKC, suggesting an involvement of PKC in tumor promotion. Bombick et al. (1988) indicated that the tumor promoter 2,3,7,8-tetrachlorodibenzo-p-dioxin owed part of its potency to its ability to stimulate the expression of a family of DNAs bearing homology to the viral oncogene v-erb-A and that one of the major actions was stimulation of various tyrosine kinases.

An active form of PKC, the product of cellular and viral genes, may also be important in carcinogenesis (Pimentel, 1986d; Tronick and Aaronson, 1988). Cytoplasmic oncoproteins raf and mos have serine-threonine kinase activity and may function in a similar manner to PKC (Druker et al. 1989). PKC appears to be a common component in mechanisms of tumor promotion (Nishizuka, 1984), chemical carcinogenesis (Verma, 1988) and viral oncogenesis (Pimentel, 1986d).

Another important receptor, the aromatic hydrocarbon (Ah) receptor is associated with gene expression in hepatic tumor promotion (Okey and Vella, 1982; Eisen et al. 1983). Specific halogenated and nonhalogenated hydrocarbons bind to the cytoplasmic Ah receptor (Poland and Knutson, 1982; Harper et al. 1988). After initial binding to the Ah receptor, the ligand-receptor complex undergoes a temperature-dependent "transformation" step. The ligand-receptor complex then interacts with specific regions of DNA (Durrin et al. 1987; Harper et al. 1988). The end result is a coordinated induction of a battery of genes including cytochrome P_1-450 and aryl hydrocarbon hydroxylase (AHH) (Poland, 1984; Cresteil et al. 1987; Durrin et al. 1987). Both cytochrome P_1-450 and AHH induction are biochemical events closely associated with hepatic tumor promotion.

Inhibition of intercellular communication

The intercellular structure which mediates the flow of substances from the cytoplasm of one cell to that of another is the membrane bound-protein, the gap junction (Spray et al. 1988). Gap junctions consist of channels that span the membrane of adjacent cells. Through these channels pass ions and uncharged molecules with molecular weights less than 1,000 daltons and sizes below that of 1.5 nm. This size range allows the diffusional exchange of K^+, Na^+, Cl^-, Ca^{2+}, cyclic AMP and protein kinase (Stewart et al. 1980; Trosko and Chang, 1989a). Gap junctions serve as a channel for metabolic and signal exchange, and are important in the control of cell growth, differentiation and physiological homeostasis (Trosko et al. 1983; Boreiko et al. 1989). Gap junctional intercellular communication helps to maintain the level of critical ions, growth stimulatory substances and second messages below that needed for cell proliferation. Inhibition of intercellular communication causes an increase in critical ions, prevents the normal exchange of regulatory signals between cells, and thus leads to disturbances of differentiation and/or proliferation (Trosko et al. 1990c).

Disruption of gap junctional communication is associated with teratogenesis (Loch-Caruso and Trosko, 1985), tumor promotion (Klaunig and Ruch, 1987; Boreiko et al. 1989) and tumorigenesis (Klann et al. 1989; Trosko et al. 1990b). Many tumor promoters including phenobarbital inhibit gap junctional communication (Ruch and Klaunig, 1986; Rezabek et al. 1988). The molecular mechanisms by which tumor promoters inhibit intercellular communication is presently unknown. Tumor promoters may inhibit intercellular communication directly by acting on gap junction proteins or the plasma membrane and/or indirectly by affecting gap junction regulators (Klaunig and Ruch, 1987). Inhibition of intercellular communication by a tumor promoter may serve to isolate an initiated cell from the growth regulatory signals of its neighbor and permit clonal expansion (Klaunig and Ruch, 1987; Trosko et al. 1990c).

An increase in intracellular pH, PKC and Ca^{2+} down-regulate gap junctions, while elevated levels of cyclic AMP upregulate the number of gap junctions within the membrane (Trosko and Chang, 1988). Klaunig et al. (1987) postulated that inhibition of intercellular communication between primary cultured B6C3F1 mouse hepatocytes by phenobarbital (PB) and 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (DDT) was mediated by transient decreases in intercellular cAMP.

Growth factors and oncogene products also modulate gap junctions (De Feijter et al. 1990; Trosko et al. 1990c). A possible correlation between loss of gap junctional communication and the action of epidermal growth factor was associated with cellular proliferation (Madhukar et al. 1989).

Oncogene products ras, src, mos, neu but not myc down regulate gap junctions (Trosko et al. 1990a; Tzen et al. 1990).

Prooxidant theory

A mechanism that involves the generation of free radicals has also been implicated in tumor promotion (Sun, 1990). Hydrogen peroxide, enzymatically generated oxygen radicals and other activated oxygen species are effective tumor promoters (Troll and Wiesner, 1985; O'Brian, 1988). Metabolism of tumor promoting compounds by the hepatic mixed function oxidase system can induce oxygen radical formation (Cerutti, 1985). Free radicals may directly attack gap junction proteins, induce membrane changes via lipid peroxidation and alter intracellular cyclic nucleotide and/or Ca^{++} levels (Ruch and Klaunig, 1986). Addition of Cu and Zn-superoxide dismutase to neonatal rat hepatocyte cultures prevented the stimulation of DNA synthesis and mitosis by liver tumor promoters PB, DDT, and lindane (Armato et al. 1984).

Peroxisomes constitute another potential source of reactive oxygen species (Reddy and Rao, 1989). Hepatic peroxisomes of rats contain at least five oxidases which utilize oxygen and generate hydrogen peroxide. The tumor promoter TCDD is an inducer of peroxisomal proliferation (Mustonen et al. 1989). After rats were treated for 2 weeks with 0.05 - 5.0 ug TCDD/kg/day, there was a maximum (25%) increase in the number of peroxisomes with no increase in catalase activity. Tomaszewski et al. (1988) failed to induce peroxisomal B-oxidation by TCDD.

Cerutti (1989) theorized that oxidant promoters induce DNA strand breaks. DNA strand breaks elicit secondary metabolic reactions, in particular poly ADP-ribosylation of chromosomal proteins (Cerutti, 1985). Nuclear ADP-ribosylation reactions are associated with various events controlling chromatin structure and function and consequently gene expression (Tsujiuchi et al. 1990). Inhibitors of ADP-ribosyl synthetase suppressed the mitogenic actions exerted by tumor promoters PB and TPA (Romano et al. 1988).

If tumor promoters damage DNA then they function like "mutagens" rather than "mitogens". Tumor promoters are generally not mutagens (Butterworth, 1987). Trosko et al. (1988) postulated that an alternative cellular target for oxidant injury is the cell membrane rather than DNA. Oxygen radicals may damage membrane molecules by altering Ca^{++}-ion regulation, gap junctional function and activation of PKC.

Suppression of immune system

The concept of immune surveillance proposes that immune mechanisms can provide a defense against spontaneous and chemically-induced tumors by recognizing and eliminating aberrant cells (Kaczmarek, 1986; Sandstrom and Chow, 1988; Updyke et al. 1988). Natural killer cells, macrophages and antibodies have been implicated in the defense against small tumor foci and metastatic spread (Cotran et al. 1989). Escape from this form of surveillance would create an environment favorable for tumor cell progression.

Suppression of natural immune resistance may play a role in tumor promotion (Sandstrom and Chow, 1988; Updyke et al. 1988). *In vitro*, TPA induced a reversible decrease in the natural immune surveillance (Sandstrom and Chow, 1988). It was concluded that reversible TPA-induced reductions in sensitivity to mediators of natural resistance may be an integral component of promotion, contributing to tumor survival *in vivo* and increasing the probability that the tumor would progress to a more malignant phenotype. In SENCAR mice, topical application of TPA at doses relevant to the two stage model of carcinogenesis suppressed natural killer cell activity (Updyke et al. 1988).

Oncogenes and carcinogenesis

Over the last few years steps towards an understanding of the molecular basis of cancer have been made, due largely to the discovery of proto-oncogenes. Proto-oncogenes (cellular oncogenes) are normal cellular genes associated with cell growth, proliferation and differentiation (Bishop, 1987; Tronick and Aaronson, 1988). At present some 40 proto-oncogenes have been identified. Many of these genes are expressed at specific times and in selected tissues during growth and differentiation (Seemayer and Cavenee, 1989). c-onc gene products reside in the plasma membrane, cytosol or nucleus and may play a role in signal transduction pathways responsible for cell growth (Druker et al. 1989; Travali et al. 1990). Cellular oncogenes consist of exons and introns and differ from retroviral oncogenes which consist of only exons. The fact that the expression of proto-oncogenes is highly regulated has led some to propose that a mechanism operative in neoplasia may be an inappropriate timing and/or amount of proto-oncogene products (Pimentel, 1986; Seemayer and Cavenee, 1989).

Oncogenes on the other hand are altered or overexpressed versions of their normal cellular counterparts, proto-oncogenes (Pimentel, 1986a; Paul, 1988). They encode proteins called oncoproteins which are very similar to the normal products, except that they have lost important regulatory constraints on their activity and do not need external activation signals (Pimentel, 1986c).

Four basic mechanisms may be associated with the activation of proto-oncogenes. These include (1) DNA rearrangements, (2) gene amplification, (3) chromosomal translocation and (4) point mutations (Bishop, 1987; Seemayer and Cavenee, 1989). Activation of a proto-oncogene may give rise to increased production of a normal protein or an altered protein product.

Oncogenes can be categorized into six general classes based on the proteins which they encode: growth factors (sis), receptors (neu, erbA, fms, kit and mas) or truncated receptors (erbB), tyrosine kinases (src, abl, and fps), cytoplasmic serine and/or threonine kinases (mos and raf), guanosine nucleotide-binding proteins (ras) and nucleus localized proteins (myc, myb, fos and jun) (Tronick and Aaronson, 1988; Druker et al. 1989; Walker, 1989). The function or malfunction of these genes can have dramatic effects on DNA synthesis, transcription of genes, secondary messenger regulation and expression of growth factors and growth factor receptors (Bell, 1988).

Five different oncogenes (c-erb-B, c-mos, c-myc, c-myb and c-Ha-ras) have been implicated in a mechanism operative in animals known as promotion insertion which results in gene transcription (Bishop, 1987). Promotion insertion occurs when a retrovirus is positioned upstream, within or down stream of c-onc in the genome of an infected animal. The retrovirus acts as a promoter and enhances the expression of c-onc genes. In transgenic mice, juxtaposition of c-myc genes next to enhancer segments for heavy or light chain immunoglobulin genes is sufficient to induce lymphomas within a few months of birth (Adams et al. 1985). Enhancer segments function in a similar manner to viral promoters.

A strong association exists between cellular growth factors, expression of proto-oncogenes and normal and neoplastic growth control (Travali et al. 1990). As indicated before, proto-oncogenes code for growth factors and growth factor receptors. Growth factors affect transcription of nuclear proto-oncogenes, which in turn regulate the transcription of other genes involved in cell proliferation (Druker et al. 1989). The creation of autocrine or paracrine loops of growth stimulation and inappropriate expression of signal substances may give a presumptive cancer cell growth advantages by disrupting the normal regulation of cell proliferation and differentiation during the course of carcinogenesis (Su et al. 1989).

Multidrug resistance has also been linked to oncogenes. Neoplastic transformation of rat liver epithelial cells with v-raf or v-H-ras, independently of chemical exposure, resulted in multidrug resistance (Burt et al. 1988). Induction of P-glycoprotein and glutathione-S-transferase-P is thought to play an important role in the phenomenon of multidrug resistance. In another study, ras oncogenes increased the resistance of NIH 3T3 cells to ionizing radiation (Sklar, 1988a) and to the anticancer drug cis-diamine-dichloroplatinum (Sklar, 1988b). The mechanism by which ras oncogenes provided a survival advantage to NIH 3T3 cells remains unclear. This observation has potential clinical significance since ras is the oncogene most commonly involved in human tumors. The problem with multidrug resistance is that it often limits the potential effectiveness of current chemotherapeutic agents. This may be one of the reasons why human hepatocellular carcinomas are not very responsive to chemotherapeutic agents.

ras genes and chemical carcinogenesis

ras genes have attracted a great deal of attention because of their prominent role in malignancy (Gibbs and Marshall, 1989). Recent studies have provided an indication that ras activation may be a causative event in human tumor formation.

The ras gene family

The acronym ras was derived from the words rat sarcoma because these genes were first identified as the transforming principle of Harvey and Kirsten strains of sarcoma viruses (Campana, 1989). ras genes make up a family of highly conserved sequences. In mammalian cells, three members of the ras gene family have been described (Gibbs and Marshall, 1989). These include the N-ras gene, located at chromosome 1, Harvey (Ha)-ras gene, located at chromosome 11, and the Kirsten (K)-ras gene located at chromosome 12.

ras genes share a similar structure of 4 exons separated by 3 introns that code for a 21,000 dalton protein of 189 amino acids (ras p21) (Taparowsky et al. 1983). These exons are transcribed and form part of the processed ras mRNA, and may be involved in regulating translation of ras mRNA.

The ras protein

The proteins encoded by ras genes have been the subject of extensive biochemical study (McGrath et al. 1984; Pimentel, 1986c; John et al. 1988). Translation of ras mRNA gives rise to precursor pro-ras p21 in the cytoplasm which migrates to the inner surface of the plasma membrane, where it is processed, phosphorylated, and acquires palmitic acid residues (Shih and Weeks, 1984)

The biochemical properties of ras proteins include binding, exchange and hydrolysis of guanine nucleotides (Santos and Nebreda, 1989). Normal and transforming ras proteins bind guanine nucleotides GTP and GDP with similar affinities (Yu et al. 1988; McCormick, 1989). The intrinsic GTPase activity is impaired in transforming alleles of ras gene (Barbacid, 1987). Mutated forms of these proteins differ from their normal homologs by having amino acid substitutions at specific positions 12, 13, 59, and 61 (Clanton et al. 1987; Shih et al. 1988). The cellular location, structural and biochemical similarities to G proteins suggest that ras proteins participate in signal transduction (Hurley et al. 1984; Sigal et al. 1988).

Mechanisms of activation of ras genes

ras genes can be activated through quantitative or qualitative mechanisms (Barbacid, 1987). The quantitative mechanism states that increased expression of a normal ras gene is sufficient for activation (Chang et al. 1982; Mulcahy et al. 1985; Cichutek and Duesberg, 1986). Enhanced expression can be obtained by insertion of a strong promoter or enhancer in the vicinity of ras genes, by amplification of a normal gene or deletion of the first noncoding exon (Schwab et al. 1983; Pulciani et al. 1985; Bos, 1988). Support for the quantitative theory comes from experiments in NIH 3T3 cells in which cellular proto-oncogenes linked to viral promoters induced certain manifestations of the malignant phenotype (Chang et al. 1982; Pulciani et al. 1985).

Activation of ras genes can also occur by a qualitative mechanism. Qualitative mechanisms include point mutations that cause a single amino acid substitution, larger deletions or rearrangements (Pimentel, 1986; Barbacid, 1987; Shih et al. 1988). Mutations identified in NIH 3T3 cell assays or nude mouse tumorigenicity assays are commonly located in codons for amino acids 12, 13, 59 or 61 (Clanton et al. 1987; Guerrero and Pellicer, 1987). Point mutations at these specific sites within the coding exons seem to be the most frequent method of activation (Spandidos, 1988).

In addition, ras gene activation may occur by hypomethylation. Dietary methyl deficiencies were associated with hypomethylation of ras proto-oncogenes (De Feijter et al. 1990; Brockenbrough et al. 1991). Preneoplastic lesions from animals fed methyl deficient diets had hypomethylated ras genes (Bhave et al. 1988). Hypomethylation appears to be a relatively early event in carcinogenesis (Rao et al. 1989). Although the mechanism by which dietary methyl deficiency induces liver cancer is not clearly understood, hypomethylation seems to be involved either directly or indirectly, in the induction of carcinogenesis.

Proto-oncogenes regulating liver growth

Proto-oncogenes important in liver growth include fos, myc and ras, and the antioncogene p53 (Kaczmarek, 1986). These genes are expressed at specific times, and in sequence during the cell cycle. During liver regeneration, increases in fos and myc occur four hours after partial hepatectomy which corresponds to the passage of hepatocytes from G_0 to G_1 of the cell cycle. The transit of hepatocytes from phase G_1 to S of the cell cycle occurs 4 to 6 hours after partial hepatectomy with an increase of p53. Hepatocyte DNA synthesis and the major wave of cell division occurs 24 hours after partial hepatectomy and are associated with increased expression of ras genes (Hsieh et al. 1988; Fausto and Mead, 1989; Porsch-Hallstrom et al. 1989). After the peak of DNA synthesis at 24 hours, growth in the liver is controlled through the activation of a positive autocrine effector circuit involving transforming growth factor alpha and an inhibitory paracrine circuit in which transforming growth factor beta acts to prevent unrestrained cell proliferation (McMahon et al. 1986; Liu et al. 1988; Fausto and Mead, 1989).

Oncogenes in hepatocarcinogenesis

Expression of proto-oncogenes at unscheduled times in the liver result in abnormal cell growth, proliferation and differentiation (Seemayer and Cavenee, 1989). Oncogenes associated with hepatocarcinogenesis in animals and man are primarily *myc* and *ras* and to a lesser extent *fos* and *raf*.

myc genes in hepatocarcinogenesis

The *myc* gene is a classic example of an oncogene which is activated by gene amplification (Weinberg, 1985; Pimentel, 1986a; Bishop, 1987). Gene amplification could occur through a series of unequal sister chromatid exchanges in different cell cycles. Moreover, chemical rearrangement may be an essential and intimate step in the amplification process.

High expression of c-*myc* may represent the first proliferative marker of early preneoplastic cells (Porsch-Hallstrom et al. 1989). Increased levels of c-*myc* transcripts have been found in both experimental and spontaneous liver tumors (Jian-Ren et al. 1986; Hsieh et al. 1988; Nagy, 1988; Beer and Neveu, 1990; Pitot, 1990). Northern blot analysis of c-*myc* expression during 3'-methy-4-dimethylaminobenzene induced rat hepatocarcinogenesis disclosed an enhanced level of c-*myc* transcripts throughout neoplastic development (Cote et al. 1985). Makino et al. (1984) using the same experimental model compared the levels of c-*myc* transcripts in primary hepatomas versus the surrounding liver tissue and found significantly higher levels of c-myc in tumors. Yanswen et al. (1985) examined the expression of c-myc during early stages of hepatocarcinogenesis induced by a choline-deficient diet and observed increased levels of c-myc transcripts in oval cells two weeks after the beginning of the protocol. Beer et al. (1986) using the initiation-promotion protocol developed by Pitot et al. (1980b), could only detect increased levels of c-myc transcripts in primary hepatomas and questioned the proposed causative role of c-myc in early stages of chemical hepatocarcinogenesis.

ras genes and hepatocarcinogenesis

In mouse or rat liver tumor models several investigators have identified ras oncogenes (Strom and Faust, 1990; Beer and Neveu, 1990). Two different groups reported that approximately 80% of spontaneously occurring hepatocellular carcinomas in B6C3F1 mice contained activated H-ras genes (Fox and Watanabe, 1985; Stowers et al. 1988). Using the same mouse strain, Weiseman et al. (1986) detected activated H-ras in 100% of hepatocellular carcinomas induced by repeated injections of 3 structurally diverse carcinogens N-hydroxy-2-acetylaminofluorine, vinyl carbamate and 1'hydroxy-2',3'-dehydroestragole.

In F344 rats, an activated H-ras gene was identified in a single hepatocellular carcinoma induced with 2-amino-3-

methylimidazo[4,5-f]quinoline (Ishikawa et al. 1985a), while only 1 of 28 N-nitroso-diethylamine induced liver tumors contained transforming sequences (Boukamp et al. 1990). Hepatocellular carcinomas induced in rats by multiple injections of aflatoxin B_1 had an activated K-ras gene at a frequency of 20% (McMahon et al. 1986).

After long term feeding of a choline deficient diet to rats there was an increase in tumor transcripts of K-, H- and N-ras (Yaswen et al. 1985). In other studies, elevated levels of various ras gene transcripts were demonstrated in hepatic tumors and carcinogen treated livers (Makino et al. 1984; Cote and Chiu, 1987; Zhang et al. 1988). Elevated H- and K-ras transcripts also occur after partial hepatectomy (Goyette et al. 1983).

K- and H-ras genes were activated in 7 hepatocellular carcinomas from a series of 93 tumors in F344 rats initiated with methyl(acetoxymethyl) nitrosamine (DMN-OAc) and promoted with PB (Watatani et al. 1989). Whether or not the chemical caused activation of ras genes or activation was spontaneous was not known. If a single exposure of DMN-OAc affects c-ras genes directly as an obligatory initiating agent, it would seem unlikely that less than 10% of the tumors in this model would have a mutation in the ras gene.

Cooperation with other oncogenes

There is increasing evidence which indicates that cooperation of two classes of oncogenes may be necessary for transformation of cells in culture (Land et al. 1983). *myc* and *ras* oncogenes are examples of this cooperation phenomenon. *In vitro*, *myc* is responsible for immortalization, whereas *ras* is important in signal transduction, morphological transformation and anchorage independent growth. *In vitro*, cells co-transfected with *myc* and *ras* expanded into vigorously growing cultures and seeded rapidly growing tumors in nude mice. Acting together *myc* and *ras* were able to do what neither could do independently. Sandgren et al. (1989) using transgenic mice showed that the expression of *ras* or *myc* alone altered liver morphology. However, when *ras* and *myc* were coexpressed in the liver each cooperated with the other in the induction of hepatic tumors. Transgene induced hepatic lesions included altered hepatocellular foci, hepatic nodules and carcinomas.

Chemical and physical properties of dioxins

Polychlorinated napthalenes, polychlorinated biphenyls, halogenated biphenyl esters, dibenzo-p-dioxins and dibenzofurans include a group of structurally related aromatic compounds of considerable environmental concern because of their fat solubility, resistance to biological degradation, ubiquity, biomagnification potential and high toxicity

(Kimbrough, 1974; Rappe et al. 1979).

Dioxins belong to the group of highly chlorinated aromatic compounds (Firestone, 1984). The name dioxin refers to their basic structure; two oxygen atoms joining a pair of benzene rings (Tschirley, 1986). Substitution of chlorine atoms for hydrogen on the rings produce chlorinated dioxins. The chlorinated dioxin of interest in this study is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

TCDD is a by product in the manufacture of the herbicide trichlorophenol (Firestone, 1984; Tschirley, 1986). The amount of TCDD formed increases as the temperature of the reaction and pH increases. The half life of TCDD residues in humans is approximately seven years (Fingerhut et al. 1989). Mean residues in human fat from a number of North American and European studies is roughly 8.0 nanograms per kilogram (ppt) (Rappe et al. 1984). There is a strong positive correlation between lipid residues and serum concentrations.

The process by which TCDD is degraded in soil is poorly known (Tschirley, 1986). Microorganisms degrade TCDD, but at a low rate. A wood decaying fungus (Phanerochaete chryosporium) breaks down TCDD without observable mortality of the organism. Sunlight degrades TCDD rapidly by splitting off chlorine atoms. This reaction requires a hydrogen donor which is usually available in water or wax on leaves.

Environmental contamination by dioxins

TCDD was first recognized in 1957 as a contaminant in the manufacture of the herbicide 2,4,5-trichlorophenol, when 31 workers involved in the manufacture of the herbicide in West Germany developed dermatologic lesions called chloracne (Tschirley, 1986). Trichlorophenol was one of the ingredients in the herbicide Agent Orange which contained up to 40 mg/kg TCDD. TCDD was also identified in soot from chimneys of wood furnaces, residues in river fishes, eggs of herring gulls and recently in adipose tissue from more than 100 people in Canada, the United States and Vietnam (Rappe et al. 1979; Tschirley, 1986). In the chemical industry about 2,000 workers have had high exposure to TCDD (Tschirley, 1986). Low levels of exposure have undoubtly been experienced by people who handle 2,4,5-trichlorophenol and Vietnam veterans exposed to Agent Orange. Bioaccumulation of TCDD occurs primarily through the food chain and secondarily through contact with contaminated sediment (Batterman et al. 1989).

Municipal solid waste incinerators were also identified as major sources of dioxins (Murphy, 1989). Chlorine-containing materials in waste, especially polyvinyl chloride plastics provide precursors for dioxin formation. Fly ash surfaces catalyze the formation of these compounds via condensation reactions. In addition bleached kraft paper mills were reported by several researchers to be major sources of chlorinated dibenzo-p-dioxin and chlorinated dibenzofuran discharges to aquatic and marine environments (Amendola et al. 1989; Clement et al. 1989).

Pathotoxicologic effects of dioxins in animals

TCDD is the most toxic of the class of chlorinated dibenzo-p-dioxins (CDD) and chlorinated dibenzofurans (CDF) (Safe, 1986). Other 2,3,7,8-substituted CDDs and CDFs appear to elicit similar toxic responses to TCDD but at doses ranging from 2 X to 1000 X the dose level of TCDD. Studies of various congeners *in vitro* and *in vivo* clearly demonstrate that the number and position of halogenated atoms within the molecule are of critical importance for determining the toxicologic potential of a given congener (Poland and Knutson, 1982; McConnell, 1984). In general, the most toxic isomers of CDDs and related compounds are those in which the lateral positions of the molecule are fully halogenated. Removal of one or more halogen atom significantly reduces the toxicity of the molecule.

The pathotoxicologic effects of dioxins will be discussed in relation to general toxicology, receptor binding, dermal effects, hepatic effects, immunologic effects, the wasting syndrome and enzyme induction.

General toxicology

The phenoxy herbicides are readily absorbed via the respiratory and digestive systems in both rats and humans

(Lilienfeld and Gallo, 1989). The liver is the principal depository site in the rat and guinea pig (Safe, 1986; Pohjanvirta et al. 1989). Recent evidence from biopsy specimens indicate that liver and fat are major repositories of TCDD in humans (Lilienfeld and Gallo, 1989). The major route of excretion in all mammalian species is feces (Neal et al. 1984).

There are marked differences in species-specific median lethal dose (LD_{50}) levels; the most commonly used means of assessing acute toxicity. TCDD is highly toxic with LD_{50} values ranging from 0.6 ug/kg in guinea pigs to 5,000 ug/kg in hamsters (McConnell, 1984). Susceptibility to TCDD toxicity is highly species and strain specific and is thought to be causally related to the presence of an available cellular receptor, the aryl hydrocarbon (Ah) receptor (Vickers et al. 1985).

Receptor binding

It is hypothesized that the mechanism of action of TCDD is mediated via a cytoplasmic protein, the TCDD/Ah receptor (Poland, 1979; Safe, 1986). The TCDD receptor functions in a similar manner to the steroid receptor (Ringold, 1985). Polycyclic hydrocarbons including 3-methylcholanthrene compete with TCDD for Ah receptor sites. Overall evidence indicates that 3-methylcholanthrene and TCDD both induce aryl hydrocarbon hydroxylase by acting through the same receptor site (Okey and Vella, 1982). The aryl hydrocarbon hydroxylase system is responsible for metabolic activation and detoxification of polycyclic aromatic hydrocarbons (Eisen et al. 1983; Safe et al. 1984). Although both ligands have similar affinity for cytosolic Ah receptor sites, the relatively long half life of TCDD may account for its greater potency in vivo.

At least three and preferentially four halogens in positions 2,3,7 and 8 are required for high binding affinity to the Ah receptor (Poland, 1984; Safe et al. 1984). The dioxin skeleton is not essential for binding to the Ah receptor since other molecules such as anthracene and biphenylene, substituted with four chlorines at similar positions as TCDD bind to the receptor with high affinity (Gillner et al. 1989)

TCDD produces a diverse set of biological responses which in some cases reflect the expression of specific genes (Durrin et al.1987). A number of events occur prior to gene expression (Vickers et al. 1985; Cresteil et al. 1987; Durrin et al. 1987). These events include binding of TCDD to the cytoplasmic Ah receptor, ligand induced transformation to a state with high affinity for the cell nucleus and translocation of the TCDD/Ah-receptor complex to the nucleus (Whitlock, 1990). The TCDD-receptor complex is a DNA binding protein. Similar to steroid-receptor complexes, it appears that the TCDD-receptor complex activates gene expression by interacting with a genomic regulatory element termed dioxin responsive element (DRE). In mouse hepatoma cells, the DRE is located upstream of the transcriptional promoter for the cytochrome P1-450 gene. DREs have also been identified upstream in rat and human genes that correspond to the mouse cytochrome P1-450 gene (Neuhold et al. 1986). In addition to the DREs, other regulatory elements are present in DNA that flank the 5'-end of the cytochrome P1-450 gene. These include promoter and inhibitory elements. The DRE together with the TCDD-receptor complex constitute a dioxin-responsive enhancer system.

The mechanism(s) by which enhancer systems augment transcription from a distance is unknown. Two plausible models envision that binding of the *trans*-acting factor (i.e., the TCDD-receptor complex) to the *cis*-acting element (i.e., the DRE) produces (1) a change in chromatin structure that is propagated to the promoter and converts the nucleoprotein to a "transcriptionally active" form, or (2) "looping" of the DNA which brings the receptor binding site close to the promoter and results in activation of gene expression. These models imply that the TCDD-receptor complex interacts with the dioxin responsive element to activate the transcription of specific genes (Durrin et al. 1987).

Dermatologic effects

Chloracne and accompanying proliferative changes in the epidermis are the most sensitive and widely known toxic responses to TCDD in humans (Caramaschi et al. 1981). In animals such as rabbits, nude mice, nonhuman primates, cattle and horses, TCDD and a number of halogenated aromatic compounds produce typical skin lesions termed hyperkeratosis (Kimbrough, 1984). Available evidence indicates that the human epidermal Ah receptor has many functional properties in common with its murine counterpart, specifically in mediation of hyperkeratinization (Greenlee et al. 1984b,1987; Osborne et al. 1988).

Abnormal growth patterns in keratinocytes result in part from regulatory actions on receptor systems for at least three of the physiologic mediators of keratinocyte proliferation; epidermal growth factor (EGF), glucocorticoids, and cyclic AMP (cAMP) (Greenlee et al. 1987; Ryan et al. 1989). TCDD decreases the level of binding of EGF to high-affinity receptors in both human and animal epidermal cells (Madhukar et al. 1984; Matsumura et al. 1984; Hudson et al. 1986). Decreased EGF binding correlates with TCDD-induced proliferation and differentiation of epidermal cells (Hudson et al. 1986). Madhukar et al. (1988) proposed that the most logical cause for TCDD evoked changes in the EGF receptor was activation of intracellular protein kinases.

Immunologic effects

TCDD acts on selected targets within the immune system to produce a characteristic profile of responses including thymic atrophy, suppression of cellular immunity and inhibition of antibody production. Impairment of B cell differentiation was a direct result of the interaction of TCDD with B lymphocytes (Luster et al. 1984). On the other hand Clark et al. (1984) indicated that TCDD did not exert a direct toxic effect on cells of the immune system but rather acted by an indirect mechanism which promoted the generation of suppressor T cells.

Susceptible animals exposed to TCDD develop thymic atrophy. In the guinea pig, a fatal wasting condition occurs after very small doses of dioxin and atrophy of the thymus is a prominent postmortem finding (Clark et al. 1984). Thymic atrophy is characterized by depletion of cortical thymocytes (Greenlee et al. 1984a). Current data indicates that TCDD, rather than acting directly on thymocytes, acts through a receptor on thymic epithelial cells (Vos, 1984). The interaction of TCDD with the receptor on thymic epithelium alters the capacity of these cells to support intrathymic maturation and differentiation of lymphocytes. This may be the basis for the well documented TCDD-induced thymic atrophy and immunosuppression.

Studies in inbred murine strains, which differ in their sensitivity to TCDD, indicate that TCDD-induced thymic atrophy is mediated by the Ah receptor (Poland and Knutson, 1982a). Other immunologic effects of dioxins include focal hyperplasia of kupffer cells in the liver (Lilienfeld and Gallo, 1989) and an increase in the density of Langerhans cells in murine skin (Puhvel et al. 1989).

Hepatic effects

Toxicity of TCDD is most clearly manifested in the liver (Poland and Knutson, 1982a; Sassa et al. 1984). Histologic changes caused by TCDD in rodent livers include accumulation of neutral fat and cell necrosis. These processes have been linked to lipid peroxidation. Activation of oxygen species and initiation of peroxidation by Fe_2^+ remains the most attractive hypotheses for the progressive damage to the liver caused by TCDD (Sweeney et al. 1984; Stohs, 1990). Lipid peroxidation by TCDD may also occur as a result of inhibition of enzymes responsible for the elimination of peroxides (Stohs et al. 1984). TCDD administration to rats significantly inhibited selenium-dependent glutathione peroxidase activity, a major enzyme within cells responsible for removal of peroxides. Albro et al. (1988) stated that while TCDD may slightly increase hepatic lipid peroxidation in rats, the extent of such stimulation appeared too slight to account for the toxicity of TCDD.

Other changes in the liver of rats due to dietary doses as low as 7 ug/kg of TCDD include hypertrophy of hepatocytes with occasional increases in ploidy (Lilienfeld and Gallo, 1989). In rats and rabbits exposed to TCDD, aspartate aminotransferase, serum bilirubin and serum cholesterol were elevated while albumin was decreased. Ultrastructurally the primary hepatic changes observed at a dose of 0.1 ug/kg/day was proliferation of rough and smooth endoplasmic reticulum (Kociba et al. 1978).

Enzyme induction

Specific biochemical effects of TCDD in rat liver include dose-dependent induction of the enzymes aldehyde dehydrogenase, DT-diaphorase, UDP-glucuronosyl-transferase and glutathione-S-transferase (Poland and Knutson, 1982a; Goldstein and Hardwick, 1984; Vickers et al. 1985; Dunn et al. 1988). Inhibition of uroporphyrinogen decarboxylase following TCDD intoxication has been reported (Sassa et al. 1984).

TCDD is a potent inducer of aryl hydrocarbon hydroxylase (AHH) an enzyme associated with the mixed function oxidase system (Eisen et al. 1983; Whitlock et al. 1984; Durrin et al. 1987; Dunn et al. 1988). TCDD induces cytochrome p450IA1 (P-448, P1-450, P-450c). Excellent correlations exist between TCDD receptor binding, AHH induction and toxicity of various analogs of TCDD (Poland and Knutson, 1982a). The ED_{50} of TCDD for induction of AHH activity appears to be much lower than the ED_{50} for induction of lipid peroxidation, suggesting that the microsomal mixed function oxidase system may contribute but is not critically responsible for TCDD-induced oxidative damage (Stohs, 1990).

Wasting syndrome

The wasting syndrome is one of a protracted response to TCDD which resembles starvation and often culminates in death (Poland and Knutson, 1982a; Vickers et al. 1985). The syndrome consists of weight loss in adult animals or reduced weight gain in the young, accompanied by depletion of adipose tissue. Treatment of Sprague-Dawley rats with a single dose of TCDD caused dose-dependent reductions in body weight, feed and water intake and resting oxygen consumption (Peterson et al. 1984). Reduced food intake is the major cause of weight loss (Peterson et al. 1984). Other factors contributing to the wasting syndrome include depression of serum thyroxine (Rozman, 1984; Aust, 1984) and vitamin A levels by TCDD (Thunberg, 1984; Rubin and Rice, 1988).

Carcinogenic and tumor promoting activity of TCDD

TCDD is a potent teratogen (Birnbaum et al. 1989), carcinogen (Kociba et al. 1978; Greenlee et al. 1990) and tumor-promoter (Pitot et al. 1980; Goldstein et al. 1990). Carcinogenicity of TCDD has been documented in rats, mice, hamsters and monkeys at levels as low as 25 pg/kg/day (Rao et al. 1988; Lilienfeld and Gallo, 1989). Tumors induced by TCDD include hepatocellular carcinomas, cholangiocarcinomas and tumors of the skin, lung, palate, tongue, and thyroid (Poland and Knutson, 1982a).

The bulk of evidence indicates that TCDD acts as a nongenotoxic carcinogen (tumor promoter) and has little or no initiating (genotoxic) activity (Poland and Glover, 1979b). In various two-stage models of hepatocarcinogenesis TCDD is a potent tumor promoter (Pitot et al. 1980; Hebert et al. 1990; Flodstrom et al. 1991). Additional studies by Poland et al. (1982b) provide evidence for the promotional activities of TCDD. Papillomas developed on the skin of HRS/J hairless mice initiated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and promoted with TCDD. In vitro, TCDD promotes formation of transformed foci in MNNG-initiated C3H10T1/2 cells. Comparison of promotion by TCDD and TPA in mouse skin and C3H10T1/2 cells indicate that TCDD is 100- and 10,000-fold more potent than TPA (Lilienfeld and Gallo, 1989).

In the absence of convincing evidence that TCDD is a "mutagen" and in light of present documentation that TCDD induces hepatocellular carcinomas in rats initiated with diethylnitrosamine, it seems reasonable to hypothesize that tumors which develop from chronic administration of TCDD may arise from its promoting ability of cells already "initiated" by environmental factors (Pitot et al. 1980a). As stated before, the characteristic toxic responses of TCDD are postulated to be mediated via the Ah receptor. Studies are needed to determine if tumor promotion by TCDD is also mediated by the Ah receptor.

Altered hepatocellular foci in hepatocarcinogenesis

Altered hepatocellular foci (AHF) are evaluated in short-term and mid-term rat liver models to determine if chemicals have tumor promoting or carcinogenic potential (Jensen et al. 1982; Goldsworthy et al. 1984; Rezabek et al. 1987). Potent experimental tumor promoters and hepatocarcinogens cause an increase in the number and size of AHF prior to the appearance of liver tumors (Scherer, 1984; Goldsworthy and Pitot, 1985; Saeter et al. 1988). Based on the results over several decades, it is generally believed that AHF are precursors of hepatocellular tumors (Emmelot and Scherer, 1980; Peraino et al. 1983; Bannasch, 1986a; Popp and Goldsworthy, 1989a). Evidence for the precursor relationship of AHF to tumors is partially derived from studies examining the cytology, dose-dependency, sequence of appearance, clonality, and biological behavior of AHF (Scherer and Emmelot, 1975b; Emmelot and Scherer, 1980; Popp and Goldsworthy, 1989a). In studies where conversion rates have been determined, it is established that neoplastic conversion is a rare event (Popp and Goldsworthy, 1989a). In most cases, only 1 carcinoma develops for every 1,000 to 10,000 AHF. While AHF may be precursors to liver tumors, they are clearly not *in situ* tumors.

Altered hepatocellular foci are defined solely by morphologic characteristics (Bannasch et al. 1985d,1988;

Peraino et al. 1988). The relative shape is important for sterologic quantitation since available sterologic programs assume a spherical shape (Campbell et al. 1982). The most distinguishing feature of AHF is the difference in tinctorial staining between hepatocytes in AHF and those in the surrounding hepatic parenchyma (Pitot et al. 1978; Farber, 1980,1984b). Cells in AHF may be larger or smaller than hepatocytes found in surrounding tissue (Bannasch et al. 1985d). When focal cells are larger, compression of adjacent liver tissue may occur (Harada et al. 1989). Other morphological changes in AHF such as spongiosis hepatis or peliosis hepatis may also result in enlargement of AHF with compression of adjacent hepatocytes (Popp and Goldsworthy, 1989a).

Preneoplastic cells can be recognized by different kinds of alterations including enzymatic and antigenic changes, chromosomal abnormalities, and morphological characteristics (Bannasch et al. 1980; Maronpot et al. 1986; Pitot et al. 1989c). On a routinely prepared paraffin section stained with hematoxylin and eosin, AHF may be identified by cells having a basophilic, eosinophilic, vacuolated or clear cell appearance (Squire and Levitt, 1975; Maronpot et al. 1986; Bannasch, 1988). Basophilic cells are generally equal to or smaller than cells in the surrounding liver, while cells in eosinophilic and clear cell foci are generally equal to or larger than cells in the surrounding tissue.

Approximately 40 different special stains, positive or negative for specific proteins or enzymes are used to identify AHF (Peraino et al. 1983,1984; Sato, 1988; Pitot et al. 1989c). The most commonly used enzymatic markers for identifying AHF include positive markers gamma glutamyltransferase (GGT), placental form of glutathione-S-transferase (GST-P) and DT-diaphorase, and negative markers adenosine triphosphatase (ATPase) and glucose-6-phosphatase (Pitot et al. 1978; Ishikawa et al. 1980; Hanigan and Pitot, 1982; Tatematsu et al. 1987; Ward and Henneman, 1990). The marker exhibiting the greatest efficiency for scoring the largest number of AHF is GST-P (Tatematsu et al. 1987; Sato, 1988; Pitot et al. 1989c). The number of markers per focus are related to relative growth rates of AHF (Goldsworthy and Pitot, 1985; Tanaka et al. 1986). Cells with multiple markers may possess an increased likelihood of developing into tumors (Popp and Goldsworthy, 1989a).

Enzymatic markers such as GGT and GST-P may be influenced by environmental factors such as diet, strain, age and sex of rat, as well as by the type of chemical used (Russell et al. 1987). For example, AHF induced by chemicals which are classified as peroxisomal proliferators are generally negative for the positive markers GGT or GST-P (Cattley and Popp, 1989; Yeldandi et al. 1989). For this class of chemicals, GGT and GST-P provide little help in identifying or establishing the pathogenesis of AHF. Identification and quantitation of AHF induced by peroxisome proliferators are best pursued by using H&E staining characteristics and negative markers such as ATPase (Cattley and Popp, 1989; Kraupp-Grasl et al. 1990).

A most interesting response to carcinogenic agents in AHF is a decrease in enzymes of phase I metabolism and an increase in phase II enzymes (Sato, 1988; Stenius and Hogberg, 1988). In particular the phase II enzymes GST-P and GGT are increased dramatically in preneoplastic and neoplastic lesions (Hanigan and Pitot, 1985; Pitot et al. 1989c).

Under natural conditions most preneoplastic lesions are either destroyed or revert to normalcy (Pimentel, 1986b). In experimental carcinogenesis regression of AHF occur after removal of the inducing agent (Bursch et al. 1984; Garcea et al. 1989). This demonstrates a lack of commitment of most preneoplastic lesions to neoplastic development. Some AHF and nodules however persist (Farber and Sarma, 1987).

Other lesions used to evaluate the carcinogenic potential of chemicals in rat liver bioassays include hepatic nodules and hepatocellular carcinomas (Squire and Levitt, 1975; Farber, 1980; Saeter and Seglen, 1990). Hepatic nodules cause compression of adjacent liver parenchyma and lack normal hepatic lobular architecture (Stewart et al. 1980; Brooks and Roe, 1985). Diagnostic criteria of importance in defining hepatocellular carcinomas include cellular atypia, local invasiveness, haphazard arrangement of cells, trabecular patterns and gland-like formations (Popp, 1985).

Models of initiation, promotion and progression

Most rat liver short- and mid-term bioassays are based on the concepts of initiation and promotion and use AHF as an endpoint in evaluating chemicals for tumor promotion and carcinogenic potential. Multistage hepatocarcinogenesis in the rat has been studied in a variety of model systems. Representatives of these models will be reviewed briefly.

Pitot initiation-promotion assay

In this model of hepatocarcinogenesis the stages of initiation and promotion are clearly defined. Twenty four hours after 2/3 partial hepatectomy, 5-8 week-old rats are initiated with a single subcarcinogenic dose of 10 mg/kg diethylnitrosamine (DEN). Two weeks after partial hepatectomy rats are given the test chemical or tumor promoter (0.05% phenobarbital) in the diet (Emmelot and Scherer, 1980; Goldsworthy et al. 1986; Pitot, 1988).

An advantage of this model system is that single, nonnecrogenic doses of DEN prevent cytotoxicity and minimize overlapping of initiating and promoting events (Goldsworthy et al. 1986). In addition, partial hepatectomy increases "fixation" of the initiation event. A disadvantage of this model is the length of time required for tumor promotion to occur.

Pitot initiation-promotion-progression assay

This model is an extension of the initiation-promotion assay (Pitot et al. 1988c,1989c) and a modification of the initiation-promotion-initiation protocol of Scherer et al. (1984). It offers an advantage of identifying putative "progressor" agents as well as those acting at the stages of initiation and promotion. In this model, initiation is carried out during the first week of life as described by Peraino et al. (1981). At 3 months of age, following a partial hepatectomy, one or more doses of a second initiating agent such as ENU is administered. In this model foci-in-foci represent lesions of the progression stage. Malignant neoplasms develop within 6 months of administration of the second "initiating" or "progressor" agent.

Resistant hepatocyte model (RH model)

The RH-model has a major advantage of rapidly inducing preneoplastic lesions (Goldsworthy et al. 1986). In this model rats are initiated with a high dose (200 mg/kg) of DEN. "Initiated" cells are selected by the combined action of feeding a low, subcarcinogenic dose (0.02%) of 2-acetylaminofluorine (2-AAF) for a short period and partial hepatectomy (PH). 2-AAF is mitoinhibitory to normal liver cells whereas initiated cells continue to proliferate. PH acts as a mitogenic stimulus for initiated cells. AHF can be identified at the time of cessation of 2-AAF treatment (4 weeks after initiation). The majority of AHF and nodules redifferentiate to normal hepatocytes. A few AHF and nodules however continue to grow and proliferate to form persistent nodules. Persistent nodules are considered precursors of hepatocellular carcinomas and develop within a year (Peraino et al. 1988; Emmelot and Scherer, 1980; Leonard et al. 1982).

In this model AHF develop faster in males compared to females during 2-AAF/PH treatment. Males also develop hepatocellular carcinomas much earlier than females (Blanck et al. 1986; Saeter and Seglen, 1990). This difference in response to 2-AAF seems to be due to a pituitary influence mediated by growth hormones known to be sex differentiated in the rat (Mulcahy et al. 1985). The RH model may provide intriguing possibilities to study the influence of hormones on the expression of genes involved in the regulation of cell proliferation.

Peraino neonatal rat assay

This bioassay takes advantage of normal hepatocyte proliferation in neonates as the mode of "fixation" during the initiation phase of treatment, thereby eliminating the need for treatment with a toxic agent or partial hepatectomy (Goldsworthy et al. 1986). In this model one day old Sprague-Dawley rats are given a single "subcarcinogenic" dose of an initiating agent and repeatedly exposed to a dietary promoter starting at the time of weaning (21 days of age) (Peraino et al. 1983; Goldsworthy et al. 1986). This protocol allows for rapid screening of initiators and promoters. However, in 1-day-old rats the capacity of the liver to metabolize xenobiotics is reduced which may limit the applicability of the model.

In conclusion, rodent bioassays provide information that is used to predict risk to humans from chemical exposure. Using bioassays, scientists are better able to determine and understand the mechanisms underlying the multiple steps necessary for the development of cancer.

CHAPTER 1

TUMOR PROMOTING EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IN INITIATED WEANLING SPRAGUE DAWLEY RATS TREATED WITH PHENOBARBITAL

CHAPTER 1

TUMOR PROMOTING EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IN INITIATED WEANLING SPRAGUE DAWLEY RATS TREATED WITH PHENOBARBITAL

Abstract

A sequential study was completed to determine if 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) would inhibit the regression of adenosinetriphosphatase (ATPase) negative altered hepatocellular foci (AHF) induced by phenobarbital (PB) and to assess the long term tumor promoting effects of a low dose of TCDD. In an initiation-promotion protocol, female weanling Sprague-Dawley rats were initiated with 10 mg/kg nitrosodiethylamine (NDEA) and after 30 days promoted with either 500 ppm of PB until day 170 or 150 ppt of TCDD until days 170, 240 or 450. Alternatively, initiated rats were promoted with PB until day 170 and subsequently with TCDD until days 240 or 450. In another group, initiated rats were promoted with PB until day 170, followed by a basal diet until day 240 and subsequently with TCDD until day 450. Rats were killed at 170, 240 and 450 days. The number of AHF/cm^3 of

liver was the same whether TCDD was given continuously to 450 days or delayed and given from 170-450 days or 240-450 days. However, the mean volume of AHF was significantly larger when TCDD was given continuously than when given following PB from days 170-450 or 240-450. Thus, TCDD had a strong promoting effect even when administration was delayed as long as 240 days after initiation. Phenobarbital at a dietary concentration of 500 ppm increased tumor incidence at 450 days. However, a significant increase in the number of ATPase negative AHF/cm^3 of liver was not found in initiated rats. Because PB did not have a significant promoting effect on the development of ATPase negative AHF in this model, whether or not TCDD inhibited the regression of PB-induced foci could not be determined.

Introduction

Previous studies in our laboratory have shown that a number of polyhalogenated aromatic hydrocarbons (PHAHs) are potent hepatic tumor promoters in initiation-promotion bioassays (Jensen et al. 1982,1984; Rezabek et al. 1987; Dixon et al. 1988; Evans, 1989). The PHAHs include polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB), and congeners 2,2',4,4',5,5'-hexabromobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl and 3,4,3'4'-tetrabromobiphenyl. Included in this group of PHAHs is the environmental contaminant 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD).

TCDD is known for its chemical stability, lipophilicity, resistance to degradation and persistence in the environment. Contamination of the food chain is of environmental concern. Recently several researchers reported that bleached kraft paper mills are major sources of chlorinated dibenzofuran discharges to aquatic and marine environments (Beck et al. 1988; Amendola et al. 1989; Clement et al. 1989). In other studies consumer paper products including shopping bags, babies' diapers, coffee filters and cigarette papers contained measurable amounts of TCDD (Beck et al. 1988; Wiberg et al. 1989). Dioxins were also found in human milk (Schecter et al. 1989) and in serum of workers exposed to contaminated products in industrialized countries (Fingerhut et al. 1989).

TCDD is the most toxic of the class of chlorinated dibenzo-p-dioxins and dibenzofurans (Rappe et al. 1979; Tschirley, 1986). Studies in animals and cell culture indicate that many of the biological and toxic effects of TCDD are mediated through a soluble intracellular protein, the aromatic hydrocarbon (Ah) receptor (Eisen et al. 1983; Safe et al. 1984; Bannister and Safe, 1987; Harper et al. 1988). Toxic and biologic effects of TCDD include a wasting syndrome, immunotoxic effects, reproductive toxicity, hepatotoxicity and porphyria, organ- and species-dependent hypo- and hyperplastic responses and induction of specific forms of cytochrome P-450 and other enzymes (Poland and Knutson, 1982a; Goldstein and Hardwick, 1984; Tschirley, 1986; Lilienfeld and Gallo, 1989;

Kerkvliet et al. 1990). In addition to its toxicologic effects, TCDD has teratogenic (Courtney and Moore, 1971; Abbott et al. 1987) and carcinogenic effects (Kociba et al. 1978; Rao et al. 1988).

TCDD (Pitot et al. 1980a; Poland et al. 1982b; Hebert et al. 1990) and PB (Periano et al. 1980; Goldsworthy et al. 1984; Betschart et al. 1988) are known nongenotoxic hepatic tumor promoters in rodents. Nongenotoxic carcinogens are chemicals whose primary action does not involve reactivity with DNA (Butterworth, 1987; Scribner et al. 1987). These chemicals function through epigenetic pathways, selectively causing clonal expansion of initiated cells (Scherer, 1984; Lutz and Maier, 1988). The effect of the promotional activity of chemicals is measured in initiation-promotion models of hepatocarcinogenesis (Peraino et al. 1983; Fitzgerald and Yamasaki, 1990). Most models use altered hepatocellular foci as an end point (Slaga, 1983; Bannasch, 1986a; Maronpot et al. 1989). Altered hepatocellular foci are strongly implicated as preneoplastic lesions, and enhancement of AHF in bioassays correlates well with subsequent tumor production in long-term studies (Rezabek et al. 1987; Popp and Goldsworthy, 1989a; Kraupp-Grasl et al. 1990).

Carcinogenesis is a multistage process (Weinstein et al. 1983; Fischer et al. 1988). It comprises at least three stages termed initiation, promotion and progression (Weinstein et al. 1984; Farber and Sarma, 1987; Weinstein, 1988; Fischer et al. 1988). Initiation, the first event in the process, is irreversible and occurs spontaneously or from the genetic damage caused by a carcinogen (Pitot and Sirica, 1980b; Columbano et al. 1981).

The stage of promotion is reversible (Hicks, 1983; Farber and Sarma, 1987). Under the influence of certain agents and environmental pollutants, clonal expansion of initiated cells occurs resulting in phenotypic changes, including preneoplastic lesions such as altered hepatocellular foci (Slaga, 1983; Farber, 1988). The mechanisms of tumor promotion are unknown. Various mechanisms have been postulated depending on the chemical properties of the compound and tissue specificity. For example, it is proposed that some chemicals act through receptor-mediated pathways (Pitot et al. 1980a; Poland et al. 1982b; Saeter and Seglen, 1990) while others function by mitogenic stimulation of the cell replication cycle (Schulte-Hermann et al. 1986) or via inhibition of gap junctional intercellular communication (Trosko et al. 1983; Ruch and Klaunig, 1988).

The stage of progression is irreversible (Pitot, 1989). It is characterized by the manifestation of a malignant phenotype by some of the cells in the expanded clone of initiated cells. During this stage hepatic nodules and hepatocellular carcinomas develop (Nowell, 1986; Pitot et al. 1988c).

Previously, most research with hepatic tumor promoters was directed toward elucidating pathologic effects of single promoters rather than combinations of promoters. Of special concern to toxicologists today is whether or not the exposure to various tumor promoters which function by different mechanisms result in inhibitory, additive or synergistic effects. The purposes of this study were to determine if TCDD would inhibit the regression of AHF induced by phenobarbital and enhance the development of AHF into hepatic nodules and hepatocellular carcinomas and to assess the long term promoting and carcinogenic effects of a low dietary level of 150 ppt of TCDD.

Methods

Female weanling Sprague-Dawley rats (Charles River, Portage, MI) initially weighing 40 g were used. The rats were housed three per polypropylene cage and fed a basal diet (Certified Rodent Chow 5002, Ralston Purina Company, St. Louis Mo.) and water ad libitum. Diets were prepared by adding TCDD in corn oil to a basal diet to make a 1.5 ppb premix. From the premix, diets containing 150 ppt were made. TCDD was kindly provided by Dr. Matsumura; a gift from Dow Company to Michigan State University. Phenobarbital was obtained from Sigma Chemical Co. (St. Louis, MO). Diets containing 500 ppm PB were prepared in a similar manner to those containing TCDD.

An initiation-promotion protocol for experimental hepatocarcinogenesis was used (Figure 1.1). Weanling rats were initiated at day 0 with an intraperitoneal injection of 10 mg/kg NDEA (Sigma Chemical Co., St. Louis MO). After 30 days, rats were randomly assigned to treatment groups and promoted with a basal diet (BD) containing 150 ppt of TCDD until days 170, 240 or 450. Alternately, initiated rats were promoted with PB until day 170 and then by TCDD until days 240 or 450. In another group, rats were fed a diet containing 500 ppm PB until day 170 followed by a BD until day 240 and subsequently TCDD from days 240-450. Controls included groups of initiated rats given the basal diet and noninitiated rats given TCDD or PB. Rats were anesthetized with CO_2 and killed at day 170, 240 or 450. Necropsy consisted of a systematic examination of organs for gross pathologic changes. Brain, kidney, liver, spleen, lung, trachea, nasal cavity, esophagus, heart, thymus and mediastinal lymph nodes were examined. Liver and body weights were recorded. Samples of liver were collected for chemical analysis, wrapped in aluminum foil and stored at $-20^{o}C$.

Five sections of liver (two sections form the left lobe and one section from the median, right lateral and caudate lobes) were taken from each rat, mounted on corks and frozen in isopentane cooled with liquid nitrogen. Sections were cut at 8 um with a cryostat and stained for adenosinetriphosphatase (ATPase) by the technique of Wachstein and Meisel (1966). Tumor-promoting ability was assessed by measuring altered hepatocellular foci (AHF) negative for

ATPase activity (Figure 1.2). ATPase negative AHF were enumerated because of the relative ease of quantification with the image analyzer and as determined from other studies, a high proportion of AHF are scored with this marker (Scherer and Emmelot, 1975a; Pitot et al. 1978; Ishikawa et al. 1980). An equal area from each liver section was evaluated and the total area of liver examined from each rat was 2.5 - 3.0 cm^2. Methods of Campbell et. al. (1982) were used to compute the number of AHF/cm^3 and the volume of liver occupied by AHF. Additional sections of liver, taken from the remaining hepatic lobes of each rat were fixed in 10% buffered formalin, sectioned and stained with hematoxylin and eosin. Tumors determined grossly at necropsy were confirmed by histologic examination as hepatic nodules and hepatocellular carcinomas. Hepatic lesions were classified histologically using previous criteria (Maronpot et al. 1986).

Portions of liver were stored at $-20^{\circ}C$ for chemical analysis of TCDD. Chemical analysis was done by Dr. Zabik of the Pesticide Research Center at Michigan State University.

Data were analyzed by one-way analysis of variance. Differences between group means were analyzed by the Student-Newman-Keul's test. Differences between groups were considered significant at the level of $P<0.05$.

Results

The number and mean volume of AHF/cm^3 of liver are given in Tables 1.1, 1.2, and 1.3. At 170 days, initiated rats fed TCDD in the diet had significantly more AHF/cm^3 of liver when compared to similarly treated rats fed PB or basal diet. The number of AHF/cm^3 were somewhat more in initiated rats given PB compared to the basal diet, however, the difference was not statistically significant (Table 1.1).

At 240 days, initiated rats fed TCDD from days 30 - 240, or rats fed PB from days 30 - 170 and subsequently TCDD until day 240 had significantly more AHF/cm^3 compared to similarly treated rats fed a basal diet or PB followed by a basal diet (Table 1.2).

The mean volume of AHF at day 170 or 240 was larger in initiated rats fed a basal diet compared to initiated rats promoted with TCDD and/or PB (Figure 1.1 and 1.2). This difference may be due to the fact that a few animals given the basal diet had very large AHF, while animals promoted with TCDD or PB had AHF within a common range. Also, the smaller number of animals in the basal diet group may have influenced this result.

At 450 days, initiated rats fed TCDD from days 30-450, or PB and subsequently TCDD until day 450, or PB followed by a basal diet until day 240 and subsequently TCDD until 450 days had significantly more AHF/cm^3 compared to initiated rats fed a basal diet or PB and subsequently a basal diet (Table

1.3). The mean volume of AHF was significantly larger in animals exposed to TCDD continuously from days 30-450. Dietary exposure to TCDD or PB did not cause significantly increased numbers of ATPase negative AHF when compared with values for rats fed a basal diet or those initiated with NDEA.

In hematoxylin and eosin stained sections of liver various types of preneoplastic lesions were present including eosinophilic, clear cell, vacuolated, mixed and basophilic. The characteristics of these foci and differences between treatment groups will be discussed in chapter two. Three rats initiated with NDEA and treated with PB had apoptotic bodies in eosinophilic AHF at day 170 of sacrifice. Apoptotic bodies were not present in noninitiated rats treated with PB. Hepatic nodules and hepatocellular carcinomas were increased in rats initiated with NDEA and fed diets containing PB or TCDD at day 450 (Table 1.4). Livers from rats fed diets containing PB had moderate hepatocellular hypertrophy in the centrilobular region. Lesions of significance were not present in brain, kidney, spleen, lung, trachea, nasal cavity, esophagus, heart, thymus and mediastinal lymph node.

Body weight gains from the time of TCDD administration to necropsy were not significantly different from rats given the basal diet (Table 1.5). The liver weight of initiated rats given PB and subsequently TCDD until day 240 was significantly different from initiated rats given a basal diet, PB or TCDD (Table 1.6). TCDD concentrations in liver were proportional to the dose and indicated persistence of the chemical in the diet (Table 1.7).

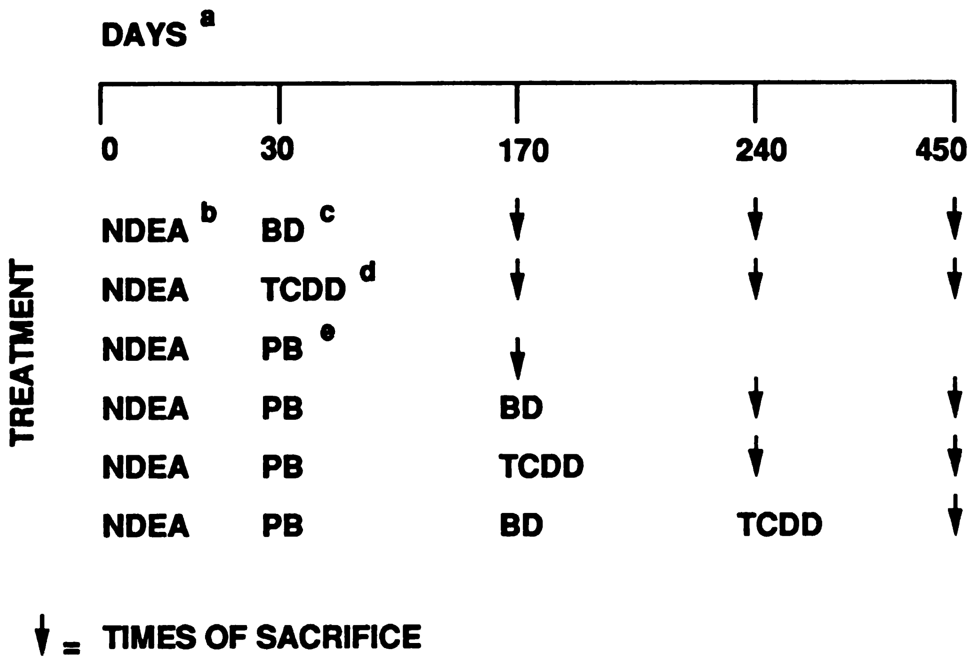

Figure 1.1. Experimental design

a - Days of treatment/sacrifice
b - Nitrosodiethylamine
c - Basal diet
d - 2,3,7,8-Tetrachlorodibenzo-p-dioxin
e - Phenobarbital

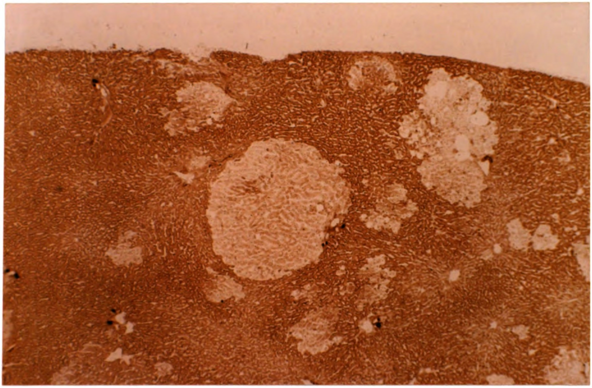

Figure 1.2. Photomicrograph of multiple adenosine triphosphatase negative altered hepatocellular foci in the liver of an initiated rat fed a diet containing 150 ppt TCDD (X 60).

Table 1.1. Altered Hepatocellular Foci Per Cubic Centimeter of Liver in Initiated and Noninitiated Rats at 170 Days[a]

Treatment	No. of rats	AHF/cm^3 liver	Mean volume (mm^3) of AHF
NDEA + BD	6	357 ± 166	0.06 ± 0.04
BD	3	6 ± 6	0.01 ± 0.01
NDEA + TCDD	6	1543 ± 296[b]	0.01 ± 0.00
TCDD	6	43 ± 16	0.01 ± 0.00
NDEA + PB	12	422 ± 121	0.02 ± 0.00
PB	3	0	0

a - Data expressed as mean ± S.E.
b - Significantly different from initiated rats which received basal diet or 500 ppm PB in the diet ($p<0.05$)

Table 1.2. Altered Hepatocellular Foci Per Cubic Centimeter of Liver in Initiated and Noninitiated Rats at 240 Days[a]

Treatment	No. of rats	AHF/cm^3 liver	Mean volume (mm^3) of AHF
NDEA + BD	6	571 ± 131	0.12 ± 0.02
BD	3	17 ± 17	0.002± 0.00
NDEA + TCDD	6	1930 ± 433[b]	0.02 ± 0.03
TCDD	7	35 ± 16	0.02 ± 0.01
NDEA + PB + BD	12	698 ± 136	0.01 ± 0.04
PB + BD	3	0	0
NDEA + PB + TCDD	12	1491 ± 223[b]	0.02 ± 0.04
PB + TCDD	3	575 ± 575	0.01 ± 0.01

a - Data expressed as mean ± S.E.
b - Significantly different from initiated rats which received basal diet or 500 ppm PB in the diet ($p<0.05$)

Table 1.3. Altered Hepatocellular Foci Per Cubic Centimeter of Liver in Initiated and Noninitiated Rats at 450 Days[a]

Treatment	No. of rats	AHF/cm^3 liver	Mean volume (mm^3) of AHF
NDEA + BD	12	709 ± 151	0.05 ± 0.01
BD	5	0	0
NDEA + TCDD	13	1344 ± 148[b]	0.13 ± 0.02[c]
TCDD	10	211 ± 44	0.06 ± 0.02
NDEA + PB + BD	12	654 ± 78	0.06 ± 0.01
PB + BD	5	15 ± 5	0.08 ± 0.07
NDEA + PB + TCDD	15	1364 ± 198[b]	0.10 ± 0.01
PB + TCDD	5	124 ± 22	0.02 ± 0.01
NDEA + PB + BD + TCDD	13	1328 ± 165[b]	0.07 ± 0.01
PB + BD + TCDD	6	34 ± 24	0.02 ± 0.01

a - Data expressed as mean ± S.E.
b - Significantly different from initiated rats which received basal diet or 500 ppm PB in the diet ($p<0.05$)
c - Significantly different from initiated rats which received basal diet, PB followed by the basal diet or PB followed by the basal diet and subsequently TCDD ($p<0.05$)

Table 1.4. Number of animals with hepatic nodules and hepatocellular carcinomas at 450 days

Treatment	Hepatic nodules	Hepatocellular Carcinomas
NDEA + BD	1	0
NDEA + PB + BD	3	2
NDEA + TCDD	2	3
NDEA + PB + TCDD	5	1
NDEA + PB + BD + TCDD	4	3

Table 1.5. Body Weight Gains in Initiated Rats[a]

Treatment	Days of sacrifice		
	170	240	450
BD	97 ± 5	125 ± 8	150 ± 1
DEN + BD	104 ± 5	124 ± 4	135 ± 1
DEN + TCDD	107 ± 6	100 ± 3	132 ± 6
DEN + PB	107 ± 6	---	---
DEN + PB + BD	---	125 ± 5	140 ± 9
DEN + PB + TCDD	---	113 ± 7	138 ± 7
DEN + PB + BD +TCDD	---	---	126 ± 7

a - Data in grams and expressed as mean ± S.E.

Table 1.6. Liver Weight in Initiated Rats[a]

Treatment	Days of sacrifice 170	240	450
BD	6.68 ± 0.68	6.40 ± 0.23	6.98 ± 0.36
DEN + BD	7.14 ± 0.48	6.47 ± 0.14	7.72 ± 0.39
DEN + TCDD	7.83 ± 0.45	7.35 ± 0.54	9.41 ± 0.60
DEN + PB	7.75 ± 0.26	---	---
DEN + PB + BD	---	6.54 ± 0.22	8.25 ± 0.47
DEN + PB + TCDD	---	7.86 ± 0.27[b]	10.21 ± 1.23
DEN + PB + BD + TCDD	---	---	8.14 ± 0.33

a - Data presented as actual weight in grams and expressed as mean ± S.E.

b - Significantly different from initiated rats which received basal diet or 500 ppm of PB in the diet or PB followed by BD and subsequently TCDD ($p<0.05$)

Table 1.7. TCDD Concentrations in Liver of Rats

Days	Promoter	No. of tissue samples	TCDD concentrations in liver (ppt)
170	TCDD	3	570
240	TCDD	3	320
240	PB + TCDD	2	380
450	TCDD	4	410
450	PB + TCDD	3	760
*	BD	4	0

* = Sample consisted of liver from days 170, 240 and 450

Discussion

Hepatocytes that lose the ability to express ATPase are generally considered precursors of liver cancer (Scherer and Emmelot, 1975b; Pitot et al. 1978). In this study TCDD fed to NDEA initiated rats at a low nontoxic dietary concentration of 150 ppt (0.007ug of TCDD/kg/day) had a promoting effect on the development of altered hepatocellular foci at days 170, 240 and 450. The increase in the number of AHF correlated with hepatic nodules and hepatocellular carcinomas at day 450.

The results confirm and extend previous findings with different initiation-promotion protocols in the liver (Pitot et al. 1980a) and skin of rats (Poland et al. 1982b). In a 2-year carcinogenicity study, TCDD was a potent carcinogen at a high dose of 2200 ppt but not at lower doses of 210 or 22 ppt (Kociba et al. 1978). In addition to hepatocellular carcinomas reported by Kociba et al., other tumors included squamous cell carcinomas of the lung, hard palate, nasal turbinates and tongue. Recently, Rao et al. (1988b) showed that TCDD may be a complete carcinogen in hamsters, the species most resistant to the toxic effects of TCDD. In people exposed to TCDD the most consistent lesion described was chloracne (Caramaschi et al. 1981).

The number of AHF/cm^3 of liver was not significantly different in rats given TCDD continuously from day 30-450, from day 170-450 or from day 240-450. However, the mean volume of AHF was significantly larger in animals exposed to

TCDD from day 30-450. This response is similar to a study done by Goldsworthy et al. (1984) using phenobarbital (PB) as the promoting agent. The number of AHF per liver increased with the duration of continuous PB feeding up to a maximum level at 3-4 months. Animals fed PB for longer periods had no further increase in the number of AHF. As expected, while the number of AHF plateaus after a long period of promotion, the volume fraction or number of cells in AHF continue to increase until carcinomas arise (Goldsworthy et al. 1984; Hendrich et al. 1986; Pitot et al. 1989c).

Both AHF and tumors are used as end points in initiation-promotion studies. In this experiment PB at a dietary concentration of 500 ppm did not significantly increase the number of ATPase negative AHF/cm^3 of liver in initiated rats compared to controls. Even though there was no significant increase in the number of AHF using ATPase as a marker, a number of tumors developed in initiated rats promoted with PB. This would suggest that initiated cells were responsive to the proliferative stimulus of PB, which allowed some cells to continue through subsequent stages of the carcinogenesis process without regression (Schulte-Hermann et al. 1986,1989). This is consistent with the fact that PB is a strong tumor promoter in the liver (Periano et al. 1980; Glauert et al. 1986; Saeter and Seglen, 1990). It may be possible that during chronic exposure to PB, multiple "hits" occurred which made cells within AHF tumor promoter independent and capable of progressing to hepatic nodules and hepatocellular carcinomas (Knudson, 1971; Emmelot and Scherer, 1980; Scherer, 1984; Farber and Sarma, 1987). Further evidence which showed that PB was effective in this study was the presence of hypertrophy of hepatocytes in zone 3 of Rappenport indicating that the cytochrome P450 enzyme system was induced (Gumucio and Chianale, 1988).

A number of factors may be responsible for a negative response in initiation-promotion protocols when AHF are used as an end point. Such factors include the type of promoting agent used, treatment too short to induce AHF by a weak carcinogen or use of insensitive markers. For example, the peroxisome proliferator WY-14,643 promoted the development of ATPase negative AHF but not gamma-glutamyl transpeptidase (GGT) positive or glucose-6-phosphatase (G6Pase) negative AHF (Cattley and Popp, 1989). In contrast, PB promoted the development of AHF detected by all three markers. Both GGT and G6Pase were insensitive markers for evaluating the tumor promoting potential of WY-14,643. In another study, C.I. Solvent Yellow 14 induced AHF that had extremely low levels of GGT, but most AHF had high levels of GST-P (Pitot et al. 1989d). In this study by Pitot et al., GGT was also an insensitive marker. On the other hand, GST-P (Sato, 1988; Ito et al. 1988) and GGT (Goldsworthy et al. 1984; Jensen and Sleight, 1986; Evans and Sleight, 1989) were effective markers when other tumor promoters such as phenobarbital were used.

In a number of studies using TCDD and PB as promoting agents, ATPase was an effective marker for identifying preneoplastic lesions (Pitot et al. 1978,1980a). This lessens the possibility that ATPase was an insensitive marker in identifying PB induced lesions in our study.

Dose and type of initiating agent and subsequent environmental alterations may also affect the phenotype of AHF. For example, a number of factors influence GGT activity including diet, strain, age and sex, as well as the choice of carcinogen used (Russell et al. 1987). Whether or not one or more of these factors may have had an effect on ATPase staining in AHF from initiated rats promoted with PB is unknown.

Even though more than 40 markers have been used to characterize AHF, most studies using AHF as an endpoint utilize a single marker for identification and quantitation of AHF (Scherer and Emmelot, 1975a; Hanigan and Pitot, 1985). At present the marker with the greatest efficiency for scoring the largest number of AHF is the placental form of glutathione S-transferase (Tatematsu et al. 1987; Sato, 1988; Pitot et al. 1989d).

It was interesting that at day 170, three animals treated with PB had apoptotic bodies in eosinophilic AHF. Schulte-Hermann et al. (1990) speculated that a relatively high apoptotic activity in AHF during early periods of promotion may help select a more persistent cell population. This persistent cell population may have been one of many factors contributing to development of tumors in initiated rats promoted with PB. In others studies, the number of apoptotic bodies increased after PB withdrawal (Bursch et al. 1984; Garcea et al. 1989). In our study, rats which were off of PB for 170 days did not have an increase in the number of apoptotic bodies. Earlier sacrifice times may have increased the chances of identifying apoptotic bodies histologically.

The persistence of AHF upon removal of the carcinogenic and/or promoting stimulus has been demonstrated under certain experimental conditions in some liver multistage models. Goldsworthy et al. (1984) showed that the number of AHF remained constant for 4 months following the cessation of exposure to PB in an initiation-promotion assay. Other investigators demonstrated regression of AHF after removal of the inducing agent (Bursch et al. 1984; Glauert et al. 1986; Garcea et al. 1989). In our study because of the negative response in the number of ATPase negative AHF, it was difficult to determine if AHF persisted or regressed after withdrawal of PB.

Noninitiated rats given a basal diet, TCDD or phenobarbital developed a low number of AHF. PBBs also enhanced the development of AHF in noninitiated animals (Jensen et al. 1984; Rezabek et al. 1987). Enhancement of AHF may result from promotion of spontaneously initiated cells or may reflect some initiating activity of the promoter.

The mechanisms of tumor promotion are unknown. One mechanism suggests that toxic promoters inhibit cell proliferation in normal hepatocytes but not in initiated cells, believed to be resistent to this effect (Farber and Sarma, 1987). A proposed mechanism of TCDD toxicity is lipid peroxidation (Stohs et al. 1984; Sweeney et al. 1984). Histologically, there was no evidence of fatty change associated with lipid peroxidation in the liver of rats treated with TCDD. Also, dietary levels of TCDD did not appear to be toxic since body weight gains of control and treatment groups were similar.

These studies suggest a different mechanism of promotion by TCDD and PB. TCDD induced a high ratio of AHF to carcinomas compared to PB using ATPase as a negative marker. Current proposed mechanisms of tumor promotion for PB include mitogenic stimulation (Schulte-Hermann et al. 1986), inhibition of intercellular communication (Ruch and Klaunig, 1988), and ADP ribosylation (Romano et al. 1988). The most widely accepted mechanism of action for TCDD and structurally related compounds is receptor-mediated and involves binding to the cytoplasmic Ah receptor with translocation of the TCDD-receptor complex to the nucleus and resultant gene expression (Okey and Vella, 1982; Eisen et al. 1983; Poland, 1984; Bannister and Safe, 1987). Many hepatic tumor promoters (Klaunig and Ruch, 1987; Trosko et al. 1987; Boreiko et al. 1989) and the classic skin tumor promoter TPA inhibit intercellular communication (Madhukar et al. 1989). Lincoln et al. (1987) and Boreiko et al. (1989) showed that TCDD was ineffective in inhibiting intercellular communication at a dose shown to affect differentiation.

The results of the current study indicate that TCDD fed to initiated weanling rats at a low dietary concentration of 150 ppt had a strong promoting and carcinogenic effect, even when administration was delayed as long as 240 days after initiation. It could not be determined whether or not TCDD inhibited the regression or enhanced the development of PB induced AHF.

CHAPTER 2

MORPHOLOGIC EVALUATION OF ALTERED HEPATOCELLULAR FOCI IN SPRAGUE DAWLEY RATS INITIATED WITH NITROSODIETHYLAMINE AND PROMOTED WITH PHENOBARBITAL AND/OR 2,3,7,8-TCDD

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Abstract

In chemically induced hepatocarcinogenesis, short term tests have been used to evaluate the tumor promoting and carcinogenic potential of various compounds. Although a large number of altered hepatocellular foci (AHF) may develop, the significance of such lesions is continuously being debated. The objectives of this study were to characterize AHF in hematoxylin and eosin stained sections, determine if unique foci are present early in the carcinogenesis process and determine whether or not there is a difference in the phenotypic characteristics of AHF induced with the tumor promoter 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or AHF induced with phenobarbital (PB) followed by TCDD. In an initiation-promotion protocol, weanling Sprague Dawley rats were initiated with 10 mg/kg nitrosodiethylamine (NDEA) and promoted with either 500 ppm of PB or 150 ppt TCDD in the diet. Alternatively, initiated rats were promoted with PB

until day 170 and subsequently with TCDD until days 240 or 450. In this study phenotypes of AHF consisted of clear cell, eosinophilic, vacuolated, mixed and various types of basophilic foci including diffuse, tigroid and atypical. The most common AHF in initiated rats promoted with PB or TCDD was the eosinophilic type. Differences in phenotypic characteristics of AHF based on treatment protocols were observed at days 170 and 240 of sacrifice. At day 170, initiated rats promoted with TCDD had a higher incidence of basophilic AHF compared to initiated rats promoted with PB. At day 240, initiated rats promoted with TCDD had eosinophilic AHF with spongiosis hepatis and basophilic AHF including diffuse, atypical and tigroid. On the other hand, initiated rats promoted with PB and subsequently TCDD had a higher incidence of atypical and diffuse basophilic AHF and eosinophilic foci with peliosis hepatis. These foci were generally not present in control initiated rats. It was concluded that critical evaluation of altered hepatocellular foci based on phenotypic characteristics in H&E sections may aid in determining which AHF are of greatest significance in evaluating chemicals for tumor promoting and carcinogenic potential.

Introduction

Altered hepatocellular foci (AHF) are studied most extensively in the liver of rats treated with chemical carcinogens (Schulte-Hermann et al. 1983; Goldsworthy and Pitot, 1985; Hendrich et al. 1986). They are considered preneoplastic lesions and used in several laboratories as indicators of chemicals with carcinogenic potential (Scherer, 1984; Goldsworthy et al. 1986; Schulte-Hermann et al. 1986). Evidence for the precursor relationship of AHF to tumors has been partially derived from studies examining the cytology, dose-dependency, sequence of appearance, clonality, and biological behavior of AHF (Scherer and Emmelot, 1975b; Emmelot and Scherer, 1980; Popp and Goldsworthy, 1989a).

Since many AHF but only a few tumors develop as a result of carcinogen treatment, it is important to determine which foci are most likely to develop into tumors. In most cases only 1 carcinoma develops for every 1,000 to 10,000 foci that are observed either prior to or concurrent with the appearance of the neoplasm (Popp and Goldsworthy, 1989a). In addition, the long latent period of nearly 2 years for the development of tumors in rats exposed to hepatocarcinogens has led to interest in identifying early endpoints that would provide an indication of future tumor development (Solt and Farber, 1976; Weinstein et al. 1983; Farber, 1984). Altered hepatocellular foci provide such an end point in various models of hepatocarcinogenesis (Leonard et al. 1982; Goldsworthy et al. 1986).

Altered hepatocellular foci are clusters of hepatocytes that appear distinct from the surrounding liver when the tissue is stained with a variety of biological and/or histochemical stains (Pitot et al. 1978; Farber, 1980, 1984b). In initiation-promotion protocols approximately 40 different histochemical and immunohistochemical stains have been used to identify AHF in rats for either an elevated or reduced stain for a specific enzyme or protein (Peraino et al. 1983; Peraino et al. 1984; Hanigan and Pitot, 1985; Sato, 1988; Pitot et al. 1989a). Quantitation of AHF has been used to determine the relative potencies of hepatocarcinogenic agents during specific stages of carcinogenesis (Campbell et al. 1982; Pugh et al. 1983). The number and size of AHF are increased in livers of rats exposed to hepatocarcinogens (Scherer, 1984; Farber and Sarma, 1987). While initiation-promotion models can be extended to allow tumor development, treatment periods are frequently abbreviated and the livers evaluated for the presence and number of AHF (Peraino et al. 1983; Farber and Sarma, 1987; Popp and Goldsworthy, 1989). Whereas a large number of AHF may develop in response to tumor promoters and carcinogens, AHF may also occur spontaneously as a function of age (Harada et al. 1989).

Recently emphasis has been placed on evaluating altered hepatocellular foci in hematoxylin and eosin (H&E) stained paraffin sections. Basophilic, tigroid and amphophilic types of AHF are more easily identified using H&E stained sections of liver than with histochemical and immunoperoxidase techniques (Weber et al. 1988; Harada et al. 1989; Bannasch et al. 1989). On a routinely prepared H&E section, cells in AHF may have a clear, eosinophilic, vacuolated, mixed or basophilic appearance (Squire and Levitt, 1975; Maronpot et al. 1986; Bannasch, 1988). Comparative light and electron microscopic studies reveal characteristic alterations in several cytoplasmic constituents of AHF, particularly in the content of glycogen, endoplasmic reticulum and ribosomes (Bannasch et al. 1980). For example, clear cell and eosinophilic AHF have abundant glycogen whereas basophilic AHF have a large number of ribosomes.

Bannasch et al. (1985d) proposed that the sequence of cellular changes during hepatocarcinogenesis progresses from clear and eosinophilic cell foci through mixed cell foci and nodules to basophilic cell populations prevailing in hepatocellular carcinomas. However, other studies show that this sequence probably is not continuous but represents random events in chemical carcinogenesis (Peraino et al. 1984,1988).

Most rat liver short- and mid-term bioassays using AHF as an endpoint are based on the concepts of initiation and promotion (Leonard et al. 1982; Goldsworthy et al. 1986; Fitzgerald and Yamasaki, 1990). Initiation is a chemically induced or spontaneous irreversible genetic event which requires cell division for fixation (Scribner and Suss, 1978; Pitot, 1988a). Generally initiated cells cannot be identified in H&E sections. However, by using immunohistochemical methods it has been possible to identify single initiated cells with the use of the placental form of glutathione-S-transferase (GST-P) marker (Sato, 1988). Unlike initiation, promotion is reversible and dependent on the continuous administration of the promotion agent for the clonal expansion of initiated cells (Farber and Sarma, 1987; Saeter and Seglen, 1990). In the liver AHF represent clonally expanded cell populations (Popp and Goldsworthy, 1989a; Harada et al. 1989). Progression is irreversible and is characterized by the development of hepatic nodules and hepatocellular carcinomas (Pitot, 1989a; Popp and Goldsworthy, 1989a).

Pitot (1989a) recently demonstrated a unique type of AHF termed foci-in-foci using an initiation-promotion-initiation model in rats. Foci-in-foci appear to represent the development of a genetically new population of cells different from AHF (Scherer, 1984; Pitot, 1989a). Estadella et al. (1988) showed that foci-in-foci had additional deficiencies that enabled them to distinguish foci-in-foci from other cells in AHF. They concluded that these foci are subclones originating from cells already modified that have developed an additional phenotypic change. Scherer (1984) suggested that the second carcinogenic agent ethylnitrosourea used in the initiation-promotion-initiation model is able to produce some rare event, probably a mutation, in the cell clone developed from a single cell altered in the first initiation.

Critical parameters in evaluating altered hepatocellular foci in rats during multistage hepatocarcinogenesis include the enumeration of AHF induced by test agents as well as those spontaneously occurring in livers of untreated animals; quantitating the volume percentage of liver occupied by AHF; and evaluating the phenotype/morphology of individual AHF as determined by multiple markers and H&E staining characteristics.

The objectives of this study were to characterize AHF in hematoxylin and eosin stained sections, determine if unique foci were present early in the carcinogenesis process, and determine whether or not there was a difference in the phenotypic characteristics of AHF based on treatment protocols.

Methods

Female weanling Sprague-Dawley rats initially weighing 40 g were used. Rats were initiated with an intraperitoneal injection of 10mg/kg nitrosodiethylamine (NDEA). After 30 days, rats were randomly assigned to treatment groups (n = 6 to 12) and fed a basal diet containing 150 ppt of TCDD until days 170, 240 or 450, or 500 ppm PB until day 170. Alternatively rats were fed a diet containing PB until day 170 and subsequently TCDD until days 240 or 450. Controls included groups of initiated rats given the basal diet and noninitiated rats given TCDD or PB in the diet. Rats were anesthetized with CO_2 and killed at day 170, 240 or 450. Please see experimental design in Chapter 1 (Figure 1.1).

Five sections of liver (two sections from the left lobe and one section from the median, right lateral and caudate lobes) were taken from each rat and stained for the enzyme adenosinetriphosphatase (ATPase). The number and volume of AHF negative for ATPase were quantitated using an image analyzer. Additional sections of liver were taken from the remaining hepatic lobes of each rat, fixed in 10% buffered formalin, sectioned and stained with H&E. Tumors determined grossly at necropsy were confirmed by histologic examination as hepatic nodules and hepatocellular carcinomas.

In H&E stained sections altered hepatocellular foci were classified as clear cell, eosinophilic, basophilic, vacuolated and mixed using previously published criteria (Squire and Levitt, 1975; Stewart et al. 1980; Bannasch et al. 1985d; Harada et al. 1989). When 70% of an AHF was comprised of a single cell type, it was classified according to the predominant cell. Mixed cell foci were comprised of 2 or more cell types with no one type occupying greater than 70% of the AHF. The minimum size of AHF documented consisted of at least 10 cells.

Results

Quantitation of AHF with the ATPase negative marker was previously reported (Chapter 1). The number of AHF/cm^3 and mean volume of AHF in the liver increased with time in initiated rats treated with TCDD or PB followed by TCDD. An increase in the number of AHF correlated with the number of hepatic nodules and hepatocellular carcinomas at day 450.

Various types of AHF were present in this study. The predominant type of AHF was eosinophilic (Figure 2.1). These foci were generally spherical in shape and confined to hepatic lobules at days 170 and 240 of sacrifice. Eosinophilic AHF occasionally occupied more than one lobule and sometimes caused slight compression of adjacent hepatocytes by day 240 of sacrifice. Compared to surrounding hepatocytes, cells in eosinophilic foci were larger, the cytoplasm was brightly eosinophilic to ground glass in appearance and nuclei contained prominent nucleoli.

Subclassifications within the eosinophilic AHF group included eosinophilic foci with peliosis hepatis or eosinophilic foci with spongiosis hepatis. Eosinophilic foci with peliosis hepatis were characterized by dilated sinusoids filled with red blood cells and separated from one another by cords of hepatocytes (Figure 2.2). Eosinophilic foci with spongiosis hepatis were characterized by multifocal cyst-like structures lined by fibroblast type cells and filled with finely granular or flocculent pale eosinophilic material (Figure 2.3).

Basophilic AHF were less commonly observed when compared to eosinophilic foci. Basophilic AHF were subclassified into the following groups: diffuse, tigroid, and atypical. Of the basophilic category diffuse basophilic AHF had a higher incidence. Diffuse basophilic foci were characterized by increased cytoplasmic basophilia, hepatocytes were smaller than surrounding hepatocytes and hepatic plates were tortuous (Figure 2.4). Tigroid foci were characterized by a basophilic cytoplasm which had a band of localized basophilia at the periphery (Figure 2.5). The cells in these foci were larger than cells in diffuse basophilic foci. Atypical basophilic foci were often associated with vessels, irregular in shape, cells were smaller than surrounding hepatocytes and the cytoplasm was strongly basophilic (Figure 2.6).

Mixed cell foci were more commonly observed at day 240 of sacrifice. They consisted of two or more combinations of eosinophilic, basophilic, vacuolated or clear cells (Figure 2.7). Vacuolated or clear cell foci were rarely observed. Vacuolated cell foci were characterized by discrete cytoplasmic accumulations of micro- and macrovacuoles which displaced the nucleus to the periphery (Figure 2.8). The vacuoles represented lipid dissolved by xylene during processing. Clear cell foci were characterized by irregular clear spaces within the cytoplasm which represented glycogen dissolved by the aqueous formalin fixative (Figure 2.9). Hepatocytes in clear and vacuolated foci were normal or moderately enlarged.

Table 2.1 and 2.2 summarizes the incidence of altered hepatocellular foci at days 170 and 240 of sacrifice and show differences between treatment groups. At day 170, initiated rats promoted with TCDD had a higher incidence of basophilic AHF compared to initiated rats promoted with PB (Table 2.1). At day 240, initiated rats promoted with TCDD had eosinophilic foci with spongiosis hepatis and basophilic foci including atypical and tigroid types. On the other hand, initiated rats promoted with PB and subsequently TCDD had eosinophilic foci with peliosis hepatis and a higher incidence of basophilic foci including atypical and diffuse types (Table 2.2).

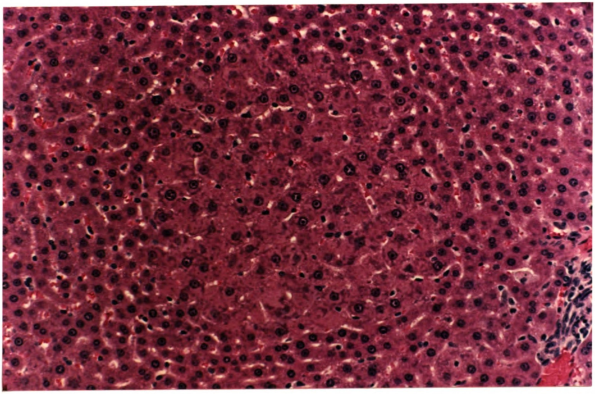

Figure 2.1. Photomicrograph of an eosinophilic altered hepatocellular focus (AHF) from a rat fed a diet containing TCDD. Notice the slightly enlarged hepatocytes. This was the most commonly observed preneoplastic lesion. H&E. (X 300).

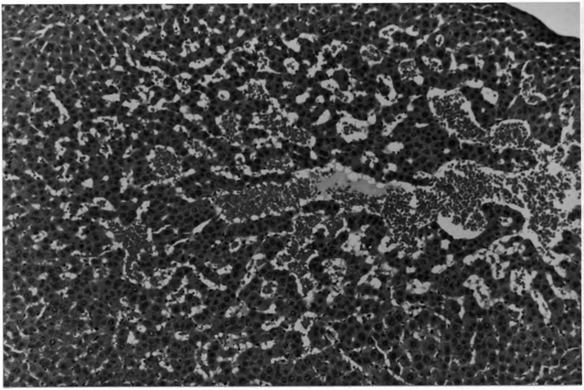

Figure 2.2. Photomicrograph of a large eosinophilic AHF with peliosis hepatis from a rat fed a diet containing PB and then TCDD. Notice the irregular focal dilations of the sinusoids containing red blood cells and separated by trabecular-like cords. H&E. (X 150).

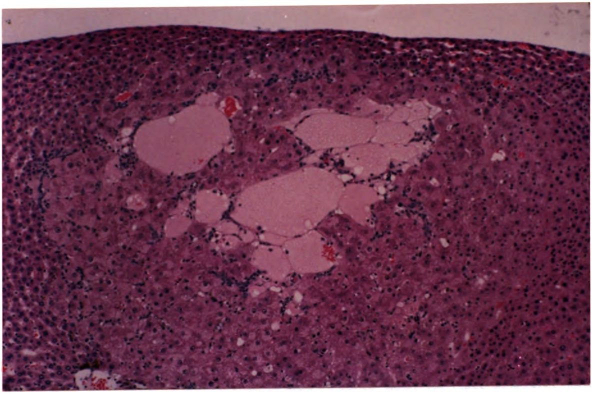

Figure 2.3. Photomicrograph of a large eosinophilic AHF with spongiosis hepatis from a rat fed a diet containing TCDD. Notice the cyst-like multilocular formations filled with a finely granular eosinophilic material. H&E. (X 150).

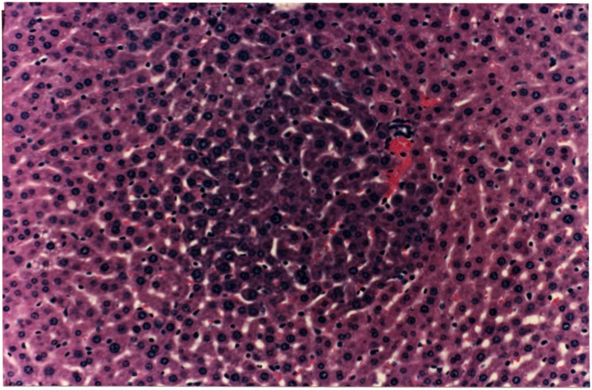

Figure 2.4. Photomicrograph of a basophilic AHF from a rat fed a diet containing PB and then TCDD. Notice the small hepatocytes in irregularly formed hepatic plates and the diffuse basophilic staining of the cytoplasm. H&E. (X 300).

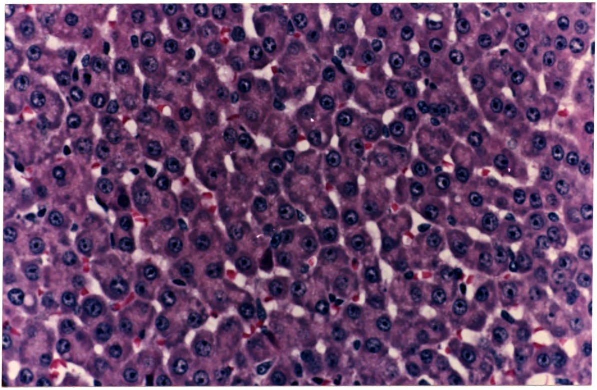

Figure 2.5. Photomicrograph of a tigroid basophilic AHF from a rat fed a diet containing TCDD. Notice the intense basophilic staining of the peripheral cytoplasm (tigroid pattern). H&E. (X 600).

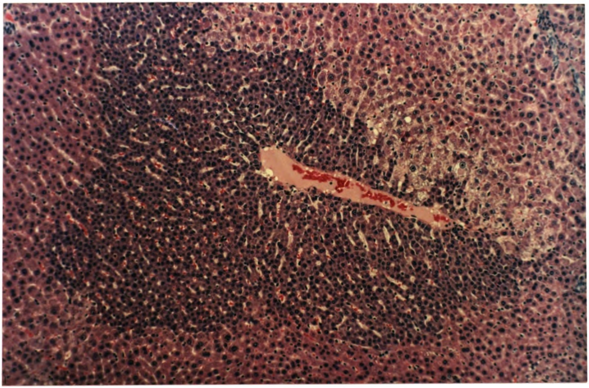

Figure 2.6. Photomicrograph of an atypical basophilic AHF present around the central vein from a rat fed a diet containing PB and then TCDD. Notice the irregular shape of the focus. H&E. (X 150).

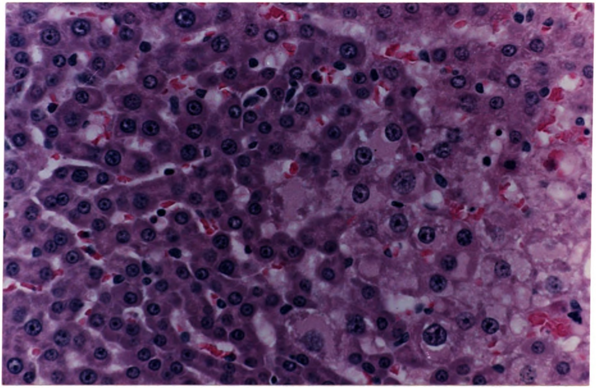

Figure 2.7. Photomicrograph of a mixed AHF from a rat fed a diet containing TCDD. Notice the mixture of pale eosinophilic and basophilic cells. H&E. (X 600).

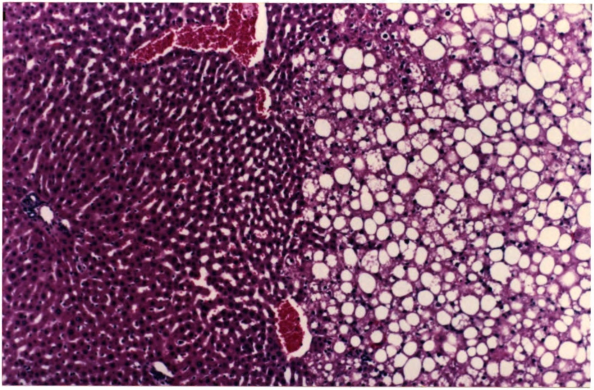

Figure 2.8. Photomicrograph of a vacuolated AHF from a rat fed a diet containing PB. Notice the micro- and macrovacuoles. H&E. (X 600).

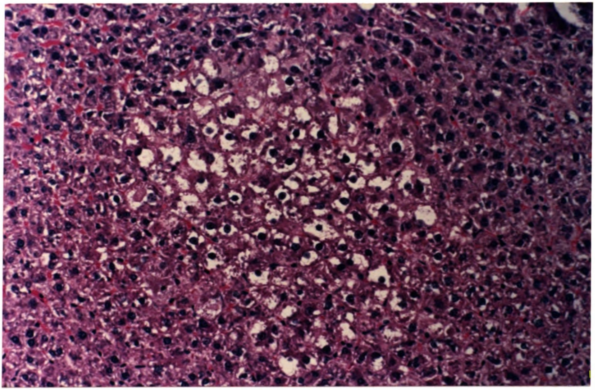

Figure 2.9. Photomicrograph of a clear cell AHF from a rat fed a diet containing TCDD. Notice the irregular clear spaces within the cytoplasm. H&E. (X 300).

Table 2.1. Incidence of Altered Hepatocellular Foci at 170 days in Initiated Rats Promoted with TCDD or PB

Altered hepatocellular foci	Treatment		
	NDEA+BD n=6	NDEA+TCDD n=6	NDEA+PB n=12
Eosinophilic foci	6	6	12
Clear cell foci	-	-	-
Vacuolated cell foci	-	-	1
Mixed cell foci	-	2	2
Basophilic foci*	-	6	4
Tigroid	-	1	-
Diffuse	-	6	3
Atypical	-	1	1

* = Some animals had multiple types of basophilic foci
n = Number of rats

Table 2.2. Incidence of Altered Hepatocellular Foci at 240 Days in Initiated Rats Promoted with TCDD and/or PB

Altered Hepatocellular Foci	Treatment			
	NDEA+ BD n=6	NDEA+ TCDD n=6	NDEA+ PB n=12	NDEA+ PB+TCDD n=12
Eosinophilic foci	6	6	12	12
Large eosinophilic foci				
with spongiosis hepatis	-	2	-	-
with peliosis hepatis	-	-	-	3
Clear cell foci	1	1	-	-
Vacuolated cell foci	-	-	-	2
Mixed cell foci	5	6	9	9
Basophilic foci*	1	3	3	6
Tigroid	-	2	1	1
Diffuse	1	2	1	4
Atypical	-	2	1	4

* = Some animals had multiple types of basophilic foci
n = Number of rats

Discussion

TCDD (Pitot et al. 1980a; Flodstrom et al. 1991) and phenobarbital (Goldsworthy and Pitot, 1985; Hendrich et al. 1986) are known tumor promoters in short term and chronic studies in rats. In previous research, quantitation and characterization of AHF was limited to histochemical stains with little interpretation of AHF using H&E stains. Most AHF induced by PB and TCDD were evaluated by the use of histochemical stains including gamma-glutamyltranspeptidase (GGT), adenosine triphosphatase (ATPase) and glucose-6-phosphatase (Pitot et al. 1978,1980).

From this study it was shown that morphologically unique types of AHF were present in sections of liver stained with H&E stains. AHF present early in the carcinogenesis process included eosinophilic foci with peliosis hepatis, eosinophilic foci with spongiosis hepatis, and atypical and tigroid basophilic foci. Since such foci of cellular alteration were not in control rats, their occurrence may be taken as presumptive evidence in support that these lesions were chemically induced and associated with their tumor promoting and hepatocarcinogenic potential.

Differences in phenotypic characteristics based on treatment were also present. At day 170, initiated rats promoted with TCDD had a higher incidence of basophilic AHF compared to initiated rats promoted with PB. At day 240, initiated rats promoted with TCDD had eosinophilic foci with spongiosis hepatis and basophilic foci including atypical and tigroid types. On the other hand, initiated rats promoted with PB and subsequently with TCDD had a higher incidence of atypical and diffuse basophilic foci and eosinophilic foci with peliosis hepatis.

It is proposed that when cells of different morphology or phenotypes arise in areas of preneoplasia or benign neoplasia, further genetic alterations may have taken place in the initial lesion, leading to the development of the secondary lesion (Pitot, 1989a). Whether or not the development of spongiosis hepatis in initiated rats promoted with TCDD, or peliosis hepatis in initiated rats promoted with PB and followed by TCDD represent additional genetic alterations is unknown but needs to be further evaluated.

Focal sinusoidal dilatation corresponding to peliosis hepatis was induced in rodents by nitrosamines, nitrosamides and nitrosomethylurea (Bannasch et al. 1985b). Popper et al. (1977) and Ward (1981) indicated that the occurrence of peliosis within hepatic adenomas was common. Wayss et al. (1979) showed that the phlebectatic form of peliosis hepatis occurred in Mastomys natalensis when a single low dose of dimethylnitrosamine (10 mg/kg body weight) was administered. In the study of Wayss et al., peliosis hepatis occurred early, followed by a proposed sequence of benign hemangioendotheliomas and finally angiosarcomas. It was concluded that these lesions represented a spectrum of changes which progressed finally to neoplasia. In our study, peliosis hepatis was present in eosinophilic foci, but there was no progression in lesions to angiosarcomas. Lee et al. (1983) showed that two-year-old Sprague-Dawley rats had a high prevalence of peliosis hepatis, however these lesions were rare in rats under one year of age. The incidence was twice as high in males when compared to females (Lee, 1983).

Bannasch et al. (1981) were the first to describe spongiosis hepatis in the liver of rats treated with N-nitrosomorpholine. In contrast to peliosis hepatitis where the sinusoids are filled with blood, the cavities of spongiosis hepatis are filled with flocculent material rich in acid mucopolysaccharides (Bannasch et al. 1985c). Cattley et al. (1989) recently described spongiosis hepatis in several neoplasms from phenobarbital-treated male Fischer 344 (F344) rats, but not in neoplasms from WY-14,643 treated rats. Spongiosis hepatis developed frequently in the livers of rats treated with N-nitromorpholine, dimethylnitrosamine, nitrosopyrrolidine and 2-acetylaminofluorene (Bannasch et al. 1981; Zerban and Bannasch, 1983; Ito et al. 1984). In old rats, spongiosis hepatis may occur spontaneously but is usually rare in younger untreated control rats (Bannasch et al. 1985c).

In stop experiments using the carcinogen N-nitrosomorpholine Bannasch et al. (1985d,1988) proposed that altered hepatocellular foci progress from clear cell through eosinophilic, mixed and basophilic to neoplastic lesions in Sprague-Dawley rats. This linear relationship among AHF was also demonstrated in rats with other chemicals such as diethylnitrosamine, dimethylaminoazobenzene and thioacetamide (Bannasch et al.1980). It is postulated that AHF, particularly the basophilic type, may be related to the development of hepatocellular neoplasms. Harada et al. (1989) suggested that most basophilic AHF are not related to the development of hepatocellular neoplasms in control F344 rats. Even though control F344 rats had a high incidence of basophilic AHF, the occurrence of hepatocellular neoplasms was very low.

In our study the progression of AHF from clear cell to basophilic was not clearly defined. Few clear cell foci were present at day 240 of sacrifice. The majority of AHF present at all sacrifice times were eosinophilic. At day 240 of sacrifice a larger number of animals had mixed cell foci and various types of basophilic foci. These findings were suggestive of a trend in the progression of lesions from clear cell to basophilic as proposed by Bannasch et al. (1985d). A study with earlier and multiple end points may provide additional information on the sequential progression of AHF.

Although it is proposed that a progression of lesions from preneoplastic to neoplastic (AHF to hepatic nodules and finally hepatocellular carcinomas) may be important in hepatocarcinogenesis it is also probable that hepatocellular carcinomas may develop directly from AHF without going through the intermediate stage of hepatic nodules. Foci-in-foci may represent lesions capable of developing into hepatocellular carcinomas (Scherer, 1984; Pitot, 1989a).

While it is evident that the number of chemically induced AHF far exceed the number of neoplasms in treated animals, some focal phenotypes may have a greater probability of progressing to neoplasms. It is proposed that cells exhibiting multiple markers possess an increased likelihood of developing into tumors (Popp and Goldsworthy, 1989a). Relative increased growth rates of AHF are related to the number of markers per focus (Tanaka et al. 1986; Goldsworthy and Pitot, 1985). Foci with multiple phenotypes in H&E stained sections such as eosinophilic foci with spongiosis hepatis, eosinophilic foci with peliosis hepatis, mixed cell foci and the various types of basophilic foci may have a similar greater likelihood of developing into tumors.

Schulte-Hermann (1981,1987) demonstrated that hepatocytes in AHF had higher rates of replication than hepatocytes in the surrounding liver, and that proliferation of AHF was increased by the administration of one of several liver tumor promoters. Increased proliferative rates in AHF increases the probability of further genetic alterations occurring (Richardson and Swenberg, 1987; Farber and Sarma, 1987). Variable degrees of proliferative activity have been demonstrated in AHF using H&E staining characteristics. Zerban et al. (1985) showed that the proliferative rate in early appearing clear and eosinophilic foci was slightly increased but not significantly different from the growth rate of hepatocytes from untreated controls. A pronounced and steadily increasing cell proliferative rate was linked to the appearance of mixed and basophilic foci, nodules and carcinomas, supporting the hypothesis that mixed and basophilic foci may be closely linked to the carcinogenesis process.

The phenotype of AHF depends on the carcinogen used in initiation-promotion bioassay. For example, peroxisome proliferators induce a greater proportion of basophilic foci compared to other AHF (Marsman and Popp, 1989). In other studies basophilic foci were negative for enzymes such as GGT and GST-P; therefore identification and quantification of AHF induced by peroxisome proliferators may best be pursued by using the H&E stain and other histochemical markers (Rao et al. 1988; Yeldandi et al. 1989). Evaluation of H&E stained slides may allow a more precise evaluation of AHF (Bannasch et al. 1989). For example, AHF such as tigroid cell foci induced with aflatoxin B_1 (Bannasch et al. 1985a) and amphophilic cell foci induced by N-nitrosomorpholine followed by dehydroepiandrosterone (Weber et al. 1988) lacked enzymatic changes frequently present in other AHF but were easily identified using the H&E stain. Conversely, some foci could only be identified with such enzymes as glucose-6-phosphatase and pyruvate kinase (Enzmann et al. 1989). Thus, in order to optimize the greatest number of AHF and appreciate their phenotypic heterogeneity it may be necessary to use H&E and histochemical markers.

Strain differences in AHF are observed in Sprague-Dawley and F344 rats. Both strains of rats are commonly used in initiation-promotion studies. In F344 rats the majority of AHF observed are basophilic. The number and size increases with age and the incidence may be nearly 100% in two-year old females (Maronpot et al. 1986; Harada et al. 1989). In Sprague-Dawley rats the majority of AHF observed are eosinophilic. Generally a high incidence of spontaneous focal lesions does not occur in this strain of rat (Bannasch et al. 1989).

During the stage of promotion, there is a transition when rare AHF develop into neoplastic lesions (Emmelot and Scherer, 1980; Saeter and Seglen, 1990). In addition to characterizing and determining morphologically which AHF have a greater chance of developing into neoplasms, it is also important to determine at the molecular level, alterations necessary for the progression of preneoplastic lesions to hepatocellular carcinomas. Critical evaluation of altered hepatocellular foci for alterations in proto-oncogenes and tumor suppressor genes may further aid in determining which foci are of greatest significance in hepatocarcinogenesis.

CHAPTER 3

IMMUNOHISTOCHEMICAL DETECTION OF RAS P21 PROTEIN IN PRENEOPLASTIC AND NEOPLASTIC LESIONS DURING CHEMICALLY INDUCED HEPATOCARCINOGENESIS

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Abstract

Oncogenes most frequently detected in human and animal tumors belong to the ras gene family. Mutations in specific amino acids and overexpression of normal proteins have been linked to altered proliferation and/or differentiation and, particularly to the neoplastic process. The objectives of this study were to determine if the ras p21 protein was consistently present throughout multistage hepatocarcinogenesis or was present in specific stages of the process. Also, we wanted to determine if individual cell types such as hepatocytes, oval cells and bile duct cells expressed the ras p21 protein and, whether or not there was a difference in ras p21 protein expression in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and phenobarbital (PB) induced hepatic lesions. Animals were initiated with 10 mg/kg BW of N-nitrosodiethylamine (NDEA) ip and given 500 ppm PB or 150 ppt TCDD in the diet. Using the avidin-biotin-

immunoperoxidase technique and a *Ras* 11 monoclonal antibody the expression of *ras* p21 protein was evaluated in paraffin-embedded sections. *ras* p21 protein was present in altered hepatocellular foci (104/124), hepatic nodules (11/13) and hepatocellular carcinomas (9/9). Cytoplasmic and membrane staining were the patterns of reactivity observed. Since translocation of *ras* p21 protein to the membrane is associated with transformation it is proposed that foci with membrane staining may have a greater propensity for developing into neoplastic lesions. There was no difference in *ras* p21 expression between PB- and TCDD-induced lesions. In regard to tumor promotion it is proposed that *ras* p21 protein expression is an early and stable event which may be associated with the proliferative process of carcinogenesis.

Introduction

Previous studies in our laboratory have used histochemical markers to demonstrate and quantify altered hepatocellular foci, and hematoxylin and eosin staining characteristics to define preneoplastic and neoplastic lesions in a two-stage initiation-promotion model of hepatocarcinogenesis (Jensen et al. 1982,1984; Rezabek et al. 1987). By the use of these procedures we have shown that relatively low doses of important environmental chemicals such as polybrominated and polychlorinated biphenyls act as promoters of hepatocarcinogenesis (Jensen and Sleight, 1986; Dixon et al. 1988; Evans et al. 1989). Since the process of multistage hepatocarcinogenesis is well documented we decided to use immunohistochemical methods to determine if the proto-oncogene product ras p21 protein is present in preneoplastic and neoplastic lesions.

Proto-oncogenes (c-onc) are normal cellular genes present in a cell which play key roles in growth control (Pimentel, 1986c; Seemayer and Cavenee, 1989). They influence cell proliferation and differentiation (Travali et al. 1990). Oncogenes are proto-oncogenes that have been altered by point mutational or transcriptional mechanisms (Guerrero and Pellicer, 1987; Paul, 1988). Transcriptional mechanisms include insertional mutagenesis, chromosomal translocations, gene amplification and hypomethylation (Bishop, 1987; Bell, 1988). When proto-oncogenes are overexpressed, mutated or deregulated they can be associated with transformation of cells (Bishop, 1987; Seemayer and Cavenee, 1989; Vorce and Goodman, 1990).

The cellular oncogenes important in hepatocellular growth include fos, myc and ras which are expressed at specific times and in sequence during the cell cycle (Kaczmarek, 1986). Oncogenes most frequently detected in human and animal tumors belong to the ras gene family which include Harvey ras, Kirsten ras and N-ras (Ha-ras, Ki-ras, and N-ras) (Fiorucci and Hall, 1988; Bos, 1988). These genes encode a group of closely related 21,000 dalton proteins termed ras p21

(Barbacid, 1987; Sigal et al. 1988). The proteins are known to be located in the cytoplasm and on the inner surface of the plasma membrane (Grand, 1987; Cales et al. 1988). They bind to GDP and GTP and possess GTPase activity (Hurley et al. 1984; Hoshino et al. 1987). Expression of ras genes at unscheduled times results in abnormal cell growth, proliferation and differentiation (Barbacid, 1987; Santos and Nebreda, 1989).

One of the mechanisms of ras proto-oncogene activation is by point mutations. Point mutations affect amino acid residues 12, 13, 59 and 61 of the ras encoded p21 protein and they impart to this protein the ability to transform cells even when present in very low levels (Barbacid, 1987; Guerrero and Pellicer, 1987; Bos, 1988; Stowers et al. 1988). Ha-ras mutations are common in both spontaneous and chemically induced liver tumors in $B6C3F_1$ mice (Reynolds et al. 1987). Loktionov et al. (1990) demonstrated Ha-ras mutations in DMBA-induced hepatomas in mice. No Ki-ras mutations were detected. The Ha-ras gene appears to be more important in liver carcinogenesis than Ki- and N-ras genes.

An alternative mechanism of ras proto-oncogene activation is by transcriptional activation (Barbacid, 1987; Bos, 1988). Elevated expression of Ha-ras and c-myc proto-oncogenes have been reported in liver tumors of rats given various carcinogenic regimens (Makino et al. 1984; Yaswen et al. 1985; Cote and Chiu, 1987). Long term feeding of a choline-deficient diet in rats caused elevated transcripts of all three ras genes in hepatocellular tumors (Chandar et al. 1987). Elevations in ras p21 proteins were associated with active cell proliferation in the tumors. Unregulated expression of proto-oncogenes via transcriptional mechanisms may provide some selective or maintenance role during the development of preneoplastic and neoplastic lesions in the rat (Beer and Neveu, 1990).

Recently attempts have been made to assign specific oncogene products to various stages of carcinogenesis which include initiation, promotion and progression (Weinstein et al. 1984; Farber and Sarma, 1987; Weinstein, 1988; Fischer et al. 1988). Work in animal models indicate that ras genes are a direct target of many initiating carcinogens including N-methyl-N'-nitro-N-nitroso-guanidine, methylnitrosourea and 3-methyl-cholanthrene (Barbacid, 1987; Brown et al. 1990). Kumar et al. (1990) showed that activation of ras oncogenes can precede the onset of neoplasia in the mammary gland.

During promotion, transcriptional activation of proto-oncogenes has been reported in preneoplastic hepatocellular lesions (Corcos et al. 1984; Cote et al. 1985; Beer and Neveu, 1990; Pitot, 1990). The mechanism of activation has not been determined. Using histochemical procedures Galand et al. (1988) detected c-Ha-ras protein product in diethylnitrosamine induced preneoplastic and neoplastic lesions in a rat hepatocarcinogenesis model. Other studies have shown increased transcripts of ras and myc proto-oncogenes in preneoplastic lesions (Makino et al. 1984; Yaswen et al. 1985; Zhang et al. 1988).

The final stage of hepatocarcinogenesis is termed progression (Guerrero and Pellicer, 1987; Pitot, 1989). During this stage altered hepatocellular foci and hepatic nodules develop into fully malignant lesions. Progression may require the cooperation of a number of factors including oncogenes, tumor suppressor genes and growth factors (Friend et al. 1988; Walker, 1989; Druker et al. 1989). The raf proto-oncogene may be important in the progression stage of hepatocarcinogenesis. Beer et al. (1988) showed that c-raf was abundantly expressed in most primary liver tumors but not in preneoplastic lesions.

The objectives of this study were (1) to determine if the ras p21 protein was consistently present throughout multistage hepatocarcinogenesis or if it was present only in specific stages of the process, (2) to determine if individual cell types such as hepatocytes, oval cells and bile duct cells expressed the ras p21 protein and, (3) to determine whether or not there was a difference in ras p21 protein expression in TCDD- and PB-induced hepatic lesions.

Methods

Female weanling Sprague-Dawley rats were initiated with an intraperitoneal injection of 10 mg/kg BW of N-nitrosodiethylamine (NDEA) and given 500 ppm phenobarbital or 150 ppt TCDD in the diet. Rats were anesthetized with CO_2 and killed at day 170, 240 or 450. Representative sections of liver were taken from each rat and fixed in 10% buffered formalin. The tissues were processed and embedded in paraffin. The immunohistochemical assay used for the detection of ras p21 protein was the avidin-biotin peroxidase system (Bourne, 1983).

Positive controls used to establish the immunoperoxidase procedure were subcutaneous tumors made in nude mice from H- or N-ras transfected human fibroblasts. The tumors were provided by Drs. John Dillberger and Calvert Louden from the Carcinogenesis Laboratory at Michigan State University.

Negative controls included a mouse monoclonal antibody against a protein unrelated to the ras p21 protein which was not present in the liver. An antibody which detects glial fibrillary acetic protein (provided by Dr. Philip Boyer) and phosphate buffered saline (PBS) were substituted for the primary antibody. Additional controls used in establishing the procedure included substituting PBS for the secondary antibody and avidin-biotin peroxidase complex.

The murine monoclonal antibody Ras 11 (NEI-704), raised against a recombinant ras protein was purchased from E.I. Du

Pont de Nemours, Inc., MA. The antibody is able to immunoprecipitate _ras_ proteins, regardless of the amino acid at position 12. It detects the proteins expressed by all members of the _ras_ gene family (Ha-, Ki- and N-_ras_). The optimal dilution of the antibody was determined using checkerboard titration.

Paraffin embedded sections of liver containing altered hepatocellular foci (AHF), hepatic nodules and hepatocellular carcinomas were used. Sections of liver 5 um thick were mounted on poly-l-lysine coated glass slides. Each paraffin section was deparaffinized in xylene, hydrated through decreasing concentrations of ethyl alcohol, and washed in PBS, pH 7.2. Following each step, the slides were washed in PBS. Endogenous peroxidase activity was inhibited by immersing slides for 10 minutes in absolute methanol containing 30% H_2O_2. Following a 20 minute rinse in double PBS, the sections were incubated for 20 minutes with diluted normal serum from the species in which the secondary antibody was made. Excess serum was blotted from the sections.

Sections were incubated with the _Ras_ 11 antibody for two hours at a temperature of 22°C and a final dilution of 1:1500 (working dilution was 1:150). After a 10 minute rinse in PBS, sections were incubated with a biotinylated secondary antibody for 30 minutes, and then the avidin-biotin-peroxidase complex solution for 45 minutes (Vectastain ABC kit, Vector Lab., CA.). Sections were incubated with the chromogen 3,3-

diaminobenzidine (DAB) for 5 minutes. Peroxidase reaction with DAB forms a stable brown reaction product, indicating a positive reaction for the ras p21 protein. Sections were then lightly counterstained with Gill's hematoxylin, dehydrated, cleared and mounted with non-aqueous coverslip resin (Permount; Fisher Scientific, Cincinnati, OH).

Results

The immunohistochemical procedure for detecting the ras p21 protein was initially done in subcutaneous tumors made in nude mice. The tumors were made from H-ras and N-ras transfected human fibroblasts. In positive controls, strong brown cytoplasmic staining consistent with the presence of the ras p21 protein was detected in tumors. In negative controls, no brown cytoplasmic or membrane staining were present.

Once the procedure was established, sections of liver containing preneoplastic and neoplastic hepatocellular lesions were evaluated. ras p21 protein was present in altered hepatocellular foci (104/124), hepatic nodules (11/13) and hepatocellular carcinomas (9/9). There was no difference in ras p21 expression between PB and TCDD induced lesions. Minimal staining of hepatocytes surrounding preneoplastic and neoplastic lesions was observed.

Altered hepatocellular foci (AHF) had two patterns of staining; diffuse cytoplasmic (Figure 3.1) and membrane (Figure 3.2). Cytoplasmic staining was predominant in AHF whereas membrane staining was occasionally observed.

Hepatic nodules had two patterns of staining. In some nodules, diffuse cytoplasmic staining was present (Figure 3.3) whereas in others, individual cytoplasmic staining of hepatocytes and oval cells were observed (Figure 3.4 and 3.5). Bile duct cells were negative for the ras p21 protein.

Hepatocellular carcinomas had diffuse cytoplasmic staining. Glandular structures within hepatocellular carcinomas were strongly positive for the ras p21 protein (Figure 3.6 and 3.7).

Table 3.1 summarizes the proportion of altered hepatocellular foci positive for ras p21 protein at day 170 of sacrifice. Table 3.2 summarizes the proportion of hepatic nodules and hepatocellular carcinomas positive for ras p21 protein at day 450 of sacrifice.

Hepatocellular lesions evaluated from animals sacrificed at days 170 and 450 were representative of AHF, hepatic nodules and hepatocellular carcinomas, thus lesions from animals sacrificed at day 240 were not included in the immunohistochemical study.

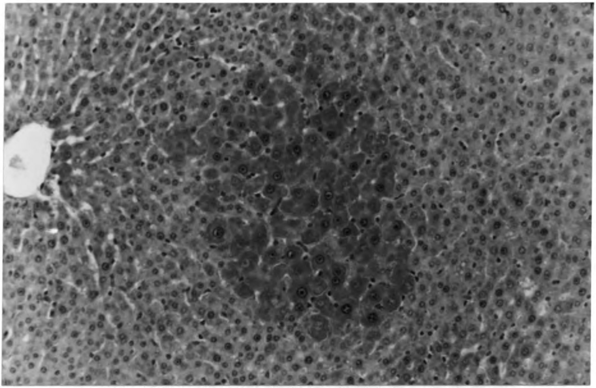

Figure 3.1. Photomicrograph of an AHF from a rat initiated with NDEA, and fed a diet containing phenobarbital (PB). Notice the diffuse cytoplasmic staining of hepatocytes with the ras p21 antibody. All sections were counterstained with hematoxylin, (X 300).

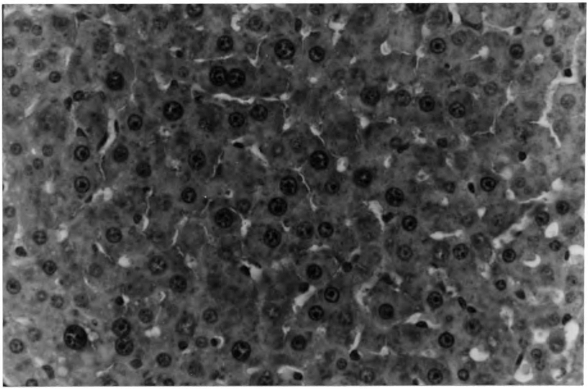

Figure 3.2. Photomicrograph of an AHF from a rat initiated with NDEA, and fed a diet containing PB. Notice the membrane staining of hepatocytes with the ras p21 antibody, (X 600).

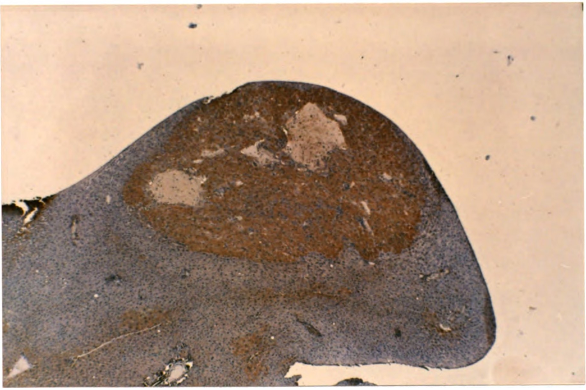

Figure 3.3. Photomicrograph of a large hepatic nodule from a rat initiated with NDEA, and fed a diet containing PB and then TCDD. The cytoplasm of the hepatocytes in the hepatic nodule stain diffusely with the ras p21 antibody. Notice the lack of positive staining in the normal hepatocytes, (X 100).

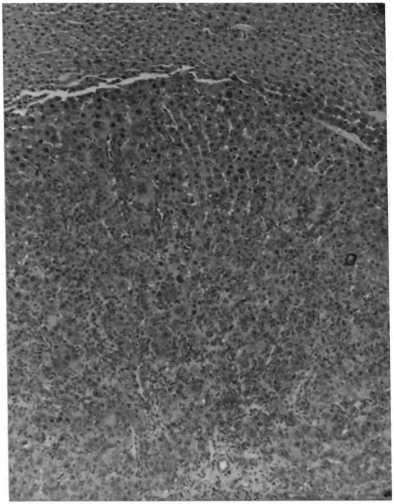

Figure 3.4. Photomicrograph of a hepatic nodule from a rat initiated with NDEA, and fed a diet containing PB. Notice the lack of positive staining in the normal hepatocytes adjacent to the hepatic nodule, (X 150).

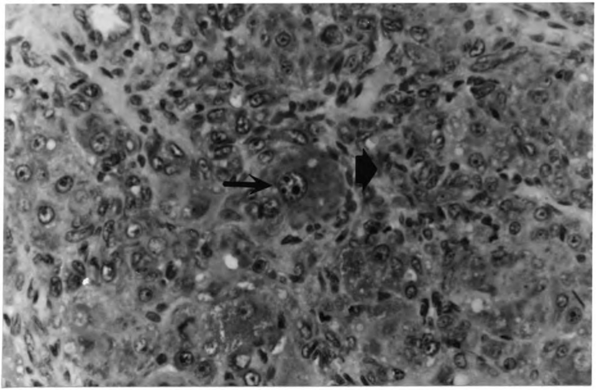

Figure 3.5. Photomicrograph of the hepatic nodule from Figure 3.4. Notice the heterogeneous cell population and cytoplasmic staining in hepatocytes (arrow) and oval cells (arrowhead) with the ras p21 antibody, (X 600).

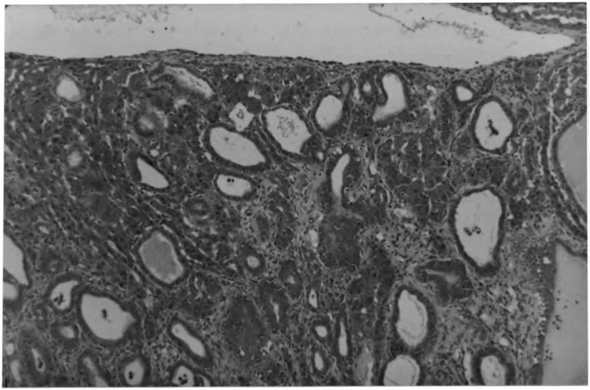

Figure 3.6. Photomicrograph of a hepatocellular carcinoma from a rat initiated with NDEA, and fed a diet containing PB and then TCDD. The glandular structures and hepatocytes in the tumor stain with the ras p21 antibody. Notice the adjacent connective tissue stroma is negative, (X 150).

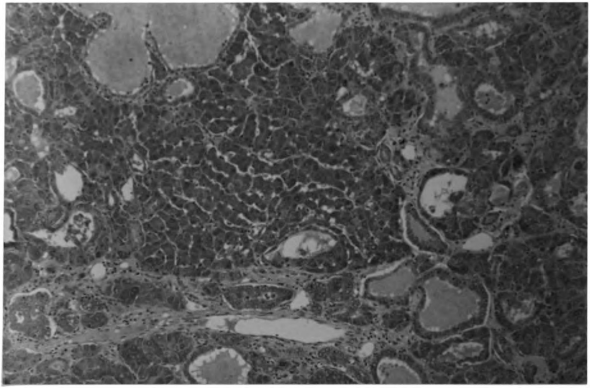

Figure 3.7. Photomicrograph of a hepatocellular carcinoma from a rat initiated with NDEA, and fed a diet containing PB and then TCDD. Notice the diffuse cytoplasmic staining of hepatocytes with the ras p21 antibody (X 300).

Table 3.1. Immunohistochemical Detection of *ras* p21 Protein in Altered Hepatocellular Foci of NDEA Initiated Rats at 170 Days

Treatment groups	Proportion of foci positive for *ras* p21
NDEA + BD	2/8 (25%)
NDEA + TCDD	42/46 (91%)
NDEA + PB	60/70 (86%)
TOTAL	104/124 (84%)

Table 3.2. Immunohistochemical Detection of *ras* p21 Protein in Hepatic Nodules (HN) and Hepatocellular Carcinomas (HC) of NDEA Initiated Rats at 450 Days

Treatment	HN positive for *ras* p21	HC positive for *ras* p21
NDEA + Basal Diet (BD)	---	---
NDEA + Phenobarbital (PB)	1/2	2/2
NDEA + TCDD	1/2	3/3
NDEA + PB + TCDD	4/4	1/1
NDEA + PB + BD + TCDD	5/5	3/3
TOTAL	11/13 (85%)	9/9 (100%)

Discussion

In our study *ras* p21 protein was present in altered hepatocellular foci, hepatic nodules and hepatocellular carcinomas with minimal expression in normal tissues. Galand et al. (1988) also showed that hepatocellular carcinomas (14/14), neoplastic nodules (8/8) and foci of phenotypic alteration had moderate to high immunostaining with an antibody raised against a peptide sequence of the Ha-*ras* p21 product. These results suggest that the expression of *ras* p21 protein may be an early and stable event in chemically induced hepatocarcinogenesis. Other studies using immunohistochemical methods have demonstrated the *ras* p21 protein in normal and neoplastic tissues (Ward et al. 1986; Nonomura et al. 1987,1988; Ward et al. 1989a; Czerniak et al. 1990).

Two patterns of staining; membrane and cytoplasmic, were observed in paraffin embedded sections of liver with the *ras* p21 antibody. Ward et al. (1986,1989a) demonstrated that the *ras* p21 protein immunoreacted on the cell membrane and in the cytoplasm of fixed sarcoma cells in Harvey virus-induced sarcomas. It was concluded that membrane staining may be diagnostic for neoplastic transformation (Ward et al. 1989). Since translocation of the *ras* protein to the membrane is associated with transformation it is proposed that AHF with membrane staining may have a greater propensity for developing into neoplastic lesions than foci with cytoplasmic staining.

The role of diffuse cytoplasmic staining in preneoplastic and neoplastic lesions is unknown. The gene may be expressed at higher levels in these lesions because of their enhanced growth rate compared to the surrounding quiescent liver tissue. Chandar et al. (1987) showed an increase in transcript levels of ras genes in tumors evaluated. It was indicated that the transcript elevations were a reflection of active cell proliferation. Increased expression of the c-myc gene is also observed in preneoplastic and neoplastic hepatocellular lesions (Nagy, 1988; Porsch-Hallstrom et al. 1989). Strom et al. (1990) proposed that while increased c-myc expression is a common and possibly obligatory event in hepatocarcinogenesis, c-myc activation may not be sufficient for inducing hepatocellular neoplasia. ras proto-oncogene expression may provide some additional selective or maintenance role during the development of carcinogenesis in the rat (Beer and Neveu, 1990).

Oval cells are postulated to be facultative stem or progenitor cells of hepatocytes (Evarts et al. 1989; Fausto, 1990). In our study, ras p21 protein was present in oval cells and hepatocytes in preneoplastic and neoplastic lesions indicating that the expression of this protein is not specific for one cell type. Yaswen et al. (1985) showed an increase in c-Ha-ras transcripts primarily in hepatocytes and abundant c-Ki-ras transcripts in oval cells throughout carcinogenesis in rats on a choline deficient diet. Makino et al. (1984) and Yaswen et al. (1985) suggested that increased expression of c-Ha-ras was related to the proliferation of hepatocytes. If the ras p21 protein is associated with proliferation of specific cell types, then oval cells and hepatocytes would be at a greater risk for developing into tumors. With increased cell proliferation, chances of additional mutations occurring is enhanced.

There was no difference in ras p21 expression between PB- and TCDD-induced lesions. In another study there was no difference in proto-oncogenes K-ras, fos and myc expression in rat liver cells treated in vitro with liver tumor promoters phenobarbital and biliverdin (Lafarge-Frayssinet and Frayssinet, 1989). The similarity in the expression of the ras p21 protein in preneoplastic and neoplastic lesions with different promoters suggests that this may be a common profile of gene expression associated with the clonal expansion of initiated cells. The exact mechanisms of ras induced proliferation is not known. It is proposed that ras proteins make contact with downstream targets which passes the signal information to other proteins, ultimately resulting in alterations of gene expression and an increase in cellular proliferation (Corton, 1990).

Several oncogenes such as src, ras, mos, neu and raf down regulate gap junctions (Trosko and Chang, 1988; Trosko et al. 1990c). There is also increasing evidence that a wide variety of tumor promoters inhibit gap junctional communication (Klaunig and Ruch, 1987; Sugie et al. 1987; Trosko et al.

1987; Boreiko et al. 1989). It is postulated that inhibition of gap junctional communication facilitates the expansion of initiated cells by allowing these cells to escape the suppressing effect of surrounding and communicating cells.

Trosko et al. (1990c) suggested that a combination of interactions of multiple oncogene products with a cell, or agents external to the cell (hormones, growth factors or tumor promoting chemicals) may cause cell proliferation associated with carcinogenesis. Whether or not the combined effects of the tumor promoting agents PB or TCDD, and the oncogene product ras p21 protein may have an additive effect on inhibiting gap junctional communication needs to be evaluated. Down regulation of gap junctions may be the unifying process linking diverse processes of carcinogenesis (Trosko et al. 1990c).

ras activation by mutational mechanisms is not consistently observed in rat liver tumors induced by several different carcinogens. Stowers et al. (1988) showed that DNA from only one of 28 DEN-induced rat liver tumors was able to transform NIH 3T3 cells. In reviewing a number of studies, Strom et al. (1990) demonstrated that only 14 of 374 (3.4%) chemically induced hepatocellular tumors in the rat contained a mutated ras gene. While mutations are rare in NDEA induced hepatic lesions, mutations are commonly found in hepatocellular carcinomas induced with aflatoxin B_1 (McMahon et al. 1986) and methyl(acetoxy-methyl)nitrosamine (Goyette et al. 1988). The type of carcinogen used for tumor induction strongly correlates with the mutated locus of the ras oncogenes.

Transcriptional, but probably not mutational activation of proto-oncogenes occur during hepatocarcinogenesis in the rat (Pitot, 1990). The presence of ras p21 protein in preneoplastic and neoplastic hepatic lesions suggest that the expression of this protein may be an early and stable event in chemically induced hepatocarcinogenesis. Further biochemical tests are needed to determine the mechanism(s) underlying increased expression of ras p21 protein in foci, hepatic nodules and hepatocellular carcinomas.

CONCLUSIONS AND FUTURE STUDIES

Conclusions

The results from the research presented in this dissertation indicate that:

1) TCDD fed to NDEA initiated rats at a low nontoxic dietary concentration of 150 ppt (0.007ug of TCDD/kg/day) had a promoting effect on the development of altered hepatocellular foci at days 170, 240 and 450. The increase in the number of AHF correlated with hepatic nodules and hepatocellular carcinomas at day 450.

2) The number of AHF/cm^3 of liver was not significantly different in rats given TCDD continuously from day 30-450, from day 170-450 or from day 240-450. However, the mean volume of AHF was significantly larger in animals exposed to TCDD from day 30-450. Thus, TCDD had a strong promoting effect even when administration was delayed as long as 240 days after initiation.

3) Phenobarbital at a dietary concentration of 500 ppm increased tumor incidence at 450 days however, a significant increase in the number of ATPase negative AHF/cm^3 of liver was not found in initiated rats. Because PB did not have a significant promoting effect on the development of ATPase

negative AHF in this model, whether or not TCDD inhibited the regression of PB-induced foci could not be determined.

4) Noninitiated rats given a basal diet, TCDD or phenobarbital developed a low number of AHF. Enhancement of AHF may result from promotion of spontaneously initiated cells or may reflect some initiating activity of the promoter.

5) Unique types of AHF in sections of liver stained with hematoxylin and eosin included eosinophilic foci with peliosis hepatis, eosinophilic foci with spongiosis hepatis and atypical and tigroid basophilic foci. Since such foci of cellular alteration were not in control rats, their occurrence may be taken as presumptive evidence that these lesions were chemically induced and associated with their tumor promoting and hepatocarcinogenic potential.

6) Differences in phenotypic characteristics based on treatment were also present. At day 170, initiated rats promoted with TCDD had a higher incidence of basophilic AHF compared to initiated rats promoted with PB. At day 240, initiated rats promoted with TCDD had eosinophilic foci with spongiosis hepatis and basophilic foci including atypical and tigroid types. On the other hand, initiated rats promoted with PB and subsequently with TCDD had a higher incidence of atypical and diffuse basophilic foci and eosinophilic foci with peliosis hepatis.

7) A progression of AHF from clear cell to basophilic was not clearly defined. Few clear cell foci were present at

day 240 of sacrifice. The majority of AHF present at all sacrifice times were eosinophilic. At day 240 of sacrifice a larger number of animals had mixed cell foci and various types of basophilic foci. These findings were suggestive of a trend in the progression of lesions from clear cell to basophilic as proposed by Bannasch et al. (1989).

8) ras p21 protein was present in altered hepatocellular foci, hepatic nodules and hepatocellular carcinomas. These results suggest that the expression of ras p21 protein may be an early and stable event in chemically induced hepatocarcinogenesis.

9) Two patterns of staining; membrane and cytoplasmic were observed in AHF with the ras p21 antibody. Since translocation of ras p21 protein to the membrane is associated with transformation it is proposed that AHF with membrane staining may have a greater propensity for developing into neoplastic lesions than foci with cytoplasmic staining.

10) ras p21 protein was present in oval cells and hepatocytes. This would indicate that the expression of this protein is not specific for one cell type. If the ras p21 protein is associated with proliferation of specific cell types, then oval cells and hepatocytes would be at a greater risk for developing into tumors. With increased cell proliferation, chances of additional mutations occurring is enhanced.

11) There was no difference in *ras* p21 expression between PB- and TCDD-induced lesions. The similarity in the expression of *ras* p21 protein in preneoplastic and neoplastic lesions suggest that this may be a common profile of gene expression associated with the clonal expansion of initiated cells.

Future studies

1) In addition to evaluating *ras* p21 protein expression in hepatocarcinogenesis, studies may be designed to determine whether or not the proto-oncogenes *fos*, *myc*, *raf* and the antioncogene p53 are present in preneoplastic and neoplastic lesions.

2) Since growth factors are important in carcinogenesis, evaluation of preneoplastic and neoplastic lesions may determine whether or not growth factors such as transforming growth factor alpha, TGF beta, EGF and insulin growth factors I and II are important in multistage hepatocarcinogenesis.

3) Immunohistochemical techniques may be used to evaluate gap junctions in paraffin sections containing AHF, hepatic nodules and hepatocellular carcinomas to determine whether or not these proteins are down-regulated during promotion. Double labelling techniques may be used to determine if there is a correlation between proto-oncogene expression and down-regulation of gap junctional

communication.

4) Immunohistochemical markers may be used to detect various proto-oncogenes and growth factors in H&E sections containing preneoplastic lesions to determine which altered hepatocellular foci are most important in hepatocarcinogenesis.

5) In terms of defining the mechanisms underlying TCDD tumor promotion, Ah receptor-responsive and Ah receptor non-responsive mice may be used to determine if the tumor promoting effect of TCDD and other PHAHs are associated with the Ah/TCDD receptor.

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VITA

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