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THE ROLE OF MAMMARY EPITHELIAL-STROMAL CELL INTERACTIONS IN RELATION TO GROWTH REGULATION BY ESTROGEN AND PROGESTIN

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THE ROLE OF MAMMARY EPITHELIAL-STROMAL CELL INTERACTIONS IN RELATION TO GROWTH REGULATION BY ESTROGEN AND PROGESTIN

By

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ABSTRACT

THE ROLE OF MAMMARY EPITHELIAL-STROMAL CELL INTERACTIONS IN RELATION TO GROWTH REGULATION BY ESTROGEN AND PROGESTIN

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Both in vivo and in vitro approaches have been used in this study to investigate how estrogen (E), progestin (P), epidermal growth factor (EGF) and insulin like growth factor-I (IGF-I) interact to promote mammary growth in relation to their interactions with epithelial and stromal components of the mammary gland.

The in vivo study focused on local vs. systemic effects of P and E on mammary growth and their relative effects on epithelial and stromal tissue. Different mechanisms of P action in the two types of mammary tissue have been revealed. The growth effects of P in epithelial cells seem to be locally mediated, whereas P stimulation of DNA synthesis in stromal cells appears to be systemically mediated. The growth effects of E on epithelial and stromal cells also appear to be systemically mediated. E+P synergistically stimulated epithelial cell proliferation via E inducible P receptor (PR), and this effect appears to be locally mediated.

An in vitro serum-free monolayer culture system was developed for culturing epithelial, fibroblast and mixed cultures, so that the effects of E or P can be examined in vitro, free of in vivo systemic influences. P stimulated proliferation of epithelial cells but had no effect on fibroblasts in cell cultures from nulliparous mice. E was able to induce PR in epithelial cells, however, E did not stimulate

proliferation of epithelial cells or fibroblasts. These results support the in vivo observations, and indicate that P has a direct effect on epithelial cell proliferation. The lack of stimulatory effect of E on epithelial cells and fibroblasts, or P on fibroblasts in serum-free media suggests that additional factors are required. EGF or IGF-I stimulated proliferation of epithelial cells but not fibroblasts, and no interaction with E, P or E+P was observed. From these in vitro studies, the influence of epithelial-stromal cell interactions on hormonal responsiveness has also been identified, such that E+P stimulated proliferation of fibroblasts in mixed cultures whereas no stimulatory effect was observed in fibroblast-enriched cultures. In contrast to cells from nulliparous mice, E or P or E+P did not stimulate proliferation of epithelial, fibroblast or mixed cultures from pregnant mice. This latter finding indicates that the developmental state of the gland can modulate hormonal responsiveness.

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List of Abbreviations

BM	basal medium
BSA	bovine serum albumin
E	estrogen
EGF	epidermal growth factor
ER	estrogen receptor
F	fetuin
HBSS	Hank's balanced salt solution
H	hydrocortisone
IGF-I	insulin-like growth factor I
I	insulin
LI	labeling index
MPA	medroxyprogesterone acetate
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P	progestin
Pg	progesterone
PR	progesterone receptor
Prl	prolactin
R	R5020
R5020	promogestone
RU486	11β–(4-dimethylamino-phenyl)1-17β-hydroxy-17α-(prop-lynyl)-estra-4,9-diene-3-one

T	transferrin

Chapter 1. Literature Review

A. Introduction

The mammary gland is a suitable system for studying the mechanisms of growth and differentiation, because of its cyclic pattern of morphological and functional development during the estrous cycle, pregnancy and lactation. Furthermore, the mammary gland is an organ where the potential for cancer is very high. In recent years, about 1 out of 9 North American women develop breast cancer (Ries et al., 1991). In this context, the understanding of mammary growth control is highly relevant to prevention and therapy in breast cancer.

The mammary gland is composed of epithelium, and adipose and fibrous connective tissue generated by fibroblasts. Adipocytes and fibroblasts are derived from embryonic mesenchyme and are referred to as stromal cells. Epithelial tissue forms ducts which lie in the adipose tissue. Fibroblasts invest the ducts and separate epithelial cells from adipocytes. The ovarian hormones estrogen (E) and progesterone (Pg) are required for normal growth and development. Both epithelial and stromal tissues contain estrogen (ER) and progesterone receptors (PR) and are responsive to E and Pg. During the menstrual cycle, pregnancy and lactation, the mammary gland undergoes cyclic changes of epithelial proliferation and degeneration, concomitant with the depletion and replenishment of stromal tissue. Morphological changes occurring during different physiological stages are the result of epithelial-stromal cell interactions and hormonal regulation (Sakakura et al., 1976; Daniel et al., 1984; Haslam, 1991). The abnormal cell proliferation which occurs in breast cancer is likely to involve changes of normal epithelial-stromal cell interactions and loss of normal hormonal control. The purpose of this dissertation research is to understand how mammary epithelial-stromal cell interaction influences E- and Pg- induced cell proliferation. Increasing our

knowledge of these mechanisms will help us to understand abnormal epithelial-stromal cell interactions and hormonal regulation in breast cancer. The mouse mammary gland is a model system which has been extensively used by researchers in studies of mammary gland biology and breast cancer. This model system will also be used in my dissertation research. Therefore, the main focus of this chapter is a review of studies on mouse mammary development and growth regulation. Growth control in breast cancer will also be discussed to elucidate our recent understanding of abnormal changes in breast cancer in relation to normal development and some critical factors involved in the process of mammary neoplasia.

B. Mammary gland development

Mammary gland development can be divided into the following stages: embryonic, prepubertal, sexually mature, pregnant, lactating and involution. In the embryonic stage, mammary epithelium derived from epidermis, forms an enlargement on both sides of the trunk. There is no apparent epithelial growth during the first 11 days of fetal life. A rapid mammary epithelial proliferation happens at late fetal life and the increased epithelial tissue penetrates into fat pad precursor stromal tissue. At birth, the mammary gland contains a few short branching ducts. From birth to puberty, development of the mammary gland occurs in two distinct phases. The first phase is isometric growth (the increase of mammary gland is in proportion to the rest of the body) in which connective tissue increases are accompanied by deposition of fat. At 3-4 weeks of age, the mammary gland starts allometric growth (the growth rate is higher than that of the body). At this stage, large club shaped end buds appear at the duct tips and these structures have been demonstrated to be the growing point of the ducts. The

growth is characterized by ductal elongation with the epithelium invading the stromal fat pad. At the time of sexual maturity, the mammary ducts extend throughout the fat pad and also give rise to side branches. The pattern of the mammary gland by now is a characteristic tree-shaped branching ductile system. Cyclic variation in mammary epithelial cell proliferation and regression occurs during the estrus cycle.

During pregnancy, the mammary gland undergoes extensive side branching and lobuloalveolar development. The interductal spaces are gradually occupied by growing epithelial tissue. As pregnancy approaches term, the epithelium differentiates towards a secretary epithelium. After parturition, active lactation begins. Proliferative activity in lactating mammary gland is very low. After lactation, the epithelium starts to degenerate, fat tissue reappears, and the mammary gland returns to the state of a branched ductal system similar to the mature nulliparous mouse. Thereafter the mammary gland reassumes the cyclic changes during the estrus cycle.

C. Hormonal regulation in mammary gland development

1. Hormonal regulation in vivo

The female pattern of fetal mammary development is a dominant phenotype and does not require hormonal stimulation. Postnatal mammary development depends on the ovarian hormones E and Pg. In female mice, ER can be detected at 3 days of age (Haslam, 1989). The mammary gland starts to grow at 3 weeks of age, when the ovaries begin to function and the mammary gland first becomes responsive to E (Haslam, 1989). The initial growth is end bud epithelial proliferation and ductal elongation. Ovariectomy causes regression of end buds and cessation of ductal growth within a few days, and this process can be reversed

by administration of E. Daniel et al. (1987) found that in ovariectomized animals, E stimulated DNA synthesis in the cap cells of the end buds. Using steroid autoradiography, they determined that the binding of E is not in cap cells but in the adjacent epithelial cells and in stromal cells. From these results, they proposed that E stimulated growth involves an indirect mechanism.

At 7 weeks of age the mammary gland acquires proliferative responsiveness to Pg (Haslam, 1988). Coincidentally, it is also at this age that E-inducible PR first appear in the epithelium. Under the influence of E and Pg, some side-branching and alveolar development occur. In the adult, the mammary gland undergoes cyclic changes during the estrus cycle as the result of cyclical secretion of E and Pg from the ovaries. Pg was found to stimulate ductal cells as well as end buds (Breciani, 1968). However the real physiological function of Pg is probably to regulate ductal cell proliferation. The effect of E on ductal branching is probably via an indirect effect of stimulation of PR in ductal epithelial tissue.

E and Pg are also major hormones involved in mammary gland development during pregnancy. E and Pg not only stimulate lobuloalveolar formation, but also are required for the maintenance of alveoli. Ovariectomy causes alveolar regression during pregnancy (Nandi 1958).

Other hormones such as thyroid hormone, prolactin, growth hormone and adrenal steroids are also involved in mammary gland development. Thyroid hormone and prolactin stimulate alveoli formation (Vonderhaar & Greco, 1979). Adrenal steroids are involved in lobule formation (Banerjee, 1976). In triply operated mice (ovariectomy, hypophysectomy and adrenalectomy), lobuloalveolar development can be induced by administration of E, Pg, deoxycorticosterone, prolactin and growth hormone (Nandi, 1958; Nandi & Bern, 1960).

There is also evidence to suggest that EGF is involved in mammary growth and E and P regulated events. Both endbuds and ductal cells are responsive to

EGF. Removal of the major source of EGF by sialodenectomy results in reduced mammary gland growth (Sheffield & Welsch, 1987). Sialadenectomy during pregnancy results in a smaller mammary gland and less milk production after parturition. Consequently, there is a higher number of pup deaths within the first week after birth. EGF replacement during pregnancy in sialadenectomized mice can restore mammary growth, milk production, and increase pup survival (Okamoto & Oka, 1984; Oka et al., 1988). During pregnancy, when there are sustained elevated levels of E and Pg, EGF concentration in the salivary glands and in the plasma is high, and EGF receptor concentration in the mammary gland increases (Ances, 1973; Kurach & Oka, 1985; Edery et al., 1985). These observations suggest a possible relationship among E, Pg and EGF. Direct evidence that E and Pg interact with EGF came from the finding that the treatment of ovariectomized mice with E and Pg increased EGF content in the salivary gland and EGF receptor concentration in the mammary gland (Vonderhaar, 1984). In addition to systemic growth factors which seems to be involved in E and Pg mediated effects in vivo, local mammary-derived growth factors have been demonstrated from E and Pg treated mammary glands (Enami et al., 1983; Vonderhaar, 1984). It is not clear, however, whether the production of the locally derived growth factors are the direct effect of E and Pg on the mammary gland or if they are also produced through systemic processes.

2. Hormonal regulation in vitro

The in vivo mammary gland developmental cycle can be mimicked in vitro by culturing whole mammary glands in serum-free hormone supplemented media. However, in vivo priming with E+Pg is required. Mammary glands from prepubertal mice pretreated in vivo with E+Pg for 9 days can exhibit full lobuloalveolar development when cultured in media supplemented with

hydrocortisone, insulin, aldosterone and prolactin for 6 days (Ichinose & Nandi 1964; 1966). Withdrawal of all the hormones from the culture media except insulin, results in regression of alveoli similar to mammary gland involution after lactation (Banerjee et al., 1976). A second round of lobuloalveolar development can be achieved by including EGF in the hormone cocktail capable of inducing the first round of growth (Tonelli & Sorof, 1980). It is possible that EGF carried over from in vivo participated in the first round lobuloalveolar formation in vitro since the in vivo E+Pg pretreatment can increase EGF binding in mammary gland and EGF concentration in submaxillary gland (Vonderhaar, 1984).

In cell culture studies, when mammary fibroblasts and epithelial cells have been co-cultured in serum-containing media, E has been shown to stimulate epithelial cell DNA synthesis (McGrath, 1983; Haslam, 1986). No effects of mammogenic hormones on growth have been demonstrated in epithelium-enriched cell cultures in serum supplemented media (Nandi et al., 1981). Serum contains unidentified growth stimulatory and inhibitory factors which possibly mask or compensate for hormonal effects. Using a serum-free culture media and growing pure epithelial cells within collagen gels, Nandi and colleagues (1985) found that Pg, prolactin and EGF stimulated proliferation of mammary epithelial cells. Pg plus prolactin had a synergistic effect. EGF at a low dose (1 ng/ml) exhibited an additive effect with Pg plus prolactin. IGF-I has also been found to synergize with EGF in the stimulation of epithelial cell proliferation. Interestingly, no direct growth stimulatory effect has been observed with E. However, E did increase PR concentration. The effects of E, Pg, EGF and IGF-I on mammary fibroblasts and co-cultured epithelial cells and fibroblasts have not been examined.

D. Epithelial-stromal cell interactions and mammary gland development

Epithelial-stromal cell interactions are important in embryonic organogenesis of the mammary gland and postnatal mammary gland development (Sakakura et al., 1976; Daniel et al., 1984). The embryonic organogenesis is hormone independent. The interactions of the epithelium and embryonic mesenchyme determine the morphological and functional organogenesis (Sakakura,1987). In postnatal mammary gland development, epithelial-stromal cell interactions influence the responses to hormones and growth factors (Haslam & Counterman, 1991). Two possible mechanisms have been proposed to describe the effects of stromal cells on epithelial cell hormone responsiveness and growth (Haslam, 1986). One is via substrate effects in which fibroblasts provide epithelium with appropriate substratum for its growth and function. Another is that fibroblasts produce soluble factors which affect epithelial cell growth. The interactions between epithelial cells and fibroblasts are believed to be reciprocal and epithelial cells can also influence stromal cells (Haslam, 1986).

1. In vivo studies

When fetal mammary epithelium is combined with fetal mammary mesenchyme or salivary mesenchyme, the subsequent pattern of morphogenesis is determined by the source of mesenchyme (Sakakura et al., 1976). This evidence indicates that embryonic mesenchymal-epithelial cell interactions are critical for normal mammary gland morphogenesis.

Mammary mesenchyme includes fibroblast and fat pad precursors. These two types of mesenchyme have been shown to have different influences on mammary epithelium. When transplanted into adult mammary gland, the fibroblast mesenchyme causes ductal hyperplasia, whereas the fat pad mesenchyme induces normal epithelial architecture (Sakakura et al., 1982). Imaguma et al. (1988)

investigated the mechanisms underlying these different effects. They found that fat pad mesenchyme produces laminin and heparin sulfate proteoglycan which are the components of epithelial basement membranes. They hypothesized that the normal basement membrane components may provide a suitable environment for normal epithelial morphogenesis. Fibroblast mesenchyme can produce tenascin. Tenascin is an extracellular matrix glycoprotein containing 13 EGF-like repeats and has been suggested to function as an autocrine or paracrine growth factor (Pearson et al., 1988). So tenascin might be a molecule responsible for the hyperplastic response induced by fibroblast mesenchyme.

Postnatal epithelial-stromal cell interactions are also important in mammary gland morphogenesis. During the initial postnatal growth, primary ducts elongate and penetrate the fat pad. The end bud is separated from adipose tissue only by the basement membrane, whereas the subtending duct is also invested by fibroblasts. When ducts reach the limit of the fat pad, elongation ceases, and the tip of the ducts are now surrounded by fibroblasts.

Transplantation experiments have demonstrated that adipose stroma is an absolute requirement for ductal growth. Interestingly, adipose stroma does not need to be mammary specific. Upon transplantation to other non mammary adipose tissue, mammary epithelial ducts can also develop. However, transplantation to any other types of stromal matrix failed to support mammary epithelial growth. Daniel et al. (1984) transplanted mammary epithelial tissue embedded in collagen gel to mammary adipose stroma. They found spike-shaped outgrowths of epithelial cells within the gel, however, normal end buds formed when the epithelial spike reached the adipose compartment. Thus the role of adipose stroma seems to be related to normal morphology of ductal elongation.

During pregnancy, the epithelial component of the gland increases concomitant with a depletion of the adipose tissue. After lactation, there is a loss of secretary

epithelium and lobuloalveolar degeneration. Adipose tissues replenish the resulting space. Thus the adipose tissue undergoes cyclic changes in accordance with epithelial function. In addition, for adequate nutritional supply in rapid growing mammary epithelium during pregnancy and for secretary activity during lactation, the glycogen synthesis and lipogenic rates are increased in adipocytes (Bartley et al., 1981). These effects can only happen in the presence of epithelium indicating interactive phenomena (Bartley et al., 1981).

Less is known about fibroblast stroma. It is a minor tissue component compared to adipose stroma. However, it is the tissue that is in direct contact with and invests the epithelial ducts. Its function seems related to epithelial ductal spacing and stabilization. Fibroblast-derived extracellular matrix components, particularly glycosaminoglycans, have been shown to play an important role in regulation of ductal branching (Bernfield et al., 1984). Thus fibroblast stroma may participate in the control of the branching ductal morphogenesis of the mammary gland.

Hormonal control of mammary growth is also influenced by epithelial-stromal cell interactions. ER are present equally in both the epithelium and stroma of nulliparous mice. ER are decreased in the epithelium during pregnancy, and in the stroma during lactation. Twenty percent of PR are located in stromal cells and are E-independent; eighty percent are in epithelial tissue and can be regulated by E. PR are also decreased in the epithelium during pregnancy. The significance of these changes in receptor levels in epithelium and stroma during different stages of mammary gland development is not known at present. However these differences in receptor distribution suggest different hormonal control programs in the two types of tissues. Recently, it has been demonstrated that mammary epithelium from 3 week old mice prematurely produce E-dependent PR when combined with mature 10 week old stroma (Haslam & Counterman, 1991). This observation

indicates that epithelial-stromal cell interactions play an important role in modulating hormonal responsiveness of mammary tissue.

EGF receptors have been detected in the outer most cell layer of end buds and in stromal cells adjacent to growing ducts (Coleman et al., 1988). EGF can stimulate end bud cell proliferation. A possible role of EGF on stromal cells may be to modify extracellular components which in turn may influence epithelial proliferation (Haslam, 1991). Recent studies have shown that EGF receptors on epithelial cells can be modulated by E and P (Haslam et al., 1992), and EGF together with E and P synergistically stimulated epithelial DNA synthesis (Haslam et al., 1993). Whether the stromal tissue contributed to these hormone- and growth factor- regulated effects needs further investigation.

2. In vitro studies

In vitro studies have revealed that fibroblasts have profound influences on epithelial responses to hormonal regulation. Using a co-culture system, McGrath has shown that epithelial cells stop proliferating when confronted with fibroblasts (McGrath, 1983). The proliferation can be reinitiated by supplying E to the culture media. Also in a co-culture system, the mechanisms of E-induction of PR and E stimulation of DNA synthesis have been examined (Haslam, 1986). E induction of PR can be achieved by culturing mammary epithelial cells with live or irradiated mammary fibroblasts or on collagen type I but not by culturing epithelial cells alone on plastic. These results indicate that E induction of PR in epithelial cells depends on an appropriate substratum provided by fibroblasts. In contrast, E stimulation of DNA synthesis in epithelial cells can only be achieved by culturing epithelial cells with metabolically active fibroblasts indicating that a paracrine mechanism might be operating in this hormone regulated pathway. In these studies epithelial cells also promoted E-induced fibroblast proliferation.

Adipose stroma has also been suggested to influence mammary epithelial proliferation and differentiation by both substrate (Sakakura, 1987) and soluble factors (Levine & Stockdale, 1984; 1985; Stockdale et al., 1986; Wiens et al., 1987). Co-culturing with 3T3-L1 cell line, which can exist as preadipocytes or fully differentiated adipocytes, resulted in maximal DNA synthesis in mammary epithelial cells from mid-pregnant mice. The stimulatory factor was present in both conditioned media and in substratum materials generated by adipocytes. Adipocyte metabolic products, such as unsaturated fatty acids, stimulated mammary epithelial cell proliferation in cell culture (Wicha et al., 1979; Bandyopadhyay et al., 1987). Mammary epithelium from non-pregnant, pregnant, lactating and involuting mice can all be induced to synthesize milk protein by mammogenic hormones when co-cultured with preadipocytes or adipocytes and soluble factors (Levine & Stockdale, 1984; 1985; Wiens et al., 1987). The effects of adipose stroma on hormonal regulation of mammary epithelial proliferation is not known.

E. hormonal regulation in breast cancer

Studies on hormonal regulation in breast cancer have been done mostly in in vitro using breast cancer cell lines or in vivo by injecting or transplanting human cells into nude mice.

While evidence supporting E as direct mitogen on normal mammary cells is lacking, several studies suggest direct proliferative responsiveness of breast cancer cells to E (Lippman et al., 1976; Chablos et al., 1982; Darbre et al., 1983; Berthois et al., 1986). E can regulate tumor growth by stimulating a large number of enzymes and proteases involved in DNA synthesis (Edwards et al., 1980; Aitken & Lippman, 1983; 1985; Dubik et al., 1987). E can also stimulate several proteins which mediate tumor cell movement across the basement membrane, release

growth factors and growth inhibitors from ECM components or from their carrier proteins (Kaufman et al., 1977; Huff & Lippman, 1984; Liotta et. al., 1986). Regulation of neoplastic growth by E is probably via modification of the normal regulatory pathway.

In MCF-7 cells, a human breast carcinoma cell line, E induces cell surface receptors for laminin, suggesting that E can mediate cell attachment to basement membranes and increase MCF-7 cell invasiveness (Thompson et al., 1988). Without E, MCF-7 cells cannot form tumors in athymic mice (Soule & McGrath, 1980). These results indicate that E is involved in both tumor cell proliferation and metastasis.

Similar to normal cells, the interactions of E with P and peptide growth factors have been identified in breast cancer cells. E regulates ER and PR expression (Saceda et al., 1988; Wei et al., 1988) and induces several growth factors (IGF-II, TGF-a, PDGF) (Dickson et al., 1986; Rozengurt et al., 1985; Yee et al., 1988; Bates et al., 1988) and growth factor receptors (EGF, IGF-I, IGF-II receptors) (Mukku & Stancel, 1985; Stewart et al., 1990; Mathieu et al., 1991). In E-free conditions, the anti-estrogen tamoxifen can totally block cell proliferation stimulated by IGF-I and EGF (Vignon et al., 1987) when ER is present. Clinically, tamoxifen has been used for breast cancer treatment.

P are growth inhibitory for human breast cancer cells (Chablos & Rochefort, 1984). P has been shown to modulate several growth factors and their receptors (Murphy et al., 1988; Murphy et al., 1988, Papa et al., 1991). Conversely, growth factors can also stimulate PR expression (Wei et al., 1988, Sumida & Pasqualini 1989).

F. Epithelial-stromal interactions in breast cancer

Stromal components of breast cancer are generally considered to be normal. However, stromal tissue can undergo morphological and chemical changes under the influence of neoplastic epithelium. In the desmoplastic reaction, a common host response to tumor invasion, fibroblasts undergo morphological changes to smooth muscle phenotype and express α-actin and extensive collagen (Sappino et al., 1988). In DMBA-induced mammary gland tumors, tumor cells are surrounded by collagen and an abnormally thickened basement membrane (Ormerod et al., 1985). It has been suggested that the abnormal changes of basement membrane and extracellular matrix may alter cell attachment characteristics increasing invasive growth (Ormerod et al., 1985).

Interestingly, the abnormal changes seem not to be restricted to breast fibroblasts. Skin fibroblasts from breast cancer patients have also been demonstrated to exhibit several abnormal behaviors: anchorage independence, overgrowth in culture and increased migratory activities (Durning et al., 1984). These abnormalities in non-malignant stromal tissue may be related to genetic predisposition. Skin fibroblasts from normal relatives of breast cancer patients also show abnormalities (Haggie et al., 1987). It is possible that an abnormal fibroblast environment offers a greater chance for mammary epithelial cells to develop malignancy. Identification of these characteristics has been shown to be helpful for the diagnosis of the disease (Schor et al., 1986).

Fibroblasts can also influence cancerous epithelial cells by substratum and soluble factors. Abnormal soluble factors secreted by tumor-derived fibroblasts have been detected in cell cultures (Adams et al., 1988). Conditioned media from fibroblasts of either benign or malignant tumors stimulated the growth of MCF-7 cells. By contrast, conditioned media from normal mammary fibroblasts inhibited MCF-7 cell growth (Adams et al., 1988). This observation suggests that a growth

inhibitory factor from normal fibroblasts is lost, or an abnormal growth factor is generated by fibroblasts from benign and malignant tumors.

Secreted stimulatory factors from breast cancer cells (malignant epithelial cells) have been identified in conditioned media from E treated cells (Butler WB, Kirkland et al., 1979; Dickson et al., 1986; Yee et al., 1988). Some of these factors might act as autocrine regulatory factors on epithelial cells (DeLarco & Todaro 1978; Osborne & Arteaga, 1990). Others might act on stromal cells and induce paracrine factors which affect epithelial cells (Lippman et al., 1986; Vignon et al., 1987). Thus identification of possible paracrine and autocrine factors produced by normal epithelial cells and fibroblasts and their effects could advance the understanding of the abnormal changes which happen in malignant cells.

G. The Objectives of Current Studies

The ovarian hormones E and Pg have been shown to be importantly involved in mammary gland development. The mammary ductal tree fails to develop when E and Pg are withdrawn by ovariectomy. However, these in vivo observations could be the net result of systemic activation of other growth factors and inhibitors by E and Pg. Alternatively, E and Pg may act on the mammary gland locally to mediate epithelial and stromal cell proliferation. In order to further understand the mechanisms of E and P action, the first specific aim of this dissertation is to determine if E and P act locally to promote mammary gland growth and to determine the relative effects on epithelial vs. stromal tissue in vivo.

As discussed above, tissues can interact to modify their hormonal responsiveness. An in vitro cell culture system is a possible approach to address the question about the direct effects of E , P and growth factors which might mediate the effects of E and P on their target tissues. So my second objective is to

establish serum-free media, monolayer culture conditions which would maintain primary cultures of mammary epithelial cells, fibroblasts or mixed cultures containing both epithelial cells and fibroblasts. Next, using the above cell culture conditions, the effects of E and P on different cell types will be studied and the interactions between epithelial cells and fibroblasts in relation to the growth regulation by E, P and growth factors will be investigated.

These studies will facilitate our understanding of the effects of E and P, and the role of epithelial-stromal interactions in normal mammary growth. Advancing our knowledge of these mechanisms will be helpful for the potential design of new therapeutic methods for breast cancer.

Chapter 2: The local action of E and P on mammary growth and the relative effects on epithelial vs. stromal tissue in vivo

Introduction

It has been shown that E and P play an important role in mammary growth and differentiation in rodents (Bresciani 1968; Edery et al., 1984) and in humans (Masters et al., 1977; Brown, 1981). E can induce PR in mammary epithelial tissue. In stromal tissue there are E-independent PR which are constitutively present. The contribution of the two types of P binding sites to mammary growth and development is not known. Even though both ER and PR are present in mammary epithelial and stromal cells, whether E and P mediated mammary growth is a local effect or mediated by systemic factors, and the relative effects of E and P on epithelial and stromal tissues are not clear. Furthermore it is quite likely that E and P synergize to promote mammary growth via E-regulated PR (Haslam & Shyamala, 1979; Haslam & Shyamala, 1980; Haslam,1986). The purpose of the present investigation was to elucidate further how P mediates mammary growth and interacts with the epithelial and stromal components of the gland as well as to assess the role of E in these interactions. Using Elvax 40P implants it is possible to confine bioactive molecules directly to the mammary gland (Silberstein & Daniel, 1982). Local vs. systemic effects of the bioactive compounds have been shown to be distinguished by using this methodology (Haslam, 1988). Therefore E and P were combined with Elvax 40P and implanted into the mammary gland. The effects of E and/or P on mammary gland morphology and DNA synthesis in both hormone implanted and control implanted mammary gland and in epithelial vs. stromal cells were investigated. PR concentration in epithelial cells is subject to E-regulation (Haslam & Shyamala, 1979), therefore E effects on PR were also assessed.

Materials and Methods

Reagents and chemicals:

[17β-methyl-^{3}H] promegestone (R5020; SA, 71 Ci/mmol) and radioinert R5020 were purchased from New England Nuclear Corp. (Boston, MA). 11β–(4-dimethylamino-phenyl)1-17β-hydroxy-17α-(prop-lynyl)-estra-4,9-diene-3-one (RU486) was a gift from Roussel Uclaf (Romainville, France). All other hormones were purchased from Sigma Chemical Co. (St. Lousis, MO). [Methyl-^{3}H]-thymidine (50 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). All other chemicals were reagent grade.

Hormone implantation:

Ten-week-old BALB/c mice were ovariectomized 1 week before Elvax implantation. Elvax pellets containing either Pg, R5020, medroxyprogesterone acetate (MPA), or the anti-progestin, RU486 (0.1 or 1 μg /pellet) alone or combined with varying amounts of 17β-estradiol (5, 50, or 500 ng/pellet) were prepared as previously described (Silberstein & Daniel, 1982; Haslam, 1988). Hormone-containing pellets were implanted into the right inguinal gland, and control pellets were implanted in the contralateral left inguinal gland.

DNA histoautoradiography:

Two or 4 days after implantation, mice received a single ip injection of ^{3}H-thymidine (2 μCi/g body wt.) 1 hour before death. The mammary glands were removed and processed for whole mount analysis as well as autoradiographic analysis as previously described (Banerjee et al., 1976; Haslam, 1988). Determination of mammary epithelial and stromal cell labeling indices (LI,

percent labeled nuclei) was accomplished with the use of a computer-interfaced morphometric digitizing system (Haslam, 1988).

Steroid hormone binding assay:

Hormone- or control-implanted mammary glands were removed, homogenized separately, and prepared as cytoplasmic extracts, as previously described (Haslam & Shyamala, 1979). Extracts were incubated with 1-20 nM [^{3}H]R5020 with or without a 100-fold excess of radioinert R5020 in the presence of 100-fold excess radioinert dexamethasone (to suppress P binding to glucocorticoid binding sites). Specific binding was determined using a dextran-coated charcoal assay procedure (Haslam & Shyamala, 1979), and binding data were analyzed according to Scatchard (Scatchard, 1947). Tissue DNA was quantitated as previously described (Ceriotti, 1952).

Statistics:

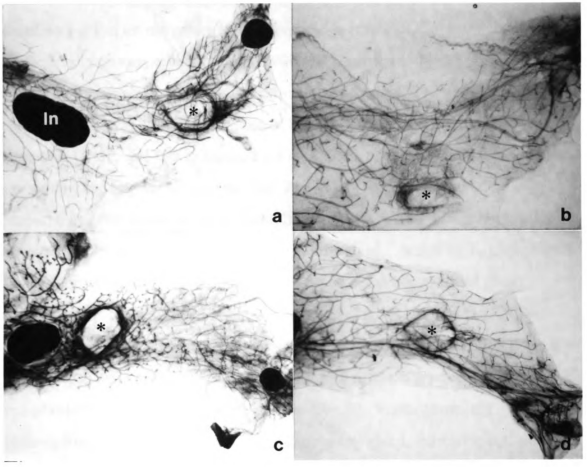
All results were analyzed by Analysis of Variance (ANOVA) and the Newman-Keuls Multiple Range Test.

Results

1. Mammary gland morphology

Fig. 1 shows that implantation of Elvax pellets containing either R5020, a synthetic progestin, or E alone had no effect on mammary gland morphology (Fig.1a, 1b). However, when R5020 and E were combined and implanted together, ductal sidebranching similar to that which occurs in early pregnancy was observed. The sidebranching occurred directly adjacent to the implant (Fig. 1c) and no sidebranching was observed in the

Figure 1. Effect of R5020 and E on mammary gland morphology. Mice were ovariectomized 1 week before implantation with Elvax pellets containing either 5ng E (a), 1μg R5020 (b), or 1μg R5020 + 5ng E (c) in the right inguinal gland and control pellets in the contralateral left inguinal gland (d). Mammary gland wholemounts were analyzed 4 days after implantation. ln = lymph node. * = pellet implant. Magnification, x 64.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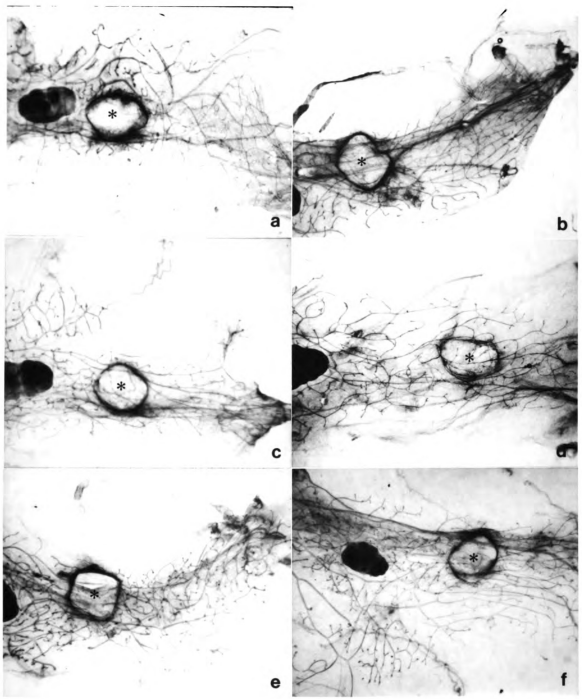
*
d

contralateral control gland (Fig. 1d), indicating the localized nature of the response. Mammary gland wholemounts were analyzed 2, 4, and 6 days after implantation with the maximal morphological response observed on day 4 (data not shown). Pg and medroxyprogesterone acetate (MPA, a synthetic P), when combined with E, were both able to produce morphological responses similar to R5020; the anti-progestin RU486 either alone or combined with E had no stimulatory effect on mammary gland morphology (data not shown).

Dose response experiments with R5020 and E revealed that the dose of E appears to be a limiting factor in determining the extent of mammary gland responsiveness to P. When a low dose of R5020 (0.1 μg) was used, increasing doses of E (5, 50, and 500 ng) caused the area and density of ductal sidebranching surrounding the implant to increase (Fig. 2). At 5 or 50 ng E no ductal sidebranching was observed in the contralateral control gland, indicating that the P response was localized to the hormone-implanted gland. At 500 ng E, plumping of the duct ends without sidebranching was observed in the contralateral gland, indicating that at this dose the effect was due to E (Fig 2), whereas the R5020 effect still was confined to the hormone-implanted gland. When a higher dose of R5020 (1 μg) was used, increasing doses of E caused much greater increases in the area of sidebranching in the implanted gland (Fig. 3). Furthermore, the sidebranching now also extended to the contralateral gland, indicating that the R5020 effect was no longer limited to the implanted gland. These results demonstrate that the extent of the effect of R5020 was only partially dose dependent and that the major factor determining the extent of the P response was the amount of E.

Figure 2. Effect of low R5020 dose plus E on mammary gland morphology. Mice were ovariectomized 1 week before implantation with Elvax pellets containing 0.1 μg R5020 + 5 ng E (a), 50 ng E (c), or 500 ng E (e) respectively in the right inguinal gland and control pellets in the contralateral left inguinal gland (b, d, and f). Mammary gland wholemounts were analyzed 4 days after implantation. * = pellet implant. Magnification, x 64.


a
b
c
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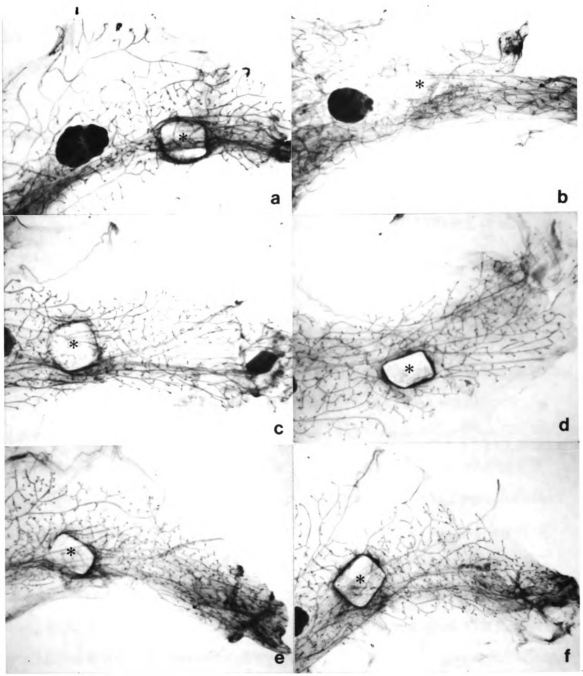
e
f

Figure 3. Effect of high R5020 dose plus E on mammary gland morphology. Mice were ovariectomized 1 week before implantation with Elvax pellets containing 1 μg R5020 + 5 ng E (a), 50 ng E (c), or 500 ng E (e) respectively in the right inguinal gland and control pellets in the contralateral left inguinal gland (b, d, and f). Mammary gland wholemounts were analyzed 4 days after implantation. * = pellet implant. Magnification, x 64.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2. Regulation of PR concentration

The effect of E on P-mediated effects as observed above could be associated with E's regulation of PR concentration. To determine if this was responsible for the limiting effect of E on P-mediated growth and morphology, the effect of varying doses of E on PR concentration in hormone-implanted and control contralateral glands was analyzed. The results shown in Table 1 demonstrate that at low dose of E (5 ng) only PR in the implanted gland is increased. However at the 50 and 500 ng doses of E, both contralateral control and E-implanted glands had elevated PR levels. These results demonstrate that the dose dependent effects of E on the degree and pattern of increased ductal sidebranching induced by R5020 + E at various doses could be determined by the amount of E-induced PR.

3. Mammary gland DNA synthesis

The effects of hormone implants on DNA synthesis of mammary epithelial cell are shown in Fig. 4. In initial experiments, DNA synthesis was analyzed at both 2 and 4 days after implantation. The greatest increase in LI was consistently observed at 2 days (data not shown), therefore all subsequent experiments were analyzed at day 2. In contrast to the effect on mammary gland morphology, P alone significantly increased DNA synthesis (Fig. 4). R5020 had a greater effect than MPA, and Pg had the least effect ($p < 0.05$). This order and magnitude of responses in the mammary gland is compatible with the P potency of these compounds and relative binding affinity to PR observed in other target organs (Feil et al., 1972, Philbert & Raynaud, 1974). When P were combined with E, greater increases in DNA synthesis were observed (Fig. 5). As shown above, R5020 and MPA had similar effects and greater effects than Pg ($p < 0.05$). The anti-progestin, RU486, alone or in combination with E, did not stimulate DNA

Table 1. Effect of varying dose estrogen implants on mammary PR concentration

E dose	Specific ^{3}H-R5020 binding (fmol/mg DNA)	
	Hormone implanted	Contralateral control
Control	400 ± 35[a]	408 ± 39
5 ng	600 ± 49*	425 ± 50
50 ng	786 ± 57	712 ± 39
500 ng	787 ± 155	735 ± 163

[a] Each value represents mean ± SEM from three experiments of sixteen animals for each treatment group.

* $p < 0.05$ that in the hormone-implanted gland, specific ^{3}H-R5020 binding is greater than that of contralateral control gland.

synthesis. However, the E + R5020-induced increase in DNA synthesis was blocked in the presence of RU486 (Fig. 6). The inhibitory effect of RU486 indicates that the R5020 effect on DNA synthesis was due to its P activity and was mediated via E-induced PR. In all cases the stimulation of epithelial cell DNA synthesis was much greater in hormone-implanted glands than in the contralateral control gland, implying a local mode of P action.

The results of P effects on stromal cell DNA synthesis are shown in Figs. 7 and 8. P alone increased stromal cell DNA synthesis, and the biological potency

Figure 4. Effect of P on mammary gland epithelial cell DNA synthesis. Mice were ovariectomized 1 week before implantation with Elvax pellets containing 1 μg R5020, MPA, Pg, or RU486, respectively in the right inguinal gland (▭) and control pellets in the contralateral left inguinal gland (▨). Two days after implantation, LI was determined in ductal epithelium by DNA histoautoradiography. Each value represents the mean ± SEM of two or three experiments; each experimental group contained three or four animals. * $p < 0.05$ that hormone treated groups are greater than controls; ** $p < 0.05$ that control-implanted gland are greater than control group but less than hormone-implanted glands.


Labeling Index
(% labeled Nuclei)
8
7
6
5
4
3
2
1
0
*
**
*
**
*
control
R5020
MPA
Pg
RU486
Hormone Treatment

Figure 5. Effect of P plus E on epithelial cell DNA synthesis. Mice were ovariectomized 1 week before implantation with Elvax pellets containing either 5 ng E alone or in combination with 1μg R5020, 1 μg MPA, 1μg Pg or 1μg RU486, respectively in the right inguinal gland ( ) and control pellets in the contralateral left inguinal gland ( ). LI was determined in ductal epithelium by DNA histoautoradiography. Each value represents the mean ± SEM of two or three experiments; each experimental group contained three or four animals. * $p < 0.05$ that P plus E-implanted glands are greater than E alone or contralateral control-implanted glands.

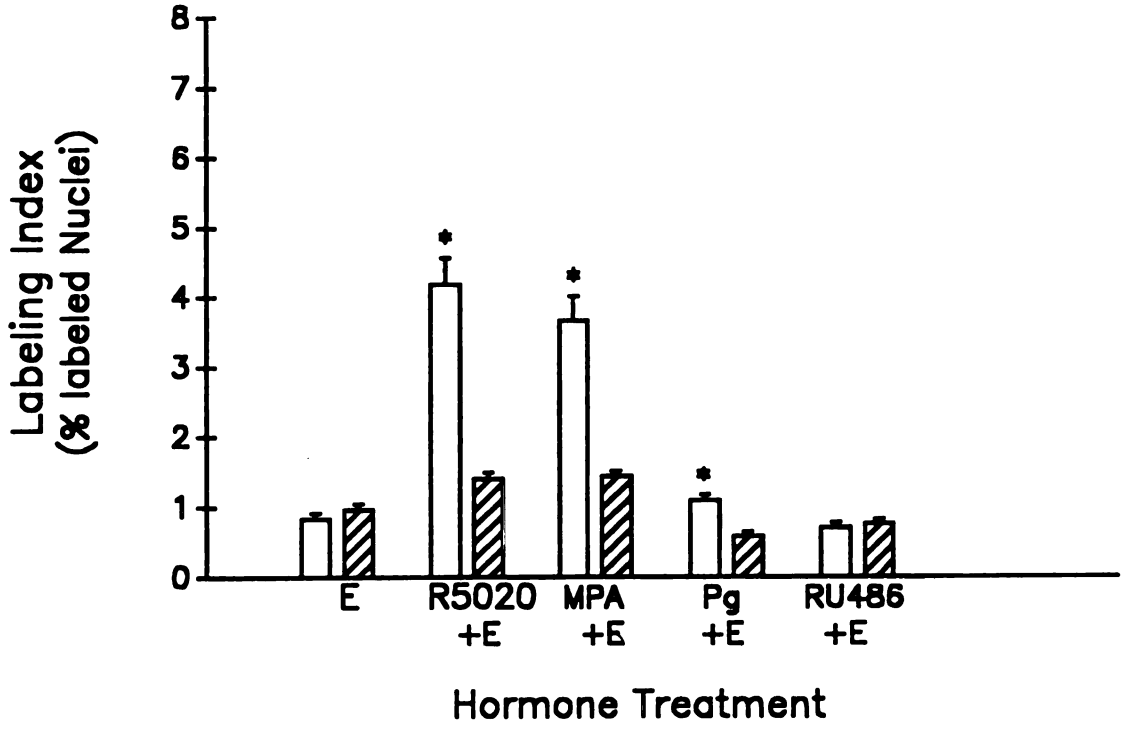
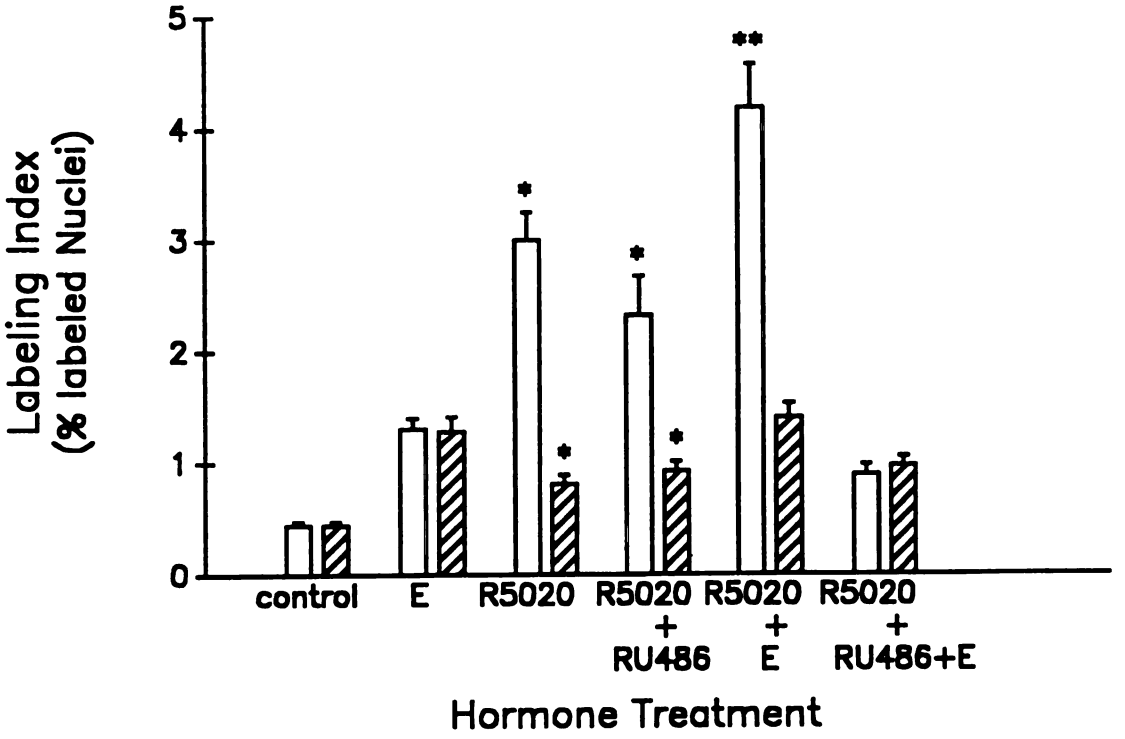

Labeling Index
(% labeled Nuclei)
8
7
6
5
4
3
2
1
0
*
*
*
E
R5020
+E
MPA
+E
Pg
+E
RU486
+E
Hormone Treatment

Figure 6. Effect of RU486 on epithelial cell DNA synthesis. Mice were ovariectomized 1 week before implantation with Elvax pellets containing 5 ng E, 0.1 μg R5020, or 1 μg RU486, in indicated combinations in the right inguinal gland (▭) and control pellets in the contralateral left inguinal gland (▨). LI was determined in ductal epithelium by DNA histoautoradiography. Each value represents the mean ± SEM of two or three experiments; each experimental group contained three or four animals. * $p < 0.05$ that R5020-implanted glands are greater than controls; ** $p < 0.05$ that R5020 + E-implanted glands are greater than E-implanted glands.


Labeling Index
(% labeled Nuclei)
5
4
3
2
1
0
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*
*
*
**
control
E
R5020
R5020 + RU486
R5020 + E
R5020 + RU486+E
Hormone Treatment

Figure 7. Effect of RU486 on stromal cell DNA synthesis. Mice were ovariectomized 1 week before implantation with Elvax pellets containing either 1μg R5020, 1 μg MPA, 1μg Pg or 1μg RU486, respectively in the right inguinal gland (▭)and control pellets in the contralateral left inguinal gland (▨). Two days after implantation, LI was determined in stromal cells by DNA histoautoradiography. Each value represents the mean ± SEM of two or three experiments; each experimental group contained three or four animals. * $p < 0.05$ that hormone and control-implanted glands are greater than control group.

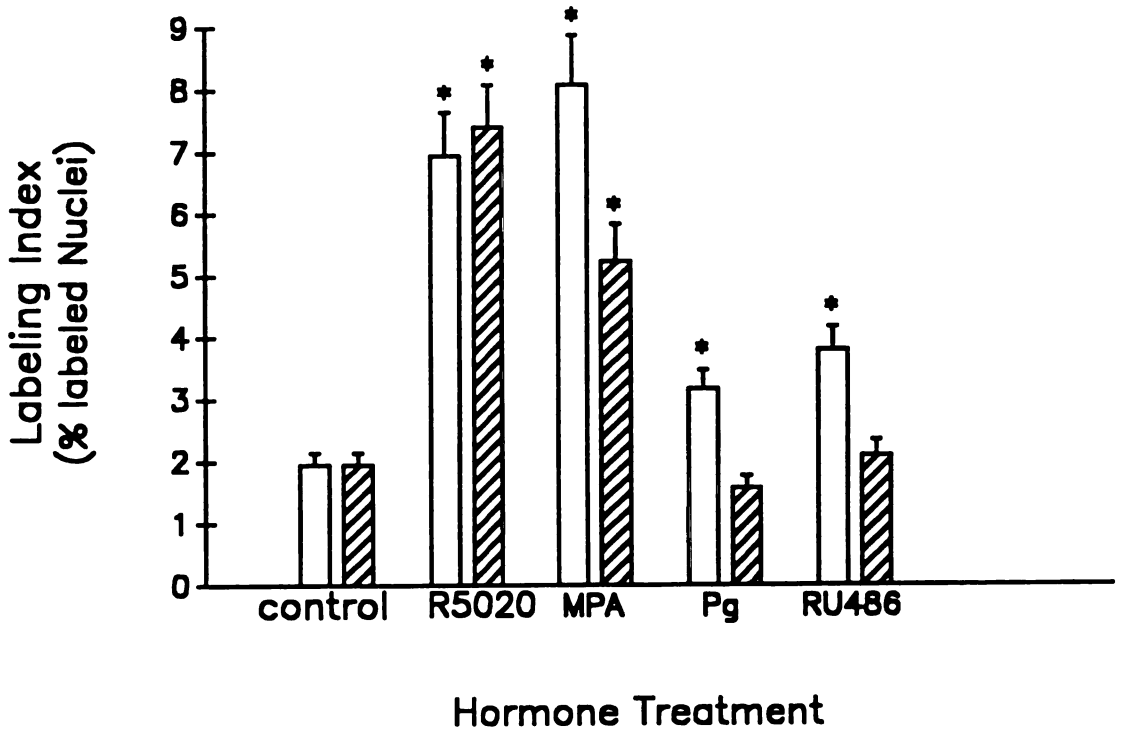
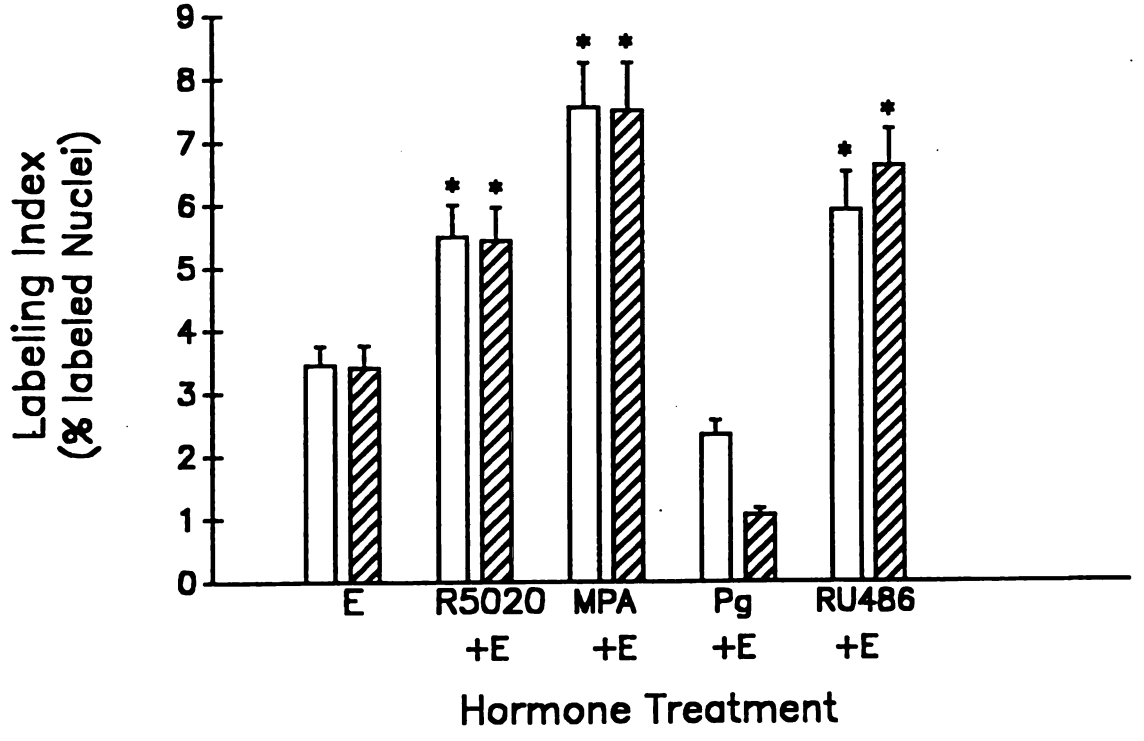

Labeling Index
(% labeled Nuclei)
9
8
7
6
5
4
3
2
1
0
control
R5020
MPA
Pg
RU486
Hormone Treatment

Figure 8. Effect of P plus E on stromal cell DNA synthesis. Mice were ovariectomized 1 week before implantation with Elvax pellets containing either 5 ng E alone or in combination with 1μg R5020, 1 μg MPA, 1μg Pg or1μg RU486, respectively in the right inguinal gland (▭) and control pellets in the controlateral left inguinal gland (▨). LI was determined in stromal cells by DNA histoautoradiography. Each value represents the mean ± SEM of two or three experiments; each experimental group contained three or four animals. * $p < 0.05$ that hormone- and control-implanted glands in P plus E-treated groups are greater than E alone group.


Labeling Index
(% labeled Nuclei)
9
8
7
6
5
4
3
2
1
0
E
R5020
+E
MPA
+E
Pg
+E
RU486
+E
Hormone Treatment

of the P followed a similar pattern as that observed for epithelial cells except that MPA and R5020 were equally potent (Fig. 7). In the case of MPA or Pg, DNA synthesis in hormone-implanted mammary glands was higher than that of the contralateral control mammary glands. However, R5020, the most stable compound, caused similar increase in DNA synthesis in both sides of the mammary glands (Fig. 7). One interpretation of the lower DNA synthesis in MPA or Pg treated groups in their respective contralateral control mammary glands may be due to the metabolic inactivation of MPA or Pg. Thus the local mode of P effect in stromal tissue was not indicated. RU486 by itself increased stromal cell DNA synthesis. In contrast to its effect in epithelial cells, E did not increase the effects of R5020, MPA, or Pg in stromal cells (Fig. 8). However, the effect of RU486 was increased by estrogen. These results further demonstrate that the regulatory effect of E on P effects observed in mammary epithelium is not operative in stromal cells.

Discussion

The ability of P to promote mammary gland growth and differentiation in vivo was assessed in two ways by their ability to: 1) stimulate DNA synthesis in epithelial and stromal cells; and 2) produce ductal sidebranching that gives rise to lobuloalveolar morphogenesis that occurs during pregnancy. Furthermore, the role of E in these events was also assessed. The results demonstrate that P tested herein produce their stimulatory effects on epithelial DNA syntheses and ductal sidebranching by virtue of intrinsic P activity. The high activity of R5020 and MPA, which have reduced sensitivity to metabolic inactivation, and the lesser activity of Pg, which is rapidly metabolized, indicates that most likely the native compounds rather than P metabolites are responsible for the activity.

It is of interest to note that whereas RU486 inhibited the increase in epithelial cell DNA synthesis resulting from E + R5020 treatment, it did not reduce the DNA synthesis increase observed with R5020 alone. One possible explanation for this observation is that RU486 interacts preferentially with newly synthesized E-induced PR. It has been previously

reported that PR, newly synthesized in response to E appears to be different from preexisting old PR with regard to molecular size and/or form (Haslam 1987). Thus, these differences may be the basis for the observed differential effect of RU486.

In combination with E, P can produce a localized effect on mammary gland morphology and epithelial DNA synthesis, implying direct action of P in the mammary gland. E enhances the stimulatory effect of P on epithelial cell DNA synthesis. Analysis of the effect of E dose on PR concentration in conjunction with the extent and patterns of ductal sidebranching indicate that the limiting factor for P-mediated effects in epithelial cells is E-induced PR concentration. Although P alone increased DNA synthesis, this does not appear to be sufficient to cause sidebranching. It has been also observed that there is significant heterogeneity in the distribution of epithelial ER (Haslam & Nummy, 1992). This suggests that topographic differences also may exist with regard to localization of DNA synthesis, and this also may be a determining factor for the occurrence of sidebranching. Thus, these results extend the previous observations and further support the concept that in normal mammary epithelial cells P has major mitogenic activity. Furthermore, the P-induced response is the result of direct/local action in the mammary gland and that it is mediated via E-induced PR. P also stimulates mammary stromal cell DNA synthesis. However, the mechanisms operative in stromal cells appear to be different from those occurring

in epithelial cells. Firstly, even at low R5020 doses the hormone effects are not confined to the implanted gland but are expressed equally in the control contralateral gland. Secondly, E does not synergize to enhance the proliferative effect of P. Thus it can be concluded that the effects of P on stromal cells are not mediated via E-induced PR. This finding is compatible with the previous observations that stromal cell PR are E-independent (Haslam & Shyamala 1981). Unexpectedly, DNA synthesis was also stimulated by the anti-progestin, RU486. Whereas E did not synergize with P, it did increase the effect of RU486. Others recently have shown that RU486 also can stimulate proliferation of certain breast cancer cell lines in vitro (Bowden et al., 1989; Hissom et al., 1989); however, the basis for this response has not been determined. RU486 also has potent anti-glucocorticoid activity (Moguilewsky & Philibert 1984; Philibert 1984), and it remains to be established whether the effect of this compound in stromal cells is due to its anti-glucocorticoid effects.

Chapter 3: Serum-free primary culture of normal mouse mammary epithelial and stromal cells

Introduction

Mammary gland growth and development are known to be dependent upon systemic hormones (Nandi, 1958). However, increasing evidence indicates that local epithelial-stromal cell interactions also play an important role in hormonal control of the mammary gland (Haslam, 1991; Haslam & Counterman, 1991). Possible mechanisms include paracrine effects caused by soluble factors produced locally by various cell types in the gland (Haslam, 1986). Identification of the specific mechanisms underlying epithelial-stromal cell interactions are difficult to approach in vivo in the whole animal and in vitro cell culture systems have provided important insights (McGrath 1983; Haslam & Levely, 1985; Haslam, 1986). However, most previous in vitro studies of mammary epithelial-stromal cell interactions have been carried out in serum containing medium (McGrath, 1983; Haslam & Levely, 1985; Haslam, 1986). Because of the presence of numerous growth factors and other undefined potential growth promoting components in serum our ability to define specific mechanisms is limited. In one study using serum-free media (Beck & Hosick, 1988), adipocyte effects on collagen-embedded epithelial cell cultures were studied; it was found that adipocyte conditioned medium promoted epithelial cell proliferation. However, the mechanisms underlying epithelial and stromal interactions remain to be identified. In the majority of studies where serum-free media have been used to study mammary cells, only the behavior of mammary epithelial cells has been investigated (Imagawa et al., 1982; 1985). Furthermore, the composition of the media was designed to support maximal epithelial growth. However, when using maximal growth conditions it has been difficult to demonstrate in vitro growth

promoting effects of mammogenic hormones comparable to their growth promoting effects in vivo (Imagawa et al., 1982; 1985). In vivo studies to investigate the mitogenic effects of hormones and growth factors have always been carried out in ovariectomized mice to reduce cell proliferation to basal levels and to remove endogenous hormones (Nandi, 1958; Haslam, 1988; 1988; Wang et al., 1990). Thus the creation of serum-free cell culture conditions which support cell maintenance rather than maximal proliferation resemble more closely the in vivo environment for the study of hormonal control of cell proliferation.

The purpose of this study was to define serum-free media and monolayer culture conditions that would support the maintenance (rather than extensive growth) of primary cultures containing only mammary epithelial cells, only mammary fibroblasts or mixed cultures containing both epithelial cells and fibroblasts. Monolayer conditions were chosen to facilitate future experimental manipulations of interactions between epithelial and stromal cells in co-culture conditions. Comparative studies were carried out with mammary cells from nulliparous and mid-pregnant mice to determine the influence of the state of mammary gland differentiation on growth in serum-free medium. The identification of suitable culture conditions should provide an approach to studies of growth factor interactions with mammogenic hormones and the investigation of potential paracrine interactions between mammary epithelial cells and fibroblasts.

Materials and Methods

Reagents and Chemicals:

Collagenase III and pronase were purchased from Cooper Biomedical (Freehold, NJ) and Calbiochem (La Jolla, CA) respectively. Culture media phenol red-free Dulbecco's modified Eagle's/ Ham's nutrient mixture F12 (DME/F12) (1:1), Hank's balanced salt solution (HBSS), bovine serum albumin (BSA)

(fraction V), penicillin and streptomycin were purchased from GIBCO (Grand Island, NY). All other culture ingredients and hormones were obtained from Sigma Chem. Co. (St. Louis, MO). All other chemicals were reagent grade.

Cell Culture:

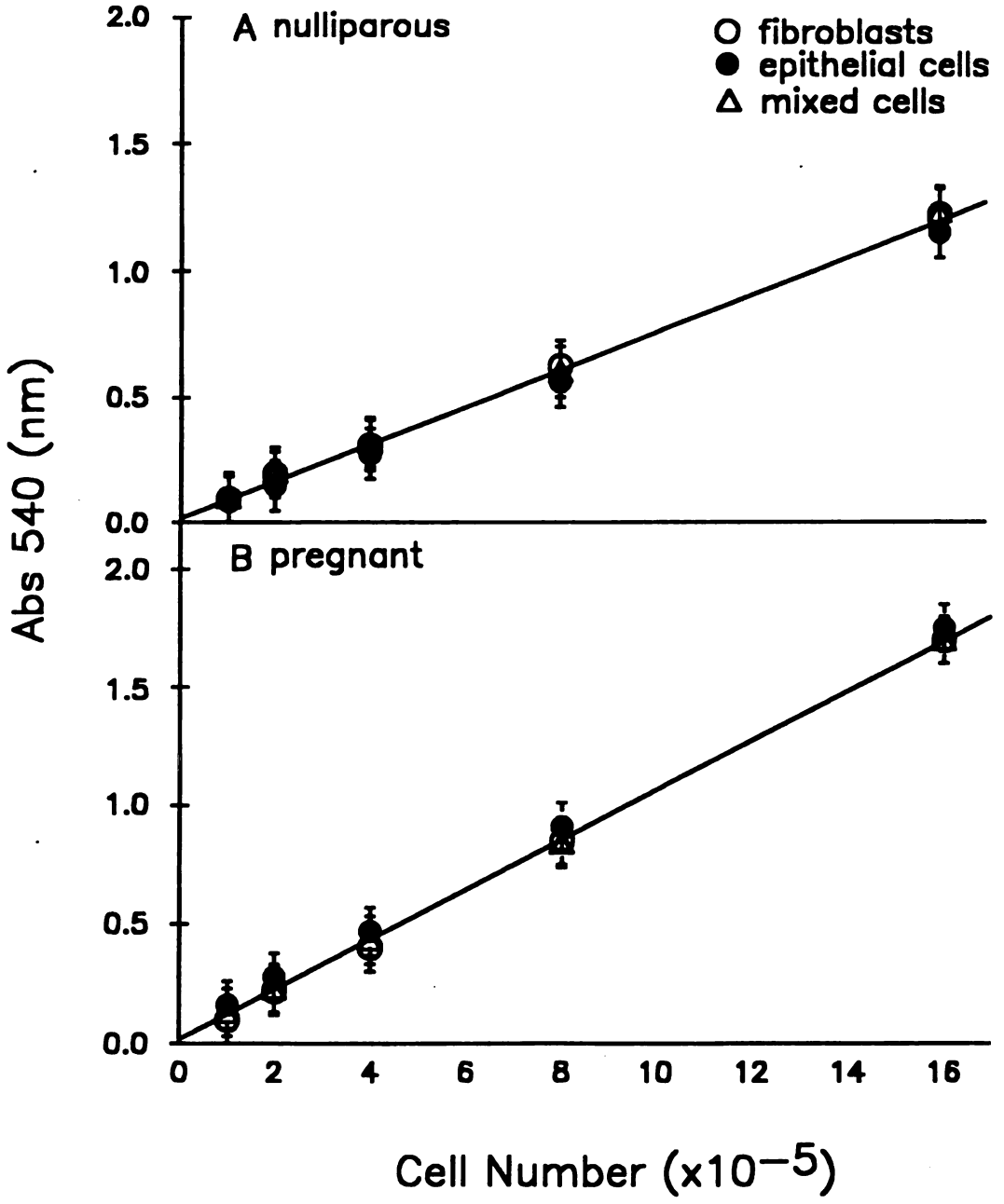
Balb/c 12-15 week-old, nulliparous or mid-pregnant (12-14 days) mice from our own colony were the source of mammary tissues. Animals were fed mouse chow (Teklad, Madison, WI) ad libitum and had free access to water. Animal facilities were temperature and humidity controlled with a light cycle of 12h on 12h off. Cell cultures containing both mammary epithelial cells and fibroblasts (mixed cultures), only epithelial cells, or only fibroblasts were obtained as previously described (Haslam & Levely, 1985). Briefly, mammary cell suspensions were obtained by enzymatic dissociation of minced mammary tissue in 0.1% collagenase. To obtain cultures enriched for mammary fibroblasts, the cell suspension was differentially centrifuged twice at 80 x g for 30 sec and the resulting pooled supernatants were the source of the fibroblasts. To obtain cultures enriched for mammary epithelial cells, the pellet remaining after the removal of fibroblasts was further purified by percoll density gradient separation. Cell numbers were determined by counting nuclei using a hemocytometer as previously described (Haslam & Levely, 1985) and were plated at a density of 4.2 x 10^5 viable cells/cm^2, or 1.4 x 10^5 cells per well of a 96-well culture dish for mixed and epithelial cultures or 9.6 x 10^5 cells per well of a 4-well culture dish for fibroblast cultures. Cell viability averaged 90% as determined by trypan blue exclusion. Both epithelial cells and mixed cells were plated on rat-tail collagen-coated culture plastic dishes to enhance cell attachment. Undiluted rat-tail collagen prepared as previously described (Emerman et al., 1977) was aliquoted (0.15 ml/cm^2) into each culture dish, air dried and washed once with HBSS prior

to plating cells. Pure fibroblast cultures were plated on tissue culture plastic since poor cell attachment was obtained on collagen-coated dishes. The basal culture medium for all cell types contained phenol red-free DME/F12 (1:1), Hepes (15mM), penicillin (0.1 mg/ml), and streptomycin (0.05 mg/ml), hydrocortisone (H, 0.2μM), and insulin (I, 0.1 μg/ml). To this medium were added indicated combinations of fetuin (F, 1, 0.5, 0.1 mg/ml), BSA (5mg/ml) and transferrin (T, 5 μg/ml). Media was changed every day.

Quantitation of Cell Growth:

Cell number was quantified using a modified 3-(4,5- dimethylthiazol-2-yl)-2,5-diphyltetrazolium bromide (MTT) assay (Carmichael et al., 1987; Dennizo & Lang, 1986; Hahm & IP, 1990). The assay is based upon the conversion of tetrazolium into blue formazan by the mitochondrial enzyme succinate-dehydrogenase. The color production is directly related to viable cell number (Dennizo & Lang, 1986). Briefly, phenol red-free DME/F12 media containing 1 mg/ml MTT was added to the cultures for 4 h at 37 ° C. Cells were then fixed with formol-saline (10% formaldehyde in 0.85% NaCl) for 30 min to prevent cell detachment and loss and then the MTT-formol-saline supernatant was removed. Isopropanol (100%) was then added to each well to dissolve the formazan crystals and absorbance at 540 nm was measured using a multiwell spectrophotometer Bio-tek EL 310 reader (Biotek Instruments, Inc., Burlington, VT). A standard curve relating cell number to absorbance for mixed cells, epithelial cells and fibroblasts from nulliparous and mid-pregnant mice (Fig. 9) was generated by counting nuclei of viable, freshly dissociated cells with the aid of a hemocytometer as previously described (Hahm & IP 1990). No significant difference in absorbance between epithelial cells and fibroblasts was observed. In all experiments, absorbance has been converted to cell number based upon the standard curves.

Figure 9. MTT assay standard curves showing the linear relation between cell number and absorbance. Dissociated mammary cells from nulliparous or mid-pregnant mice were quantified for cell number by the MTT assay and by cell counting as described in the Method section. Each point represents the mean $\pm$ SEM of triplicate determinations. Three representative experiments are plotted for each cell type.


A nulliparous
B pregnant
O fibroblasts
● epithelial cells
Δ mixed cells
Abs 540 (nm)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
Cell Number (x10⁻⁵)

Immunocytochemistry:

To establish the purity of epithelial cell and fibroblast cultures and the ratio of epithelial cells vs. fibroblasts in mixed cultures, fibroblasts were identified immunocytochemically using goat antivimentin antibody (a gift of Dr. B. Asch, Roswell Park Cancer Institute, Buffalo, NY). Fibroblasts were cultured on glass coverslips; epithelial cells and mixed cells were cultured on collagen-coated coverslips. Cells were fixed at -20°C in absolute methanol for 5 min and then in acetone for 5 min. Endogenous peroxidase activity was blocked with 0.1% hydrogen peroxide in methanol. Nonspecific antibody binding was blocked with normal rabbit serum. Antivimentin antibody binding was detected with a peroxidase labeled rabbit antigoat IgG. Peroxidase activity was detected by incubating with 3,3'-diaminobenzidine and hydrogen peroxide.

Statististics:

All results were analyzed by Analysis of Variance (ANOVA) and the Newman-Keuls Multiple Range Test.

Results

1. Optimal serum-free culture conditions for the maintenance of mixed cell cultures

Enzymatic dissociation of the mammary gland resulted in a suspension of cells comprised of multicellular epithelial organoids, single epithelial cells and fibroblasts (Haslam & Levely, 1985). The mixed cell suspensions containing both epithelial cells and fibroblasts from either nulliparous or mid-pregnant mice were plated on collagen-coated plates to facilitate attachment in the absence of serum. Serum-free medium containing hydrocortisone, transferrin, BSA and fetuin has previously been used for the successful culture of mammary epithelial cells

(Imagawa et al., 1982) and was used as a starting point for the present studies. Fetuin, a known attachment factor, is required for cell attachment in serum-free medium (Fisher et al., 1958; Hayman et al., 1982). However, fetuin along with BSA and transferrin can also contribute to epithelial cell growth in serum-free medium (Imagawa et al., 1982). The relative contributions of these components to growth were examined for cells plated on collagen. As can be seen in Fig. 10, fetuin, transferrin or BSA each contributed significantly to mixed culture growth. Thus tranferrin and BSA were deleted from future media. A fetuin dose response study carried out in medium without transferrin and BSA (Fig. 11) showed that reducing fetuin concentration to 0.1 mg/ml resulted in reduced growth stimulation while sustaining good maintenance of mixed cultures. This was true for mixed cultures derived from both nulliparous and mid-pregnant mice. Comparing the effect of fetuin on collagen vs. plastic (Fig.12) showed that optimal maintenance was obtained on the collagen-coated substratum. Hydrocortisone did not appear to be required for mixed culture maintenance since its removal from the medium did not have a negative effect on cell maintenance (data not shown).

From these experiments, the optimal conditions and minimal medium requirements for maintaining mixed cultures were determined to be basal medium containing fetuin (0.1 mg/ml), and a substratum coated with collagen I. Under these conditions good cell attachment and maintenance were obtained without high levels of proliferation.

The proportion of fibroblasts in mixed cultures was determined based upon a quantitative morphometric analysis as previously described (Haslam, 1988). Fibroblasts were identified by staining with antivimentin antibody. The cultures obtained from nulliparous mice were composed of a ratio of epithelial cells to fibroblasts of 60:40 on days 1 and 70:30 by day 7 of culture (Figs. 13, 14A). The

Figure 10. Effects of BSA, transferrin or fetuin on growth of mixed cultures from nulliparous mice and mid-pregnant mice. Dissociated mixed cells from nulliparous (A) or pregnant (B) mice were plated (4.2×10^5 cells/cm^2) on collagen in basal medium (BM) (○), BM + T (●), BM + BSA (△), BM + F (1 mg/ml) (▲), or BM + BSA, T, F (◘). Each value represents the mean ± SEM of triplicate determinations from a representative experiment. * $p = 0.05$ that all treatment groups had higher total cell numbers than the BM group.

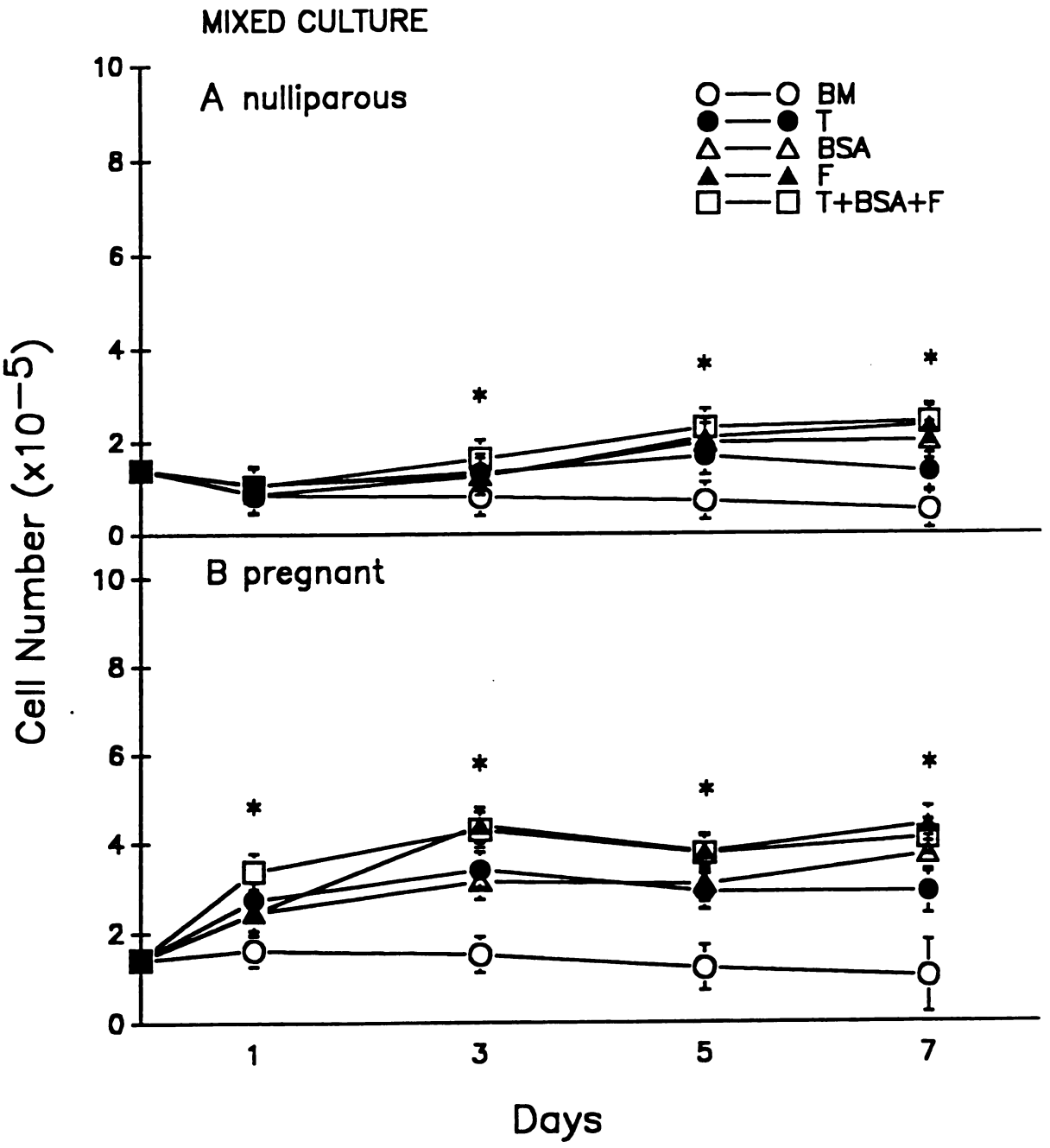

MIXED CULTURE
A nulliparous
B pregnant
BM
T
BSA
F
T+BSA+F
Cell Number (x10−5)
Days
0
2
4
6
8
10
1
3
5
7
*

Figure 11. Effects of fetuin dose on mixed culture growth. Dissociated mixed cells from nulliparous (A) and mid-pregnant (B) mice were plated (4.2 x 10^5 cells/cm^2) on collagen in BM containing 1(▲), 0.5 (△), 0.1 (●), or 0 (○) mg/ml F. Each point represents the mean ± SEM of triplicate determinations from representative experiments. * p = 0.01 that higher total cell numbers were obtained at all dosages of F than in BM without F.

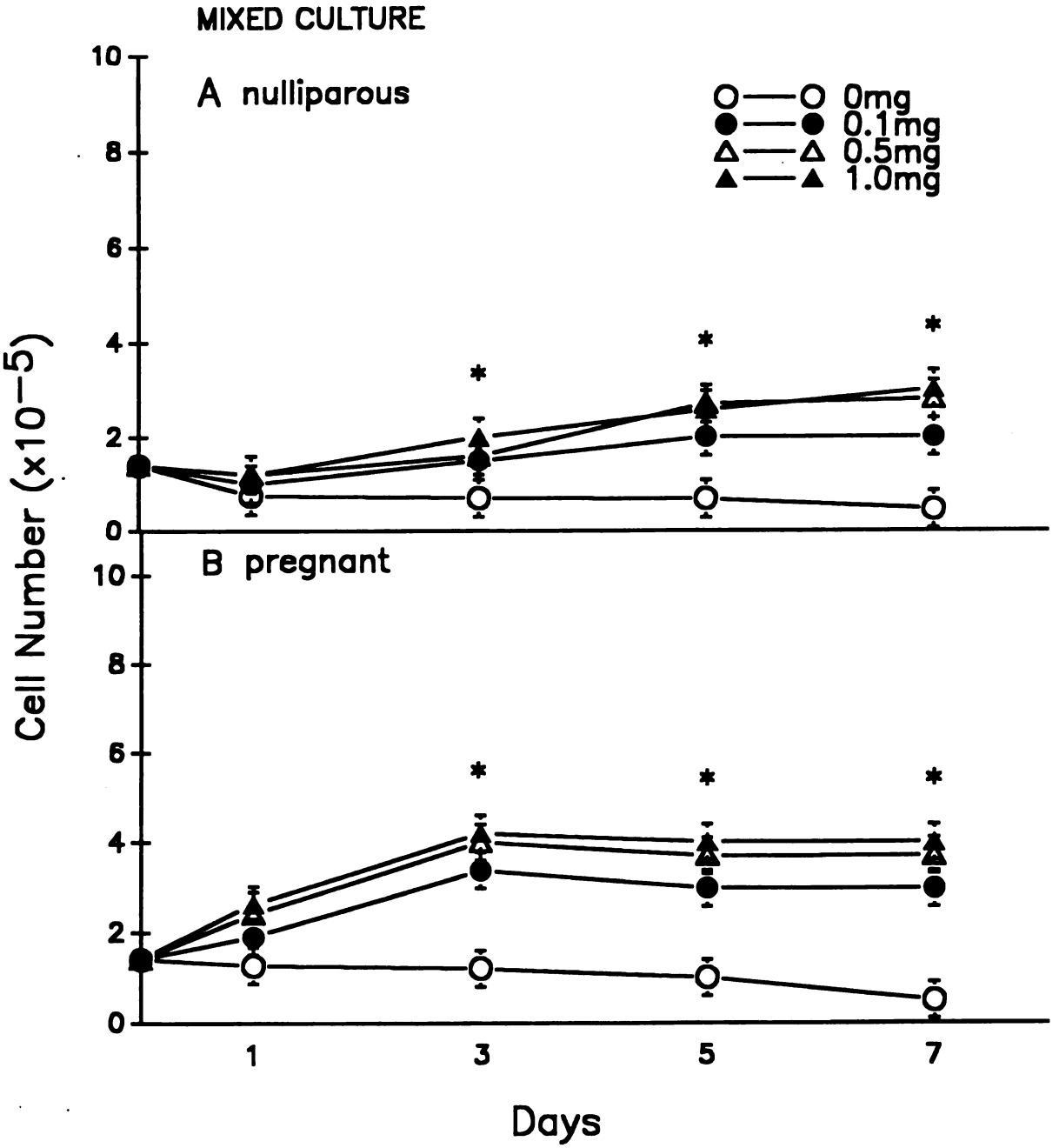

MIXED CULTURE
A nulliparous
B pregnant
0mg
0.1mg
0.5mg
1.0mg
Cell Number (x10−5)
Days
0
2
4
6
8
10
1
3
5
7
*

Figure 12. The effect of fetuin on the growth of mixed cultures on collagen or plastic. Dissociated mixed cells from nulliparous (A) or mid-pregnant (B) mice were plated (4.2×10^5 cells/cm^2) on collagen-coated (○) or plastic (●) substratum in BM + F (0.1 mg/ml). Each point represents the mean $\pm$ SEM of triplicate determinations from 2 experiments. * $p = 0.01$ that higher cell numbers were obtained on collagen.

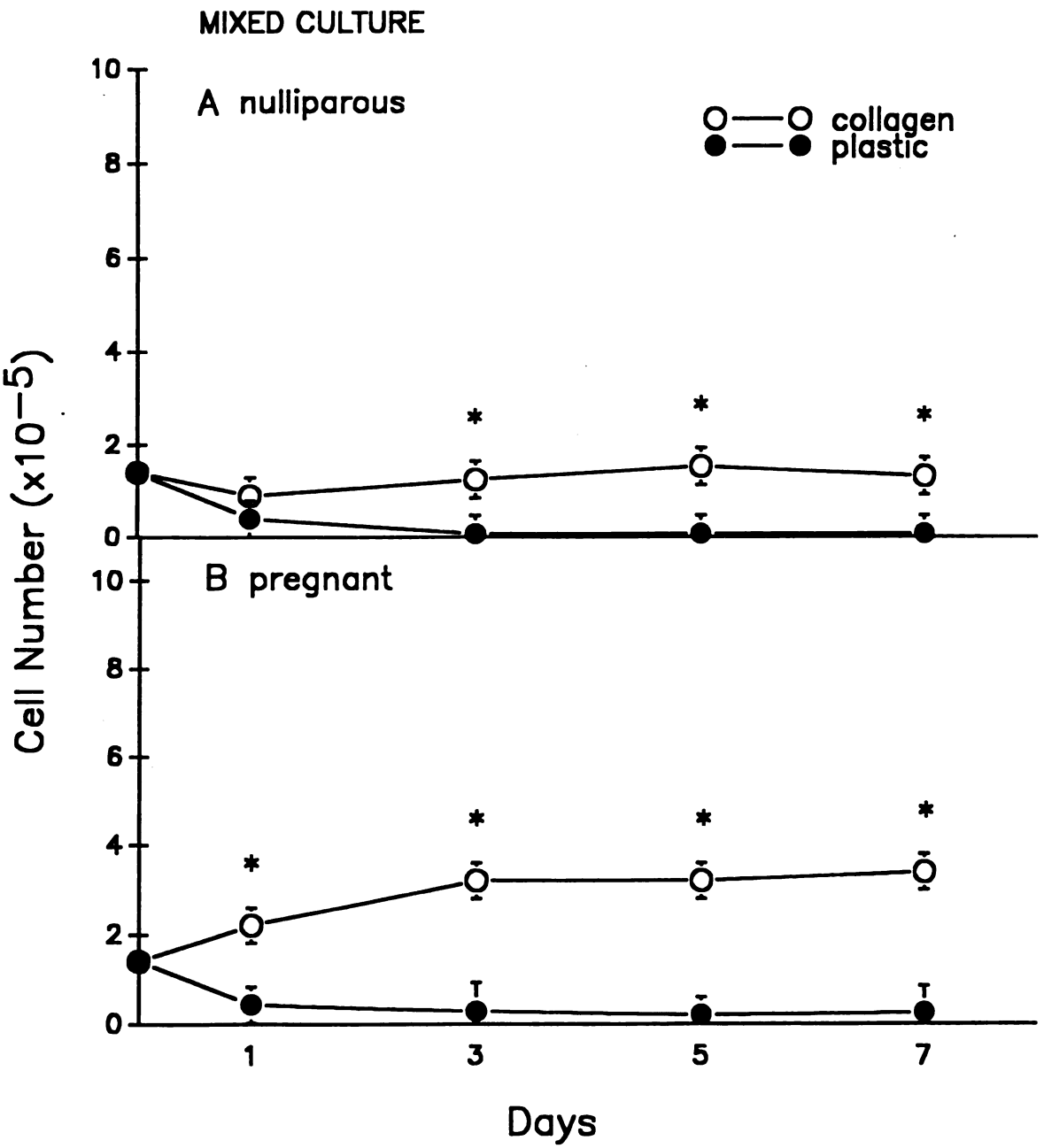

MIXED CULTURE
A nulliparous
collagen
plastic
B pregnant
Cell Number (x10−5)
Days
0
2
4
6
8
10
1
3
5
7
*
T

Figure 13. The proportional distribution of epithelial cells vs. fibroblasts in mixed cultures. Dissociated cells from nulliparous (A) or mid-pregnant (B) mice were plated (4.2×10^5 cells/cm^2) on collagen in BM + F, 0.1 mg/ml. Morphometric quantification of epithelial cells and fibroblasts was carried out at indicated times. Each value represents the mean $\pm$ SEM of 2 separate experiments.

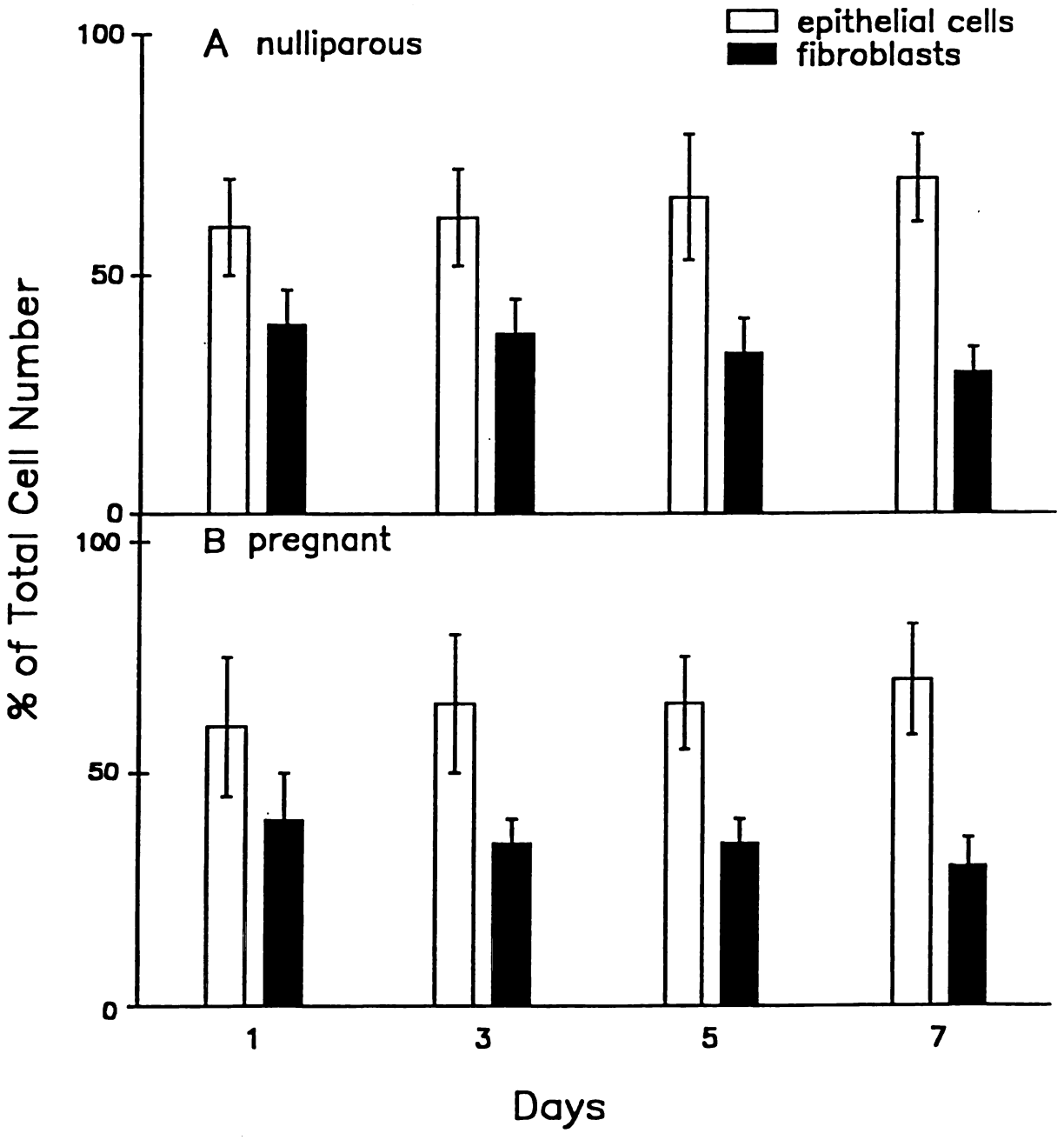
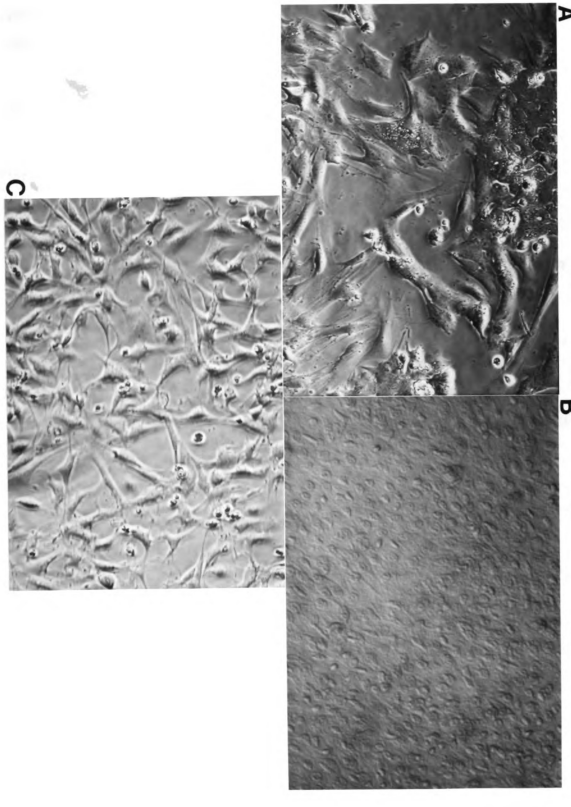

epithelial cells
fibroblasts
A nulliparous
B pregnant
% of Total Cell Number
100
50
0
100
50
0
1
3
5
7
Days

Figure 14. Photomicrographs of cultured mammary cells. Cells from nulliparous mice cultured as mixed cultures (A), epithelial cultures (B), or fibroblast cultures (C) were photographed under phase contrast after 3 days for mixed cultures and fibroblast cultures and 5 days for epithelial cultures. Mag. X 200.


A
B
C

mixed cultures obtained from mid-pregnant mice contained a higher ratio of epithelial cells to fibroblasts ranging from 65:35 on day 1 to 75:25 on day 7 of culture.

2. Optimal serum-free conditions for the maintenance of epithelial cell cultures

Cell maintenance and growth of epithelial cultures from nulliparous and mid-pregnant mice were also analyzed in maximally supplemented medium (BM plus tranferrin, BSA, fetuin, 1 mg/ml) and in media lacking transferrin, BSA and with high (1 mg/ml) or low (0.1 mg/ml) fetuin concentrations on plastic or collagen (Fig. 15, 16). Good maintenance was obtained with 0.1 mg/ml fetuin on collagen (Fig. 15A, 16A). Epithelial cultures from nulliparous mice showed similar maintenance on collegen or on plastic (Fig. 15B). Epithelial cultures from mid-pregnant mice were not maintained as well on plastic, particularly at low fetuin dose (Fig. 16B). As in the case of mixed cultures, hydrocortisone was not required for epithelial culture maintenance since its removal from medium did not negatively affect cell maintenance (data not shown). In all cases epithelial cells comprised more than 99% of the total cells (Fig. 14B).

Based upon the above experiments the minimally supplemented medium and substratum conditions identified for mixed cultures can also be used for the maintenance of epithelial cultures from mammary glands of nulliparous or mid-pregnant mice. A comparison of epithelial culture and mixed culture growth on collagen in the minimally supplemented medium is shown in Fig. 17. Under these conditions, mixed cultures and epithelial cultures from nulliparous mice exhibited identical growth behavior. In contrast, mixed cultures from mid-pregnant mice exhibited significantly better maintenance between days 3 and 7 than epithelial cultures. These results suggest that interactions between epithelial cells and

Figure 15. Effects of medium composition and substratum on growth of epithelial cultures from nulliparous mice. Dissociated epithelial cells from nulliparous mice were plated (4.2×10^5 cells/cm^2) on collagen (A) or plastic (B) in BM + BSA, T, F, 1 mg/ml (●,○), BM + F, 1 mg/ml (■,□), or BM + F, 0.1 mg/ml (▲,△). Each point represents the mean ± SEM of triplicate determinations from two experiments.

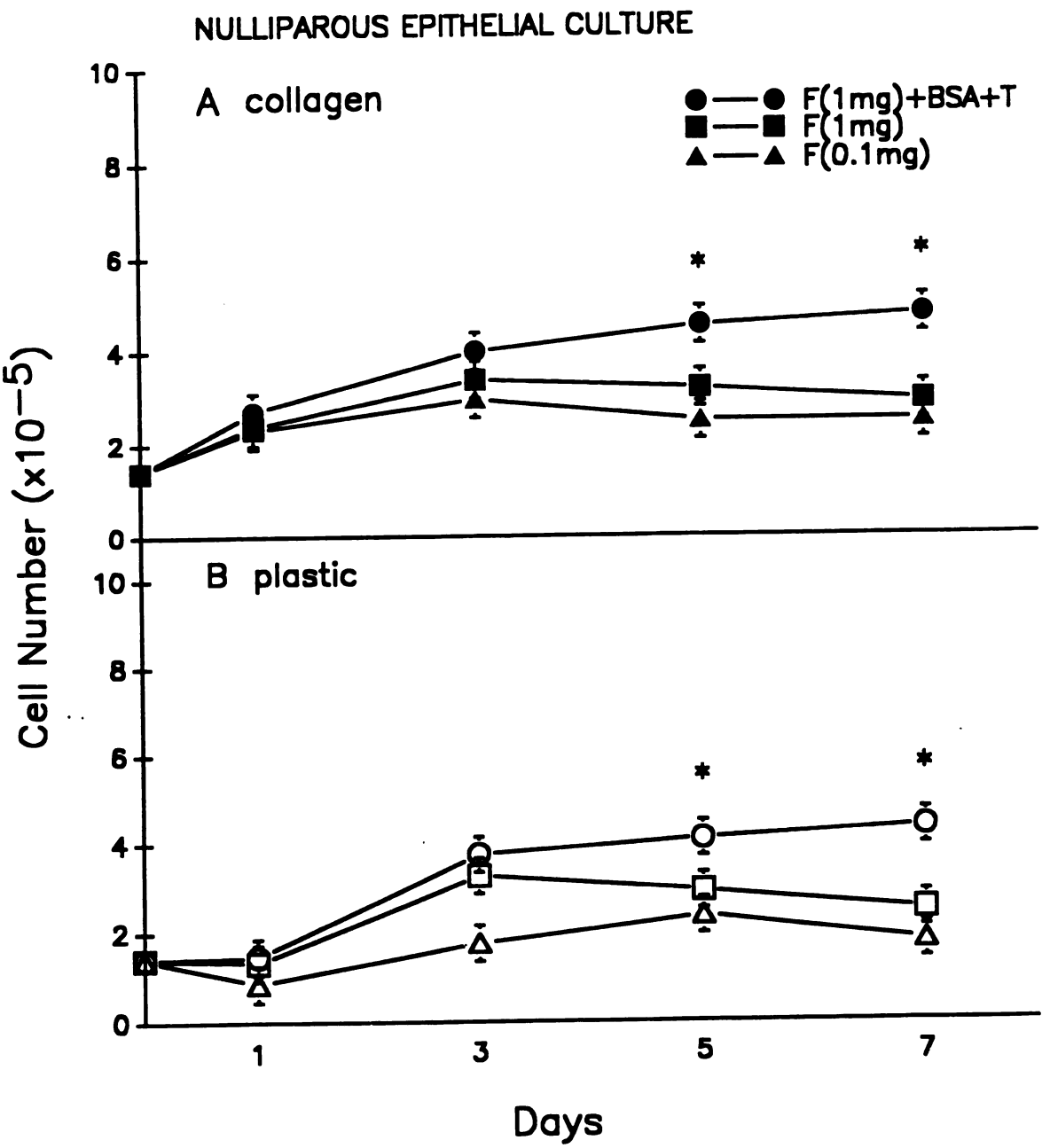

NULLIPAROUS EPITHELIAL CULTURE
A collagen
B plastic
F(1mg)+BSA+T
F(1mg)
F(0.1mg)
Cell Number (x10⁻⁵)
Days
0
2
4
6
8
10
1
3
5
7
*

Figure 16. Effects of medium composition and substratum on the growth of epithelial cultures from mid-pregnant mice. Dissociated epithelial cells were plated (4.2 x 10^5 cells/cm^2) on collagen (A) or plastic (B) in BM + BSA, T, F, 1 mg/ml (●,○), BM + F, 1 mg/ml (■,□), or BM + F, 0.1 mg/ml (▲,△). Each point represents the mean ± SEM of triplicate determinations from two experiments.

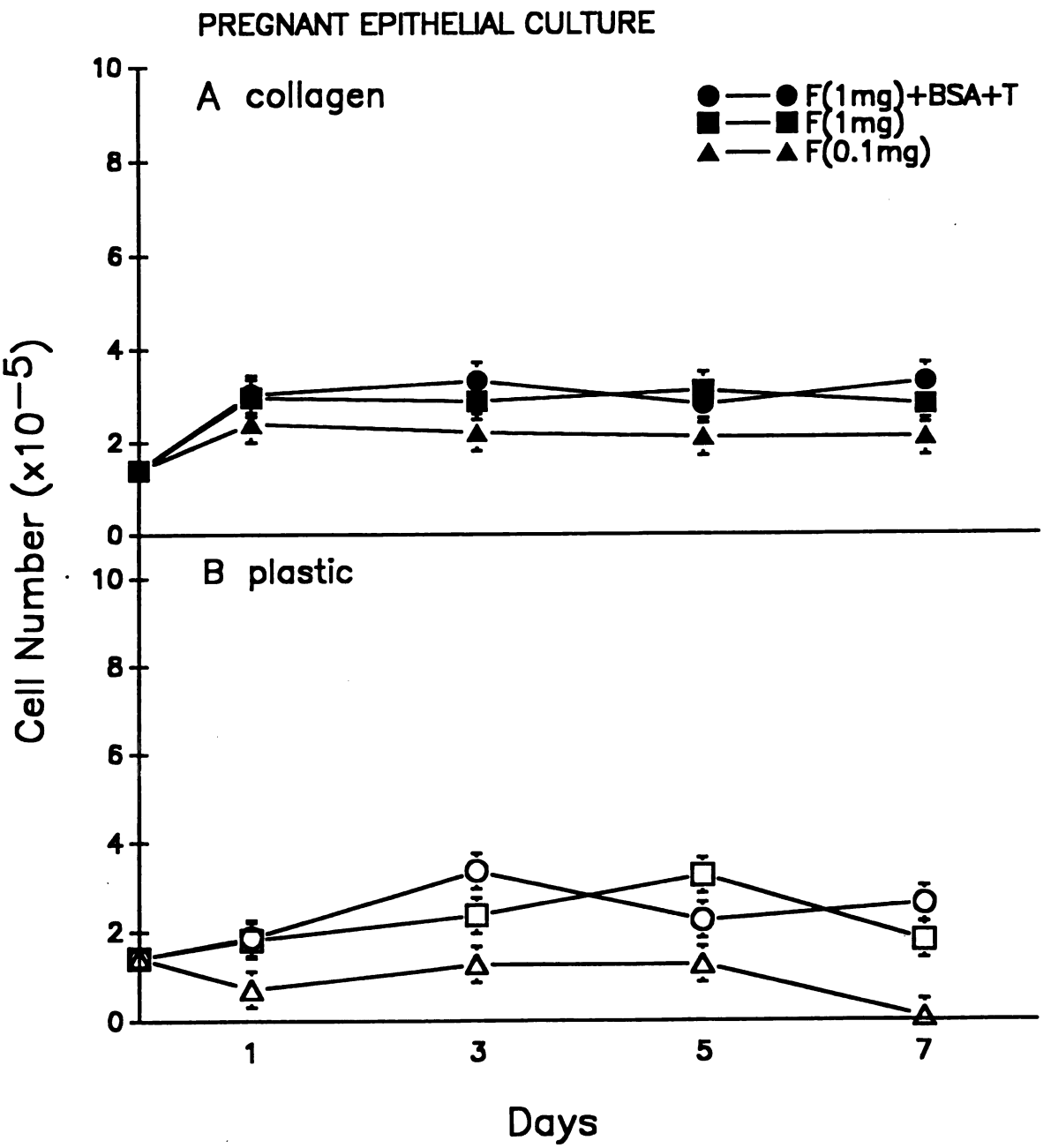
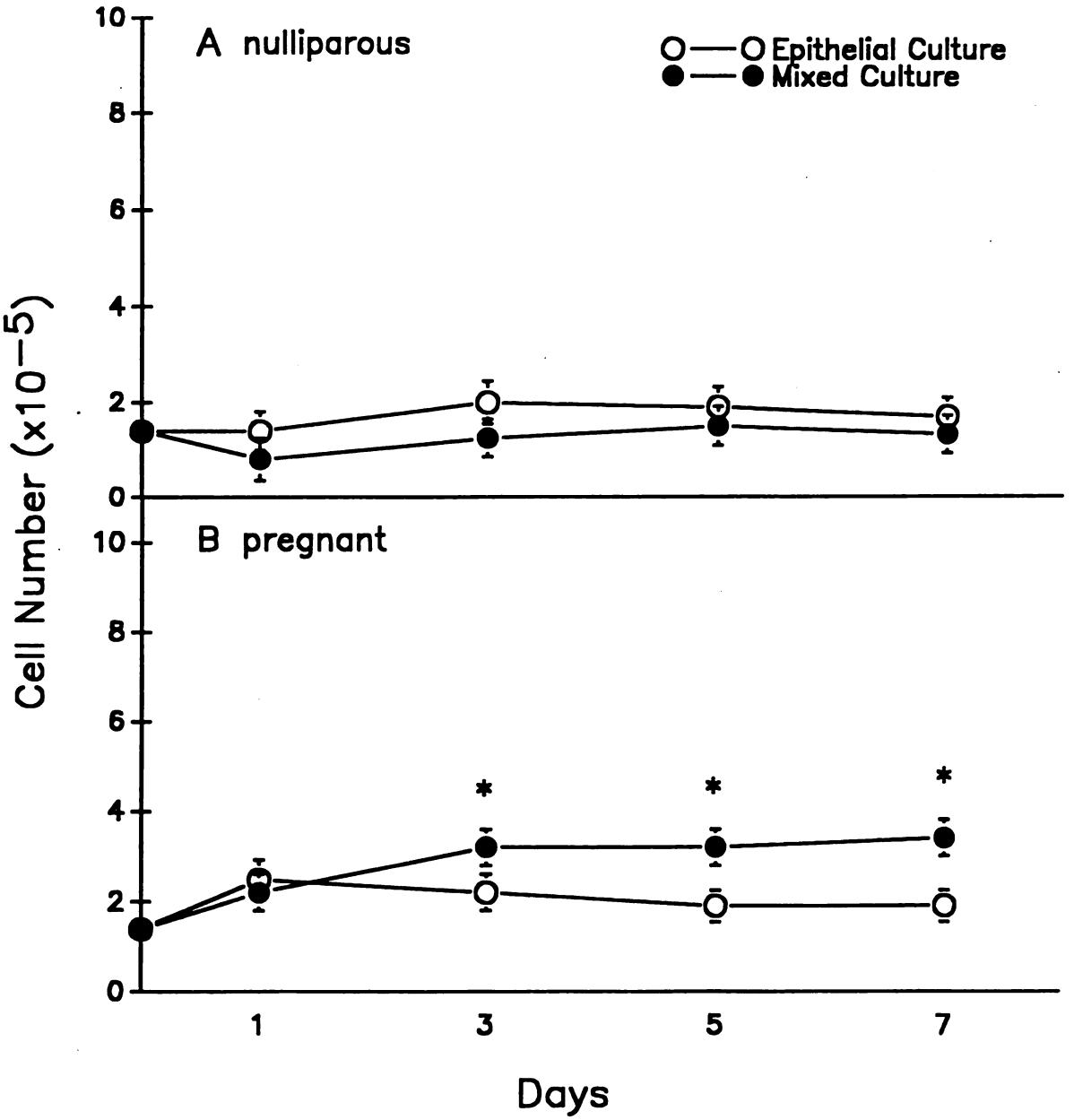

PREGNANT EPITHELIAL CULTURE
A collagen
F(1mg)+BSA+T
F(1mg)
F(0.1mg)
B plastic
Cell Number (x10−5)
Days
0
2
4
6
8
10
1
3
5
7

Figure 17. Comparison of the growth of mixed cultures and epithelial cultures from nulliparous and mid-pregnant mice in minimally supplemented medium. Dissociated mixed cells (●) or epithelial cells (○) from nulliparous (A) or mid-pregnant (B) mice were plated (4.2 x 10^5 cells/cm^2) on collagen in BM + F, 0.1 mg/ml. Each point represents the mean ± SEM of triplicate determinations from representative experiments. * $p = 0.05$ that mixed cultures total cell numbers are greater than epithelial cultures.


A nulliparous
Epithelial Culture
Mixed Culture
B pregnant
Cell Number (x10−5)
Days
0
2
4
6
8
10
1
3
5
7
*
*
*

fibroblasts in mixed cultures from mid-pregnant mice may be facilitating this effect.

3. Optimal serum-free conditions for the maintenance of mammary fibroblast cultures

Purified mammary fibroblasts attached but failed to spread and grow on collagen (data not shown). Thus all studies with fibroblasts were carried out on tissue culture plastic. Maximally supplemented serum-free media (BM + tranferrin + BSA + fetuin 1.0 mg/ml) caused a significant increase in cell proliferation (Fig. 18). The effects of fetuin, transferrin and BSA on fibroblast maintenance and growth were tested individually (Fig. 18). Since basal medium plus fetuin sustained the same degree of proliferation as basal medium plus fetuin, tranferrin and, BSA, a requirement of transferrin and BSA was not indicated. In fact, addition of BSA appeared to have an inhibitory effect on fibroblast maintenance. A dose response study with fetuin revealed that optimal fetuin dose for fibroblast culture maintenance was 0.5 and 1.0 mg/ml for cultures from mid-pregnant and nulliparous mice, respectively (Fig. 19). In contrast to mixed cultures and epithelial cultures, the presence of hydrocortisone was necessary for optimal maintenance of fibroblast cultures from mid-pregnant mice (Fig. 20). Under these conditions fibroblast cultures were comprised of greater than 95% fibroblasts (Fig. 14C).

Discussion

In this study we have determined suitable compositions of serum-free media and substratum conditions that allow for maintenance without extensive proliferation of primary, monolayer cultures containing mouse

Figure 18. Effects of BSA, transferrin or fetuin on the growth of fibroblast cultures from nulliparous or mid-pregnant mice. Dissociated fibroblasts from nulliparous (A) or mid-pregnant (B) mice were plated (4.2×10^5 cells/cm^2) on plastic in BM (●), BM + T (△), BM + BSA (▲), BM + F, 1 mg/ml (□), or BM + BSA, T, F, 1 mg/ml) (○). Each point represents the mean ± SEM of 2 experiments. * $p = 0.05$ that total numbers of cells obtained in the presence of F, or T, or T+BSA+F were greater than in BSA or BM.

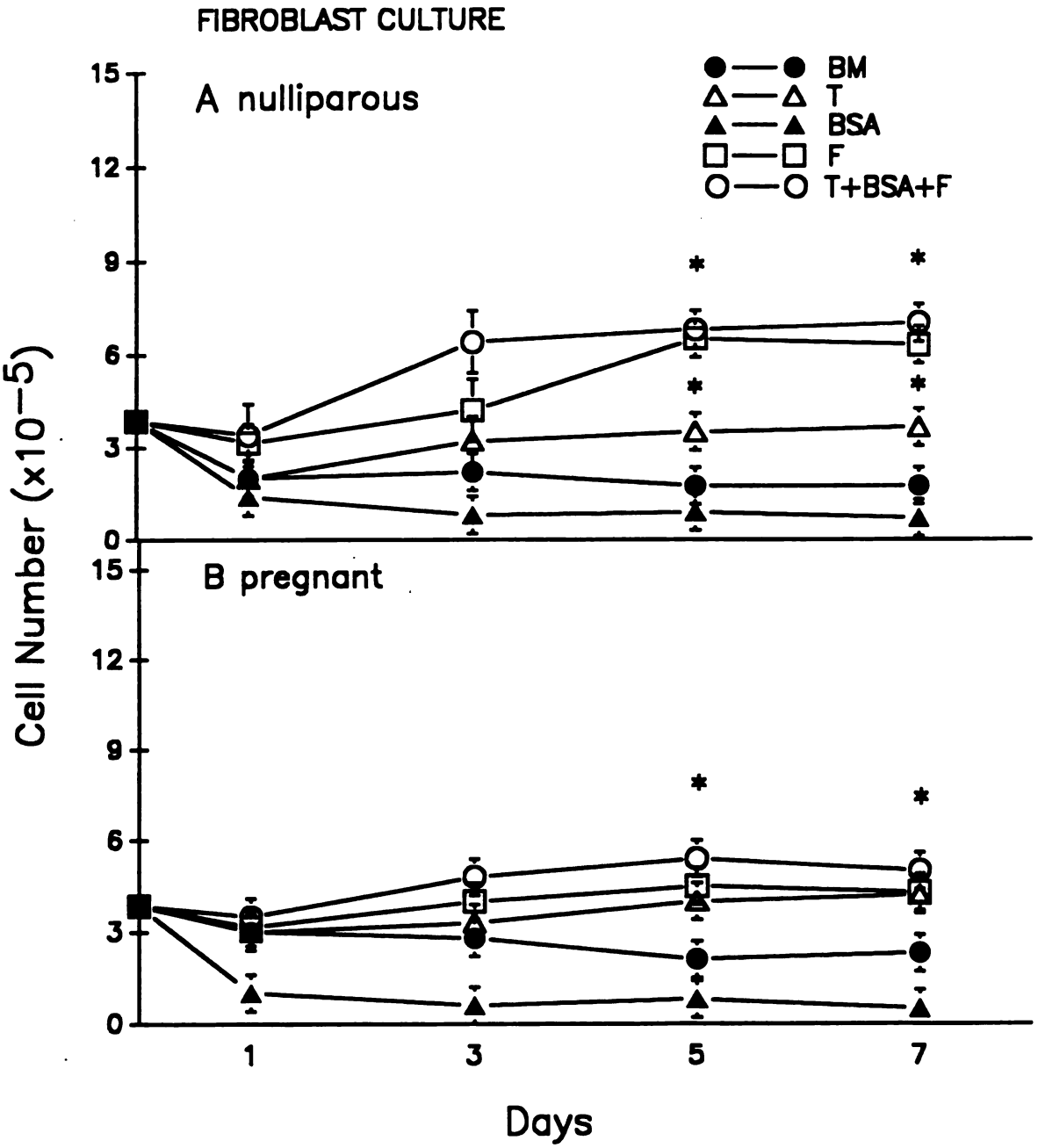

FIBROBLAST CULTURE
A nulliparous
B pregnant
BM
T
BSA
F
T+BSA+F
Cell Number (x10−5)
15
12
9
6
3
0
1
3
5
7
Days
*

Figure 19. Effects of fetuin dose on fibroblast cultures growth. Dissociated cells from nulliparous (A) or mid-pregnant (B) mice were plated (4.2 x 10^5 cells/cm^2) on plastic in BM containing 1 (▲), 0.5 (△), 0.1 (●), or 0 (○) mg/ml F. Each point represents the mean ± SEM of triplicate determinations from representative experiments. * p = 0.05 that F dose of 0.5 and 1mg resulted in higher cell numbers than a 0 or 0.1mg dose.

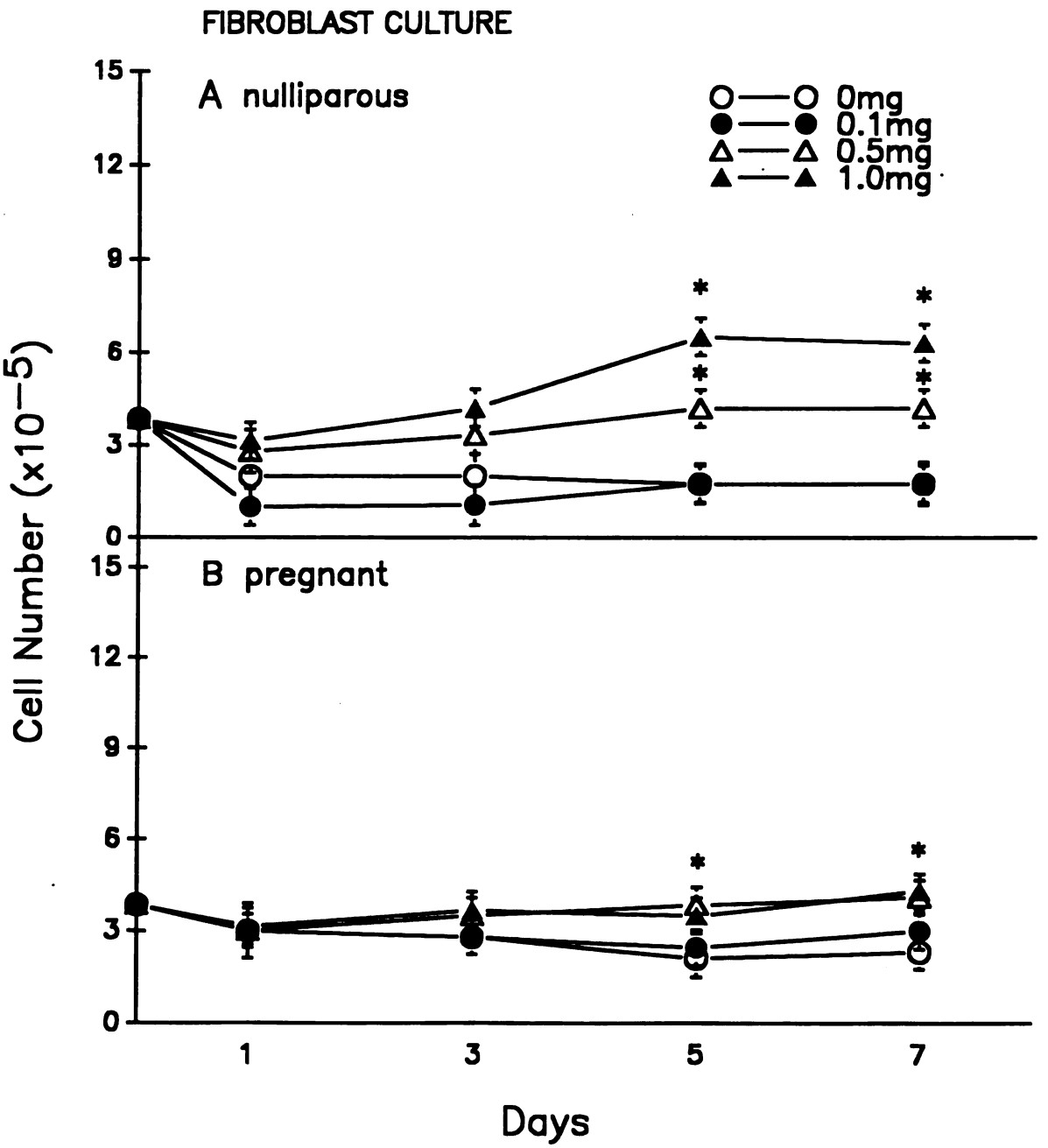
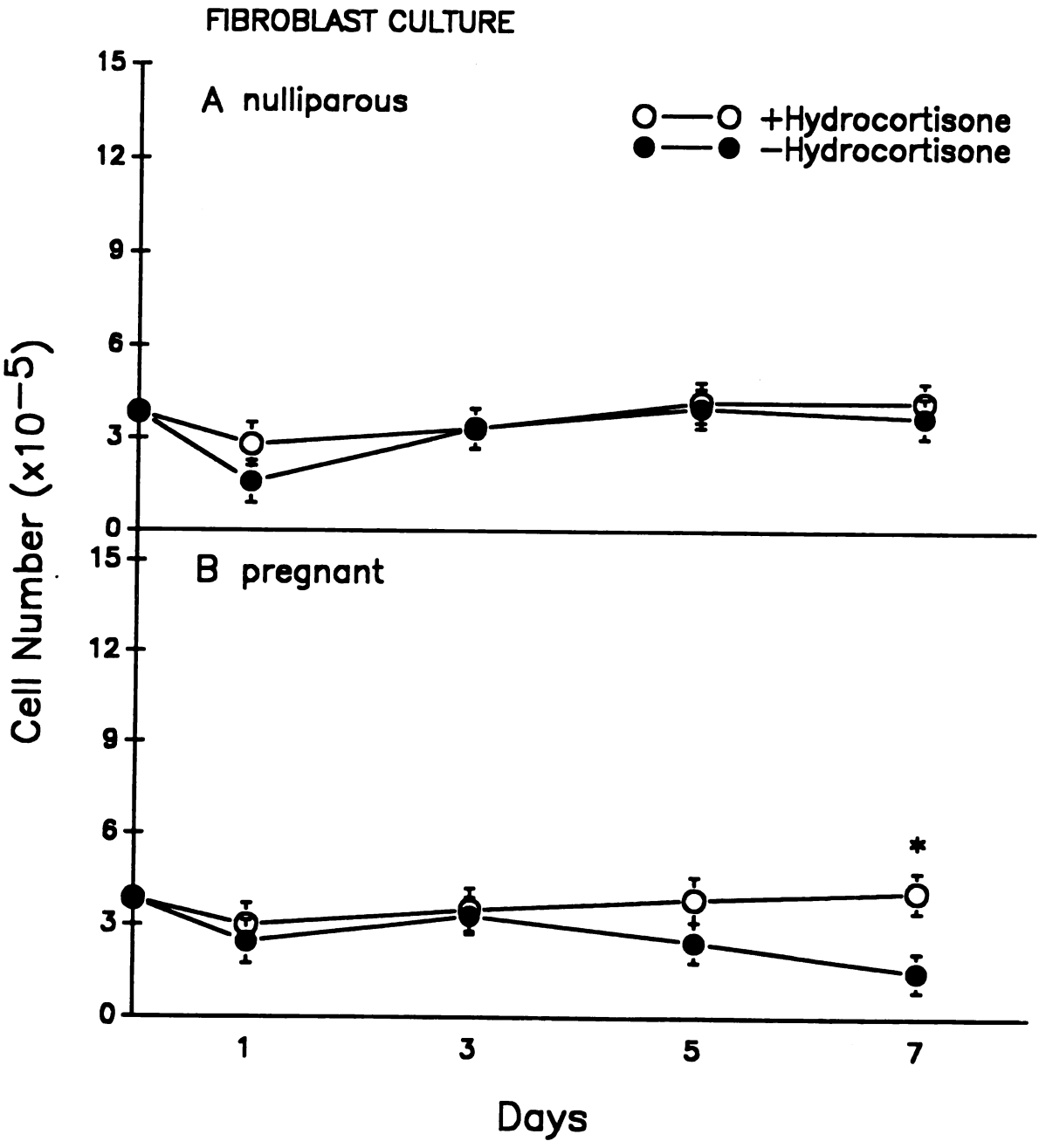

FIBROBLAST CULTURE
A nulliparous
B pregnant
0mg
0.1mg
0.5mg
1.0mg
Cell Number (x10−5)
15
12
9
6
3
0
1
3
5
7
Days

Figure 20. Effects of hydrocortisone on fibroblast cultures growth. Dissociated fibroblasts from nulliparous (A) or mid-pregnant (B) mice were plated (4.2 x 10^5 cells/cm^2) on plastic BM F, (0.5 mg/ml) with (o), or without hydrocortisone (●). Each point represents the mean $\pm$ SEM of triplicate determinations from a representative experiment. * $p = 0.05$ that the total numbers of cells grown in the presence of hydrocortisone are greater than those grown in its absence.


FIBROBLAST CULTURE
A nulliparous
+Hydrocortisone
−Hydrocortisone
B pregnant
Cell Number (x10−5)
15
12
9
6
3
0
1
3
5
7
Days
*

mammary epithelial cells, mammary fibroblasts or mixed cultures containing both epithelial cells and fibroblasts.

In the past, serum-free media have been developed for the primary culture of mammary epithelial cells (Imagawa et al.,1982). Serum factors such as fetuin, BSA and transferrin and the hormone hydrocortisone have been added to serum-free media to enhance cell attachment and maintenance. However, these serum factors and hydrocortisone can also cause such high baseline cell proliferation that the growth stimulatory effects of mammogenic hormones could not be detected using these media (Imagawa et al., 1982). In our study we have therefore examined these serum factors and hormones for their effects on cell growth.

Fetuin, a required attachment factor, also promoted mammary cell growth herein. Several studies have shown that fetuin prepared according to Pederson's method contains high molecular weight species which caused mouse embryonal carcinoma cell growth in serum-free medium (Salomon et al., 1982; 1984). We have been able to reduce the mitogenic effect of fetuin on mixed cultures and epithelial cultures without losing its attachment activity by reducing its concentration to 0.1 mg/ml. In our studies BSA promoted cell proliferation possibly due to its lipid contaminants as has been previously reported (Imagawa et al., 1982). Transferrin has also been reported to replace serum for the stimulation of mouse 3T6 cell proliferation (Rudland et al.,1977). However, in the present studies neither BSA nor transferrin were required for mixed culture, epithelial culture or fibroblast culture maintenance. Hydrocortisone is frequently included in media for the culture of mammary cells and may play an important role in the expression of differentiated functions such as casein synthesis (Emerman et al.,1977). However, the presence of hydrocortisone was required only for the maintenance of fibroblast cultures from mid-pregnant mice.

In the case of both mixed cultures and epithelial cultures, optimal maintenance was observed in basal media supplemented with fetuin (0.1 mg/ml). Although cell attachment did take place on plastic, optimal attachment and maintenance was observed on a collagen-coated substratum. The importance of an appropriate substratum for normal function of mammary epithelial cells has been previously observed and collagen has been reported to support more normal function than tissue culture plastic (Emerman et al., 1977).

Comparison of behavior of mixed cultures vs. epithelial cultures revealed that mixed cultures from mid-pregnant mice exhibited better maintenance than epithelial cultures. We have also recently observed that nulliparous epithelial cell responsiveness to ovarian hormones is altered in the presence of fibroblasts under mixed culture conditions (unpublished observations), indicating that our culture system is capable of exhibiting paracrine interactions. Taken together, these results indicate not only that interactions between epithelial cells and fibroblasts can modify growth behavior but also that the developmental state and degree of mammary gland differentiation can affect cell-cell interactions and ensuing proliferative behavior in serum-free medium.

Media composition and substratum conditions required for attachment and maintenance of fibroblast cultures were significantly different from those required for mixed or epithelial cell cultures. Optimal maintenance required a higher concentration of fetuin. This fetuin requirement for fibroblasts does not appear to be due to its function for attachment, since fibroblasts initially attached well in minimal media established for mixed cultures and epithelial cultures, but failed to be maintained. Thus as is the case for epithelial cultures and mixed cultures, fibroblast cultures also require the growth promoting activity of fetuin for maintenance. Hydrocortisone was shown to be required for maintenance of fibroblast cultures from mid-pregnant mice. Others have also reported that

glucocorticoids are essential for pregnant mammary tissue survival and secretory activity development in organ culture (Topper & Oka, 1974). With the advancing of pregnancy there was an increased tissue uptake of corticosteroid (Pearlman et al., 1981). The mechanism of this hydrocortisone requirement is not clear.

Interestingly, fibroblasts were well maintained in a less supplemented medium when cultured in the presence of epithelial cells in mixed cultures. These observations indicate that epithelial cells may be involved in the regulation of fibroblast function. Comparison of attachment and growth of fibroblasts on collagen vs. plastic revealed another major difference. Fibroblasts failed to spread out and proliferate on collagen. However, when a mixture of epithelial cells and fibroblasts were plated together, the fibroblasts did spread out and proliferate. These observations suggest another level of cell-cell interaction and indicate that in the presence of epithelial cells, fibroblast spreading and proliferative behavior is modified.

In summary, we have identified suitable serum-free conditions for the maintenance of mammary epithelial cells, fibroblasts and mixed cell cultures. We have also observed interactions between epithelial cells and fibroblasts which support previous reports that such interactions can potentially modify hormonal responsiveness and proliferative behaviors of mammary cells (Enami et al., 1983; Haslam 1986; Haslam, 1991; Haslam & Counterman 1991). The results provided herein should facilitate future studies to investigate the roles of growth factors and their interactions with mammogenic hormones and the potential autocrine and/or paracrine interactions between the various mammary cell types.

Chapter 4: The effects of estrogen, progesterone and growth factors on epithelial, fibroblast, and mixed primary cultures in serum-free monolayer culture conditions

Introduction

The media components and culture conditions for supporting the maintenance of epithelial, fibroblast, and mixed cell cultures have been carefully defined in Chapter Three. The occurrence of direct effects of E and P on mammary epithelial or fibroblast cells can now be investigated under these serum-free conditions. Identification of the effects of these hormones on epithelial cells vs. fibroblasts and on mixed cell cultures will help to elucidate the role of epithelial-stromal cell interactions in these hormone regulated effects. E and P have been shown to interact with epidermal growth factor (EGF) in the stimulation of epithelial DNA synthesis in vivo (Haslam et al., 1993) and the regulation of lobuloalveolar development in vitro organ culture system (Tonelli & Sorof, 1980). IGF-I has been shown to synergize with EGF in stimulating mammary epithelial cell proliferation in cells cultured in collagen gels (Deeks et al., 1988). However, the relationship between IGF-I, E and P and their effects on mammary fibroblasts are not clear at present. Therefore the effects of EGF and IGF-I on mammary epithelial, fibroblast, and mixed cell cultures and their interactions with E and P were examined in our serum-free monolayer culture system.

In these studies, the steroid hormones E and R5020 (a synthetic progestin) and the growth factors EGF and IGF-I were added to culture media separately or in various combinations. The effects of these hormones and growth factors on cell proliferation in epithelial, fibroblast and mixed cell cultures from nulliparous or mid-pregnant mice was compared. The maintenance of ER and the regulation of PR were also examined. These studies helped to define: a) the interactions of

steroid hormones and growth factors in regulating cell proliferation; b) the proliferative effects of steroid hormones and growth factors on different mammary cell types and the role of epithelial-stromal cell interactions and c) the effects on mammary cells at different developmental stages.

Material and Methods

Cell culture:

BALB/c 12-15 week-old, nulliparous or mid-pregnant (12-14 days) mice were the source of mammary tissues. Mixed cells were obtained by enzymatic dissociation of finely minced mammary tissue in 0.1% collagenase for 30 min at 37 C. To obtain cultures enriched for mammary fibroblasts and epithelial cells, finely minced mammary tissue was subjected to 2 hours enzymatic dissociation with 0.1% collagenase at 37 C. The cell suspension was centrifuged twice at 80 x g for 30 sec and the pooled supernatants were the source of the fibroblasts. To obtain cultures enriched for mammary epithelial cells, the pellet remaining after the removal of fibroblasts was further dissociated with 0.05% pronase for 20 min at 37 C. The cell suspension was then purified by percoll density gradient centrifugation. All cell types were plated at a density of 4.2×10^5 viable cells/ cm^2. Epithelial cells and mixed cultures were plated at 1.4×10^5 viable cells/well in a 96-well dish, and fibroblasts were plated at 9.5×10^5 viable cells /well in a 4-well dish. Epithelial cells and mixed cells were plated on rat-tail collagen-coated (collagen I) dishes to enhance cell attachment. Fibroblasts were plated on tissue-culture plastic. After 2 hours of plating, fibroblast cultures were washed 3 times with HBSS to remove any residual epithelial cells. The basal medium for epithelial and mixed cultures contained phenol red-free DME/F12 (1:1), Hepes (15 mM), penicillin (0.1 mg/ml), streptomycin (0.05 mg/ml), insulin (0.1 μg/ml) and

fetuin (0.1 mg/ml). The basal medium for fibroblast cultures contained phenol red-free DME/F12 (1:1), Hepes (15 mM), penicillin (0.1 mg/ml), streptomycin (0.05 mg/ml), insulin (I, 0.1 μg/ml), hydrocortisone (0.2 μM) and fetuin (0.5 mg/ml). Media was changed every day. Cells were kept at 37 °C in 5% CO_2 for 6 days. Hormones 17β-estradiol (E, 23nM), R5020 (promegestone; 7,21-dimethyl-19-nor-4,9-pregna-diene-3,20-dione; 10nM) and growth factors EGF (1ng/ml) and IGF-I (25ng/ml) were added to the media individually or in combinations 24 hours after plating. The concentrations of 23nM, 10nM and 25ng/ml were used for 17β-estradiol, R5020 and IGF-I respectively, based on their ability to produce a maximal proliferative effect in a dose response study (data not shown). A low dose (1ng/ml) of EGF was used because it has been previously reported that a higher dose of EGF obscures interactions with other hormones (Imagawa et al., 1985).

Quantitation of Cell Growth:

Cell number was quantified using a modified 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay is based upon the conversion of tetrazolium into blue formazan by the mitochondrial enzyme succinate-dehydrogenase. The color production is directly related to viable cell number (Hahm & IP, 1990). Briefly, 5 days after addition of hormone- and growth factor- supplemented media, phenol red-free DME/F12 media containing 1 mg/ml MTT was added to the cultures for 4 h at 37 ° C. Cells were then fixed with formal-saline (10% formaldehyde in 0.85% NaCl) for 30 min to prevent cell detachment and loss and then the MTT-formol-saline supernatant was removed. Isopropanol (100%) was then added to each well to dissolve the formazan crystals and absorbance at 540 nm was measured using a multiwell spectrophotometer Bio-tek EL 310 reader.

Immunocytochemistry:

Cells of mesenchymal origin express the intermediate filament, vimentin (Miettinen et al., 1984). Anti-vimentin antibody has been used previously to identify mammary fibroblasts in cell culture and in tissue sections (Asch et al., 1981; Haslam & Levely, 1985). Therefore to establish the purity of epithelial cell and fibroblast cultures and the ratio of epithelial cells to fibroblasts in mixed cultures, the fibroblasts were identified immunocytochemically using goat anti-vimentin antibody (a gift of Dr. B. B. Asch, Roswell Park Memorial Institute).

Fibroblasts were cultured on glass coverslips; epithelial cells and mixed cells were cultured on collagen-coated coverslips. Cells were fixed at -20°C in absolute methanol for 5 min and then in acetone for 5 min. Endogenous peroxidase activity was blocked with 0.1% hydrogen peroxide in methanol. Nonspecific antibody binding was blocked with normal rabbit serum (1:100 dilution). Cells were then incubated with anti-vimentin antiserum (1:50 dilution) for 1 hour at room temperature. Control coverslips were incubated with normal goat serum (1:50 dilution). After rinsing three times with PBS, cells were incubated with a secondary antibody, peroxidase labeled rabbit antigoat IgG (1:3200 dilution) (Cappel Research Products, Durham, NC) for 2 hours at room temperature. Peroxidase activity was detected by incubating 3 minutes with 3,3'-diaminobenzidine (25μg/ml) in hydrogen peroxide (0.01%).

Quantitation of epithelial cells and fibroblasts in mixed cell cultures:

Epithelial cells and fibroblasts in the mixed cell cultures which had been treated with ovarian steroid hormones or growth factors were quantitated, to determine their relative effects on proliferation of different cell types. After MTT assay, isopropanol was removed from culture dishes and cells were stained with methylene blue (1%) for five minutes. The percentage of epithelial cells and

fibroblasts in mixed cell cultures was determined by counting cell nuclei of cells identified to be epithelial cells or fibroblasts based upon morphological criteria under an inverted microscope. Three samples of 5000 cells from two experiments were counted for each treatment.

Steroid hormone binding assay:

The methods for measuring specific [^{3}H]estradiol binding in intact cells has been described previously (Haslam & Levely, 1985). Cells grown in culture dishes were washed three times with 37^{o}C HBSS (Hanks' balanced salt solution). Basal culture medium containing various concentrations of [^{3}H]estradiol (1-10 nM) (142.0 Ci/mmol., New England Nuclear) with or without a 100-fold excess of unlabeled estradiol was added to duplicate culture wells and incubated at 37^{o}C for 1 h. The binding reaction was terminated by the addition of ice-cold HBSS, followed by three additional washes. Cells were then extracted with 100% ethanol at room temperature for 10 minutes, and radioactivity was determined by counting duplicate aliquots of the ethanol extracts in scintillation fluid consisting of toluene, 30% Triton X-100, and 6 g/ liter Omnifluor (New England Nuclear). All samples were counted in a Beckman LCS-7000 liquid scintillation counter (Beckman, Palo Alto, CA) with a 50% counting efficiency. The amount of specifically bound estradiol was determined by subtracting the amount of [^{3}H]-estradiol bound in the presence of excess unlabeled estradiol from that bound in its absence.

To measure specific progestin-binding sites, the same procedure was used, except that cells were incubated with [^{3}H]R5020 (1-13 nM) (84.8 Ci/mmol., New England Nuclear) with or without a 100-fold excess of unlabeled R5020. Since R5020 can also bind to glucocorticoid-binding sites in mammary gland, total binding was measured in the presence of a 100-fold excess of unlabeled

dexamethasone. All binding data was analyzed by the method of Scatchard (Scatchard G, 1947).

Results

1. Cell culture morphology

Epithelial cells were plated as small organoids of aggregated cells. The cell clumps grew out into colonies containing polygonal-shaped cells and cultures reached confluence about four days after plating. Epithelial cells from nulliparous and mid-pregnant mice were morphologically similar.

After cell dissociation, fibroblasts existed as large round cells and had a granular surface. Two hours after plating, fibroblasts attached to the culture dish and had two to four cytoplasmic processes. Fibroblast cultures reached confluence by three or four days after plating. No morphological differences were observed between fibroblast cultures of nulliparous and mid-pregnant mice. Anti-vimentin antibody binding was observed only in fibroblast cultures (results not shown) indicating that epithelial cultures contained only epithelial cells.

Nulliparous mixed cultures contained two types of epithelial cell organoids. One type was a round clump of cell aggregates; the second type resembled small pieces of mammary ducts. The duct-like epithelial organoids usually attached but failed to grow and form colonies. In contrast, mid-pregnant mixed cultures contained mostly the round type of epithelial organoids which grew out into colonies. Mixed cultures also contained fibroblasts (epithelial cells : fibroblasts = 70 : 30; see Chapter Three). Epithelial cells grew in colonies and fibroblasts as single cells which surrounded the epithelial colonies. In confluent mixed cultures, epithelial cells were in contact with fibroblasts.

Addition of the hormones E and R5020 as well as the growth factors EGF and IGF-I to all the three types of cell cultures from nulliparous and mid-pregnant mice did not change cell morphology.

2. The effects of E, R5020, EGF and IGF-I on cell proliferation of cell cultures of mammary cells obtained from nulliparous mice

E, R5020 and EGF were added alone or in combination to fibroblast, epithelial and mixed cultures from nulliparous mice as shown in Fig. 21. The growth of fibroblasts was not stimulated by any of these hormones or growth factors either alone or in different combinations (Fig. 21C). Epithelial cells were stimulated by the progestin R5020, and EGF; there was no further stimulatory effect when E, R5020 and EGF were combined (Fig. 21B). Mixed cells were stimulated by E + R5020, EGF, but not R5020 alone (Fig. 21A). Thus in mixed culture there was a loss of the R5020 stimulatory effect. It is possible that this loss of a R5020 effect was due to the dilution of the epithelial cell population by fibroblasts which did not respond to R5020. Alternatively, the presence of fibroblasts may have altered epithelial cell hormonal responses.

Fig. 22 shows the effects of IGF-I and its interactions with E, R5020 and EGF. Fibroblasts were not stimulated by IGF-I alone or in any combination with E, R5020 or EGF (Fig. 22C). In epithelial cultures either IGF-I or EGF stimulated epithelial cell proliferation. When IGF-I, E, R5020 and EGF were combined there was no further stimulatory effect (Fig. 22B). In the mixed cultures IGF-I and EGF also stimulated cell growth (Fig. 22A). The stimulatory effect of IGF-I plus EGF in mixed cultures, albeit higher than IGF-I or EGF alone, was not statistically significantly (Fig. 22A).

In mixed cell cultures, the stimulatory effects of E+R5020, EGF and IGF-I on epithelial cells vs. fibroblasts have been analyzed to determine which cell types

Figure 21. Effects of E, R5020, and EGF alone or in different combinations on cell proliferation of mixed, epithelial cell and fibroblast cultures from nulliparous mice. Mixed and epithelial cells were plated on collagen, and fibroblasts were plated on plastic in basal media (C), or plus E 23nM, R5020 (R) 10nM, EGF 1ng/ml. Each point represents the mean $\pm$ SEM of triplicate determinations from two to four experiments. * $p = 0.05$ that cell numbers in hormone or growth factor treated group are greater than in control groups.

Effects of E, R5020, EGF on Cell Cultures of Nulliparous Mice

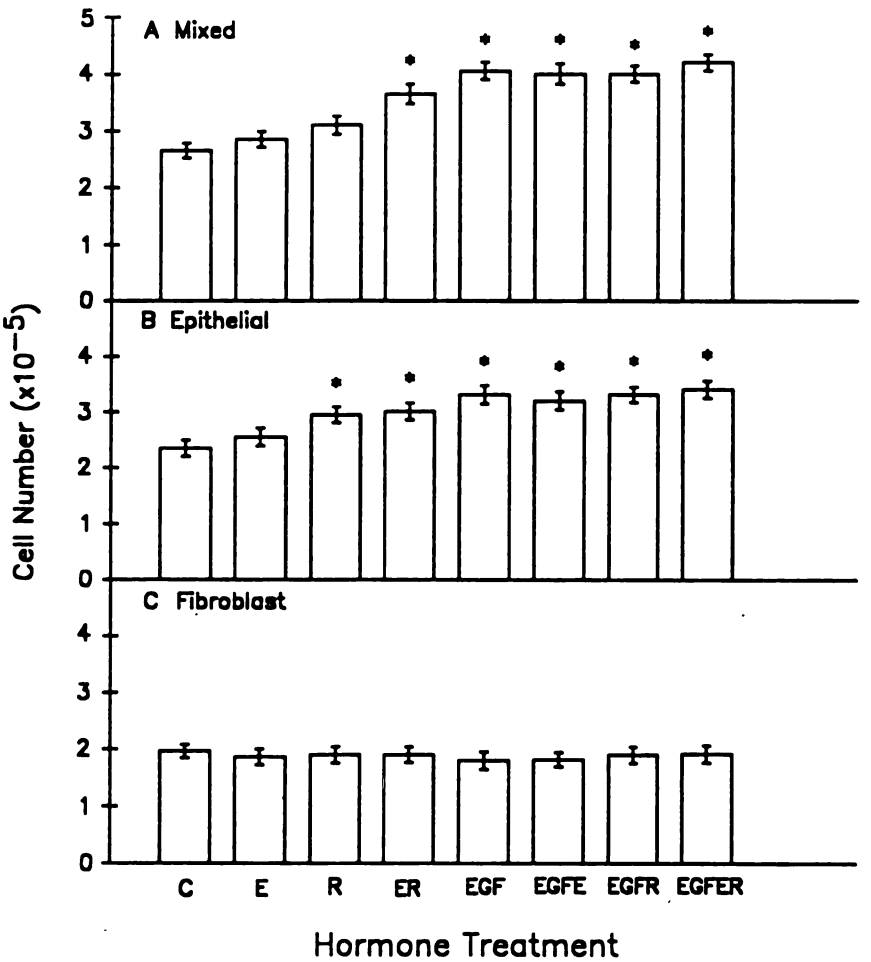


Figure 22. Effects of IGF-I and EGF alone or in different combinations with E and R5020 on cell proliferation of mixed, epithelial and fibroblast cell cultures from nulliparous mice. Mixed and epithelial cells were plated on collagen, and fibroblasts were plated on plastic in basal media (C) or, plus IGF-I 25ng/ml, E 23nM, R 10nM, or EGF 1ng/ml. Each point represents the mean $\pm$ SEM of triplicate determinations from two to three experiments. * $p = 0.05$ that cell numbers in hormone or growth factor treated groups are greater than in control group.

Effects of E, R5020, EGF and IGF–I on Mammary Cells of Nulliparous Mice

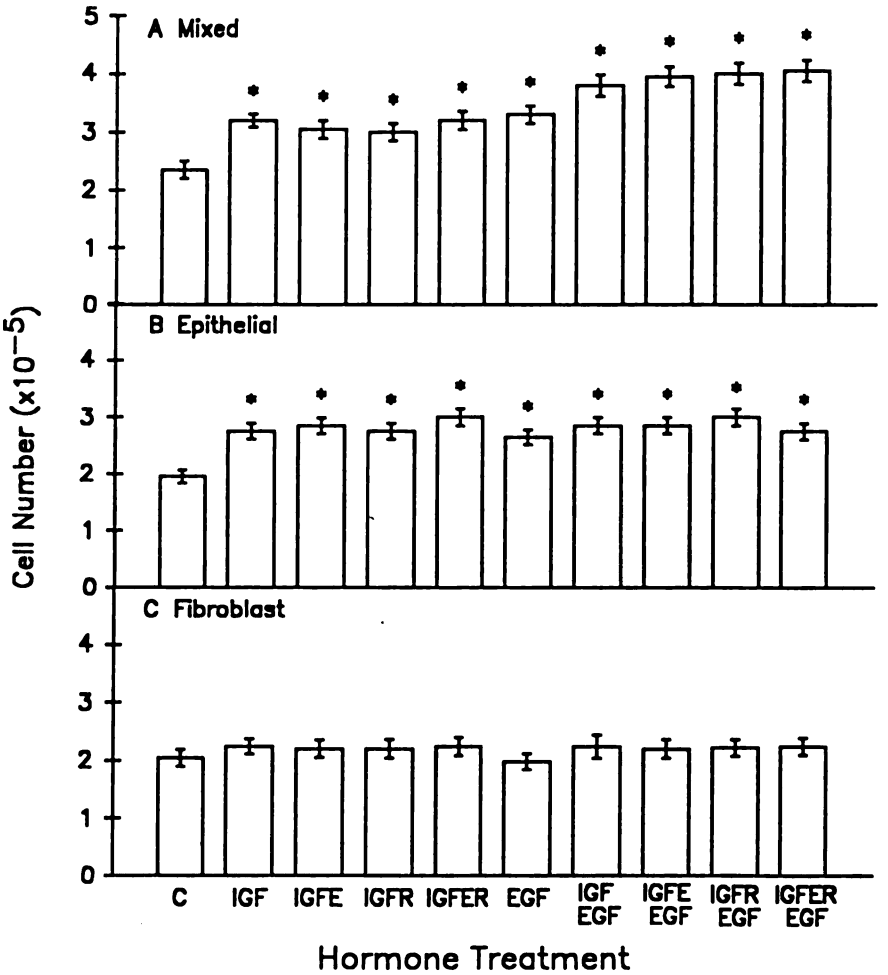


Table 2. Differential effects of R5020, E+R5020, EGF, and IGF-I on epithelial cells and fibroblasts in mixed cell cultures from nulliparous mice.

Treatment[a]	Cell Number (x 10^5)			Ratio of the % Epithelial Cells: Fibroblasts
	Total Cells[b]	Epithelial Cells	Fibroblast	
Control	1.28 ± 0.10	0.83 ± 0.04	0.45 ± 0.04	65:35
R5020	1.43 ± 0.10	1.00 ± 0.03*	0.43 ± 0.05	69:31
E+R5020	1.66 ± 0.05*	1.10 ± 0.06*	0.56 ± 0.03*	66:34
EGF	1.82 ± 0.09*	1.46 ± 0.09*	0.36 ± 0.08	80:20
IGF-I	1.66 ± 0.11*	1.29 ± 0.09*	0.37 ± 0.08	78:22

[a] Cells were cultured as described in Materials and Methods for 5 days in the presence of basal medium (control), R5020 (10 nM), E+R5020 (23 nM+10 nM), EGF (1 ng/ml) or IGF-I (25ng/ml).

[b] Cells were counted with the aid of ocular grid and a microscope. Each point represents the mean ± SEM of triplicate determinations from three experiments.

* $p < 0.05$ that there is a significant difference from the control group.

were affected. Although R5020 did not significantly stimulate mixed culture proliferation overall, the epithelial cell and fibroblast composition was also analyzed to determine if R5020 stimulated epithelial cells or if the presence of fibroblasts abrogated the epithelial cell response. As shown in Table 2, R5020 increased epithelial cell proliferation 1.2-fold but had no effect on fibroblasts. This is the same degree of stimulation that was observed in epithelial cultures

(Fig. 21). Thus, it appears that the presence of non-responsive fibroblasts diluted the epithelial cell response to R5020. E+R5020 increased both epithelial cell and fibroblast numbers, but did not change the ratio of epithelial cells and fibroblasts compared to the control. Therefore, E+R5020 appears to stimulate the proliferation of both epithelial cells and fibroblasts in the mixed cultures. In contrast, EGF and IGF-I preferentially increased only the numbers of epithelial cells, and the total cell number increases were accompanied by increases in epithelial cell number and slight decreases in fibroblasts. Therefore the growth effect of EGF and IGF-I in mixed cultures was most likely due to increased epithelial cell proliferation.

3. The effects of E, R5020, EGF and IGF-I on cell proliferation of cell cultures of mammary cells obtained from mid-pregnant mice

E, R5020 and EGF were added alone or in various combinations to fibroblast, epithelial and mixed cultures from mid-pregnant mice as shown in Fig. 23. The growth of fibroblasts was not stimulated by any of these hormones, growth factors alone or in their different combinations (Fig. 23C). In contrast to nulliparous cell cultures, epithelial cells were not stimulated by R5020 and mixed cells were not stimulated by E + R5020. Epithelial and mixed cells were only stimulated by EGF, and there was no further stimulatory effect when E, R5020 and EGF were combined (Fig. 23B, 23A). These results indicate that at different stages of mammary gland development, the hormones responsible for growth control may change.

Fig. 24 shows the effects of IGF-I alone and in combination with E, R5020 and EGF. As can be seen in Fig. 24C, fibroblasts did not respond to any of the treatments. Epithelial cells were stimulated by IGF-I or EGF, and there was no increased stimulatory effect when IGF-I and EGF were combined (Fig. 24B). E

Figure 23. Effects of E, R5020, and EGF alone or in different combinations on cell proliferation of mixed, epithelial cell and fibroblast cultures from pregnant mice. Mixed and epithelial cells were plated on collagen, and fibroblasts were plated on plastic in basal media (C),or plus E 23nM, R5020 (R)10nM, EGF 1ng/ml. Each point represents the mean $\pm$ SEM of triplicate determinations from two to three experiments. * $p = 0.05$ that cell numbers in hormone or growth factor treated groups are greater than in control group.

Effects of E, R5020, EGF on Mammary Cells of Pregnant Mice

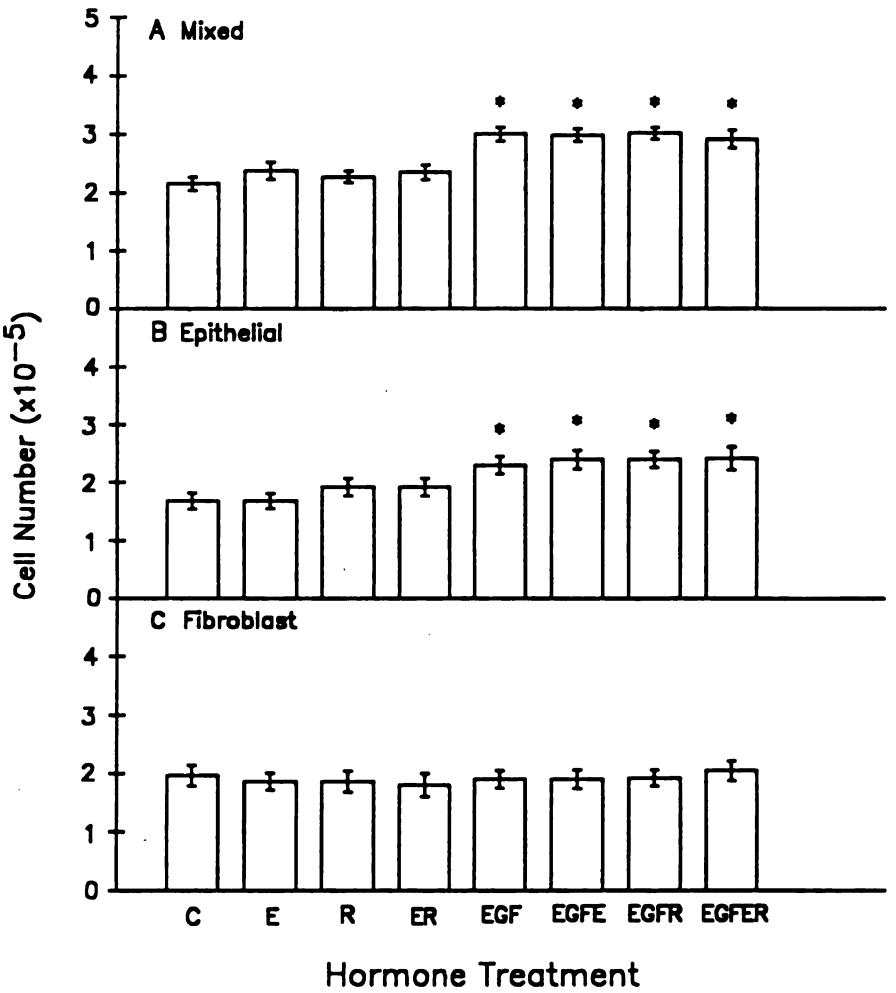
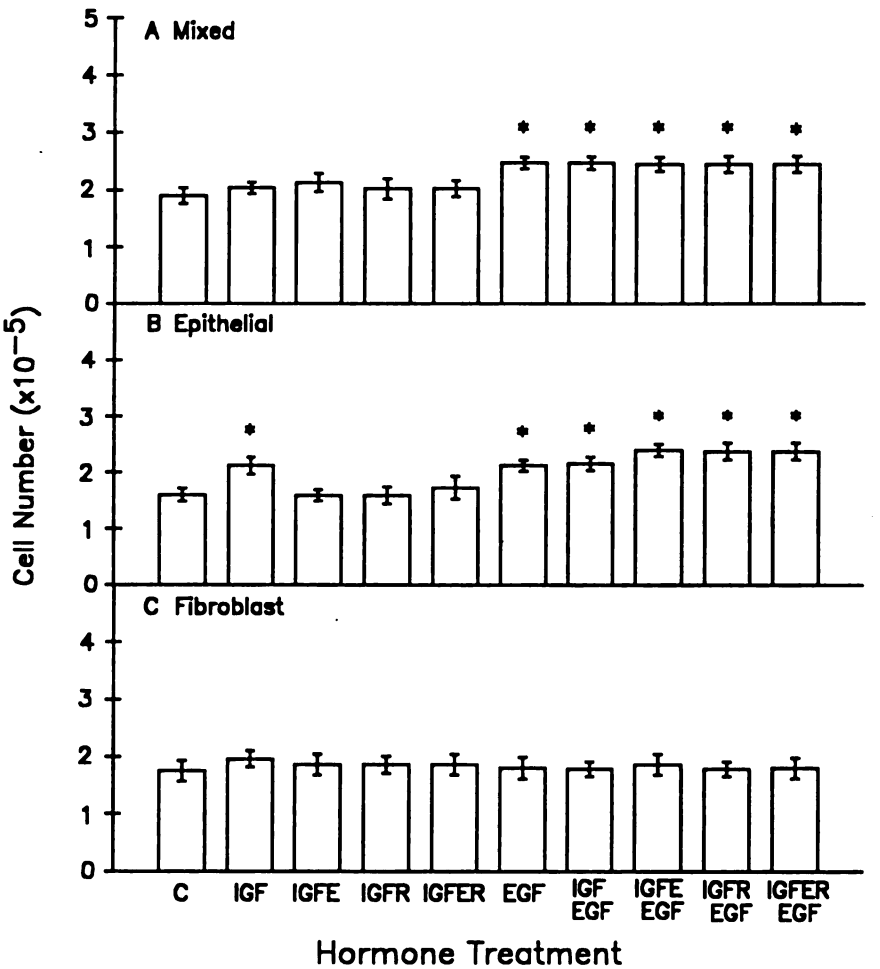


Figure 24. Effects of IGF-I and EGF alone or in different combinations with E, R5020 on cell proliferation of mixed and epithelial cells from pregnant mice. Mixed and epithelial cells were plated on collagen and fibroblasts were plated on plastic in basal media (C) or plus IGF-I 25ng/ml, E 23nM, R 10nM, EGF 1ng/ml. Each point represents the mean $\pm$ SEM of triplicate determinations from two to three experiments. * $p = 0.05$ that cell numbers in hormone or growth factor treated groups are greater than in control group.

Effects of E, R5020, EGF, IGF–I on Mammary Cells of Pregnant Mice



and R5020 appeared to be inhibitory to the effect of IGF-I in epithelial cell cultures. In the mixed cultures only EGF stimulated cell growth (Fig. 24A). IGF-I failed to stimulate mixed cell cultures. It is possible that the presence of fibroblasts in the mixed cultures either diluted or abrogated the epithelial cell response.

The proliferative effect of EGF and IGF-I on epithelial cells vs. fibroblasts in the mixed cell culture was examined further. As shown in Table 3, the presence of IGF-I slightly increased epithelial cell number. Thus, it is possible that the epithelial cell response was not detectable due to dilution by non-responsive fibroblasts, and therefore IGF-I did not show an overall stimulatory effect in mixed cultures. In contrast, EGF caused a significant increase in the ratio of epithelial cells to fibroblasts. Thus, increase in total cell number was accompanied by an increase in epithelial cells and a decrease in fibroblasts, but the decrease of fibroblasts was not significantly different from the control group. Thus these results indicate that the growth effect of EGF in mixed cultures was due to its ability to specifically increase epithelial cell proliferation.

4. Steroid hormone receptor studies

The biologic effects of steroid hormones are believed to be mediated by steroid receptors. ER and PR have been detected in mammary cells in vivo (Haslam & Shyamala, 1981) and in vitro (Edery et al., 1985; Haslam & Levely, 1985). In the present studies, in serum-free media, R5020 stimulated proliferation of nulliparous epithelial and mixed cell cultures. E alone did not show any cell proliferative effects, and E or R5020 or E+R5020 failed to stimulate the growth of cell cultures from mid-pregnant mice. To investigate whether the observed effects of E and R5020 are directly related to the presence

Table 3. Differential effects of EGF and IGF-I on epithelial cells or fibroblasts in mixed cell cultures from pregnant mice.

Treatment[a]	Cell Number (x 10^5)			Ratio of the % Epithelial Cells: Fibroblasts
	Total Cells[b]	Epithelial Cells	Fibroblast	
Control	1.8 ± 0.15	1.26 ± 0.07	0.54 ± 0.06	70:30
IGF-I	1.9 ± 0.22	1.52 ± 0.06*	0.38 ± 0.06	80:20
EGF	2.3 ± 0.18*	1.90 ± 0.10*	0.39 ± 0.05	83:17

[a] Cells were cultured as described in Materials and Methods for 5 days in the presence of basal medium (control), IGF-I (25ng/ml) or EGF (1 ng/ml).

[b] Cells were counted with the aid of ocular grid and a microscope. Each point represents the mean ± SEM of triplicate determinations from three experiments.

* $p < 0.05$ that there is a significant difference from the control group.

or absence of steroid hormone receptors, ER and PR status was examined in cultured mixed and epithelial cells from nulliparous nd mid-pregnant mice. The results obtained demonstrated that there were single classes of high affinity specific E and P binding sites in mixed and epithelial cell cultures from both nulliparous and mid-pregnant mice after 5 days of culture in serum-free basal media (Table 4). Both epithelial and mixed cultures from nulliparous mice contained about 2-fold more ER than cells from mid-pregnant mice. PR content was similar in cultured cells from both nulliparous and mid-pregnant mice. Since ER and PR were present in epithelial and mixed cultures from both nulliparous and mid-pregnant mice, the lack of cell proliferative response of nulliparous cells to E and mid-pregnant cells to E or R5020 does not appear to

Table 4. Specific ^{3}H-estradiol and ^{3}H-R5020 binding sites in mixed and epithelial cell cultures from nulliparous and mid-pregnant mice

	^{3}H-Estradiol Binding		^{3}H-R5020 Binding	
	fmol/mg DNA	Kd (nM)	pmol/mg DNA	Kd (nM)
Nulliparous:				
Mixed[a]	815	1.5	3.56	5.3
Epithelial[b]	800	1.02	3.42	5.3
Mid-Pregnant:				
Mixed	417	2.5	3.22	4.6
Epithelial	428	2.8	3.12	4.1

[a] Mixed: Epithelial cells and fibroblasts mixed cultured together.

[b] Epithelial: Epithelial cell culture.

be due to the lack of the ER or PR in the cell cultures.

In the In vivo studies described in Chapter Two, E (5 ng) induced a 1.5-fold increase of PR in nulliparous mice, and this increase of PR led to the synergistic stimulatory effects on E+R5020 in epithelial cell proliferation. To determine whether E was able to increase PR in the present in vitro serum-free conditions, E inducible PR were examined in nulliparous epithelial and mixed cell cultures. As shown in Table 5, E increased PR about 1.5-fold in both epithelial and mixed cell cultures of nulliparous mice. In nulliparous epithelial cultures,

Table 5. Regulation of ^{3}H-R5020 binding sites in mixed and epithelial cell cultures from nulliparous and mid-pregnant mice.

	Specific ^{3}H-R5020 Binding (pmol/mg DNA)			
	Nulliparous		Mid-Pregnant	
Treatment[a]	Mixed[b]	Epithelial[c]	Mixed	Epithelial
Control	3.26±0.11	2.82±0.31	3.30±0.91	3.42±0.63
E	5.17±0.15*	3.95±0.18*	4.58±0.80	4.13±0.68
IGF-I	6.01±0.52*	4.10±0.09*	4.83±0.50	5.26±0.88
IGF-I+E	5.35±0.50*	4.27±0.56*	5.28±0.78	5.39±0.52
H+Prl+I	4.91±0.37*	4.24±0.08*	5.14±0.66	5.09±0.63
H+Prl+I+E	6.55±0.61*	5.01±0.28*	4.74±0.13	4.81±0.59

[a] E 23nM, IGF-I 25ng/ml, I 6μg/ml, H 10nM, Prl 1μg/ml were added in indicated combinations to the cultures after 24 hours platting. The receptor binding assay was performed on day five of the culture.

[b] Mixed: Epithelial cells and fibroblasts in mixed cultures.

[c] Epithelial: Epithelial cell culture.

* $p < 0.05$ that the treatment groups are higher than the control group.

E did not show a proliferative effect, while R5020 stimulated cell proliferation. The effect of E+R5020 was not higher than R5020 alone. Thus the observed E-induced increase in PR did not enhance epithelial cell proliferation. In the mixed cultures, E alone had no effect, and R5020 alone stimulated only epithelial cell proliferation. E+R5020 stimulated cell proliferation of both

epithelial and fibroblasts. The stimulatory effect of E+R5020 on epithelial cells was about the same as R5020 alone. Thus, no synergistic effect was observed. This implies that E regulated PR may not play any role in E+R5020 mediated stimulatory effect on epithelial cells in the mixed culture.

In mid-pregnant epithelial and mixed cell cultures, E regulation of PR concentration was also examined. As shown in table 5, E did not significantly increase PR. Previously, in studies of mixed and epithelial cells of mid-pregnant mice cultured in media supplemented with serum, PR were increased 1.8 to 2.0- fold by E (Haslam & Levely, 1985; Haslam, 1986). However in those studies the culture medium was also supplemented with hydrocortisone (H), prolactin (Prl) and a high level insulin (I) (6 μg/ml) (Haslam & Levely, 1985; Haslam, 1986). To determine if the lack of H+Prl+I in the media was the cause of the lack of the E induction of PR in the present serum-free conditions, PR concentration was examined in epithelial and mixed cells cultured in presence of H+Prl+I or H+Prl+I+E. The supplementation with H+Prl+I without E enhanced PR concentration in mixed and epithelial cell cultures from nulliparous but not from mid-pregnant mice (Table 5). The magnitude (1.4 to 1.6-fold) of the increase in PR was about the same as the E-mediated increase in nulliparous cultures, and E did not show any further increase in PR when combine with H+Prl+I (Table 5). Thus, it does not appear that the lack of H+Prl+I was responsible for the lack of E induction of PR in mid-pregnant cells cultured in serum-free media herein.

IGF-I has been shown to increase PR levels in the MCF-7 human breast carcinoma cell line (Cho et al., 1994). To investigate if IGF-I can regulate PR in our cell culture system, PR concentration was determined in epithelial and mixed cells cultured in media supplemented with IGF-I. As can be seen in Table 5, IGF-I increased PR levels in nulliparous, but not mid-pregnant

epithelial and mixed cell cultures. There was no further increase in PR when IGF-I combined with E. In this regard, since a high concentration of I (6 μg/ml) was used in H+Prl+I treatment group, it is possible that the increase in PR is being mediated by insulin acting through the IGF-I receptor pathway (Imagawa et al., 1986).

In summary, in epithelial and mixed cell cultures from nulliparous mice, PR can be regulated by E, H+Prl+I and IGF-I. PR in cell cultures from mid-pregnant mice were not increased by any of the hormones and growth factors tested. These results indicate that there are differences in hormonal regulation of PR concentration in nulliparous vs. mid-pregnant mammary cells.

Discussion

I. Hormonal regulation of cell proliferation:

1. studies in nulliparous mammary gland:

a. In vivo studies:

E and P are known to be involved in mammary growth and differentiation. Ovariectomy of adult nulliparous mice, which removes the major source of E and P, caused regression of the mammary ductal tree (Nandi 1958). Using a DNA historadiography technique, it has been shown that injection of E can stimulate DNA synthesis in epithelial cells at the end of mammary ducts (Bresciani, 1968). Injection of P stimulated DNA synthesis in the epithelial cells of the duct proper. Injection of both E and P increased DNA synthesis in ductal epithelial cells and led to the formation of side-branching. Previously, in immature mice, by implanting hormones directly into mammary gland E was demonstrated to increase epithelial DNA synthesis via a local effect in mammary gland (Haslam, 1988). In contrast, the ability of E to increase

epithelial DNA synthesis in adult nulliparous mice appears to be systemically mediated.

In the present study, by implanting a small dose of P alone or combined with E directly into mammary gland, local vs. systemic effects of P have been distinguished. This is based on the observation that in the P-implanted mammary gland, epithelial DNA synthesis was much higher than the contralateral control-implanted mammary gland. E+P synergistically stimulated epithelial cell proliferation, and this effect was also locally mediated via an increase in E-induced PR. In stromal tissue, DNA synthesis was stimulated by E, P and E+P. However, these stimulatory effects appeared to be systemically mediated. These results indicate that different mechanisms of P action may be operating in the two types of mammary tissue.

b. In vitro studies in serum-supplemented media:

Much effort has been devoted to study the mechanisms of action of E and P on nulliparous mammary gland in vitro, so that the in vivo systemic influences and the direct hormone effects can be separated. Previously in organ culture, both E and P have been demonstrated to stimulate rat mammary epithelial tissue growth (Koyama et. al., 1972). E, P and Prl together stimulated proliferation of rat mammary epithelial cells cultured in collagen gels (Pasco et al., 1982). However, in that study, the effects of individual hormones were not evaluated. Since those studies were done in serum-containing media, the interpretation of hormonal effects is complicated by the presence of serum factors.

c. In vitro studies in serum-free media:

By studying the effects of E and P in serum-free conditions, the complications of serum factors are eliminated, and the direct influence of E and

P on target cells can be characterized. In the case of cells from nulliparous mice, P or Prl were shown to stimulate mammary epithelial cells cell growth in collagen gels, and P+Prl had a much higher effect than P or Prl alone. E had no effect either alone or in combination with P and Prl (Imagawa et al., 1985),

Since in vivo epithelial cells exist in association with fibroblasts, fibroblasts may also play a role in some of the hormonal effects on epithelial cells (McGrath, 1983; Haslam, 1986). Thus it is necessary to examine the effects of E and P in epithelial cell cultures and also in mixed cell cultures containing both cell types in serum-free conditions. In the present studies, a serum-free monolayer cell culture condition has been developed to culture mammary epithelial, fibroblast and mixed cultures containing both epithelial cell and fibroblasts. Under this culture condition, P stimulated proliferation of epithelial cells in both epithelium-enriched and mixed cell cultures. E alone did not exhibit any cell proliferative effect. These results are in agreement with the previous in vivo observation that E-mediated increase of epithelial DNA synthesis in adult nulliparous mice appears to be systemically mediated, and involves unknown factors. P alone stimulated epithelial cell proliferation in vitro in serum-free media. This result supports the in vivo observation that P can act directly to stimulate mammary epithelial cell growth. Neither E nor P or E+P stimulated growth in fibroblast-enriched cultures. However, in the mixed cultures, fibroblast growth was stimulated by E+P. This result implies that the presence of epithelial cells is probably necessary for E+P-induced fibroblast proliferation.

As mentioned above, an indirect growth stimulatory effect of E on both epithelial and stromal cells and P in stromal cells has been indicated from the in vivo and in vitro studies. To investigate the possible mediators for these E and P effects, EGF and IGF-I have been examined herein in vitro, in serum-free

cultures. EGF and IGF-I both stimulated epithelial cell proliferation, however, neither EGF nor IGF-I stimulated the growth of fibroblasts. Thus, it is possible that neither EGF nor IGF-I are the in vivo growth mediators of E or P in stromal cells. It is also possible that EGF or IGF-I may not be sufficient to mediate E and P effect, and that additional factors may be required. Furthermore, EGF and IGF-I receptor status needs to be examined in cultured fibroblasts to see if the lack of the responses are due to the lack of the respective receptors. Even though EGF and IGF-I have shown to have direct stimulatory effects in mammary epithelial cell cultures, further studies are needed to determine if they are responsible for the in vivo E-mediated cell proliferation.

2. Studies in mid-pregnant mammary gland:

a. In vivo studies:

Little is known about the mechanisms of E and P action in pregnant mammary gland in vivo. E+P+Prl have been identified as the minimum requirement for lobuloalveolar development, a characteristic of pregnancy, in hypophysectomized+ovariectomized+adrenalectomized mice (Nandi, 1958).

b. In vitro studies in serum-supplemented media:

Previously, E was demonstrated to be able to stimulate proliferation of epithelial cells and stromal cells from early pregnant mice when the two types of cells are in contact (McGrath, 1983). In another study, mammary epithelial cells from mid-pregnant mice were cultured on collagen I coated surface, or in fibroblast conditioned media, or co-cultured with dead or live mammary fibroblasts. Only in presence of live fibroblasts did E stimulate cell proliferation, and the proliferative effect of E was on both epithelial cells and fibroblasts (Haslam, 1986). Thus, the presence of fibroblasts that are

metabolically active seems to be important for E regulated epithelial cell proliferation.

c. In vitro studies in serum-free media:

In serum-free culture conditions, Nandi's group had previously found that epithelial cells from mid-pregnant mice were only slightly stimulated by P, and that E had no effect (Imagawa et al., 1985). In the present studies, experiments were carried out to investigate the effects of E, P and E+P in cultured epithelial, fibroblast and mixed cell cultures from mid-pregnant mice. E, P and E+P did not show any significant proliferative effects in any types of cell cultures. In the mixed cultures where two type cells were present, E failed to stimulate cell proliferation. Thus, the proliferative effect of E which had been demonstrated previously in serum-supplemented media may involve the presence of serum factors. It should also be noted that the hormones I, HC and Prl were also present in the serum-supplemented media in those previous studies. Whether or not these hormones were involved in E-mediated cell proliferation needs further investigation. In contrast to nulliparous cells, P did not stimulate proliferation of mid-pregnant epithelial cells in serum-free media. It is possible that under the present culture conditions, mid-pregnant cells are more differentiated, and are more oriented to differentiated functions such as secretion of milk, and have less proliferative potential overall. Another difference in E and P effects on epithelial cells from mid-pregnant mice and nulliparous mice was that E and/or P inhibited IGF-I effects on epithelial cells from mid-pregnant mice but not from nulliparous mice. Thus, E and P act differently on epithelial cells of mid-pregnant vs nulliparous mice.

II. Steroid hormone receptors:

1. Studies in nulliparous mammary gland:

The effects of steroid hormones are mediated through steroid receptors. In vivo, adult mouse mammary glands contain ER and PR. ER and PR are present in both epithelial and stromal tissue (Haslam & Shyamala,1981). PR in the epithelial compartment can be regulated by E. As demonstrated in Chapter Two, E (5 ng) implanted into the mammary gland increased PR about 1.5-fold in vivo, and this increase of PR led to the synergistic cell proliferative effect of E+P.

In this study in serum-free media, ER and PR were detected in epithelial and mixed cultures. E (23 nM) induced an 1.5-fold increase in PR. However, no cell proliferative effect mediated through E-induced PR was observed. Thus, it is possible that some component of the extracellular microenvironment which exists in vivo may be necessary for the biologic effect of E-induced PR and may not be present in vitro.

2. Studies in mid-pregnant mammary gland:

During pregnancy, there is a decrease in ER and PR. The cause of the decrease in ER is not clear. The decrease in PR might be due to the negative feedback of elevated P concentration (Haslam & Shyamala,1980). In ovariectomized and hysterectomized pregnant mice, E was able to regulate PR concentration in the epithelial compartment.

In in vitro studies, most of the work on ER and PR of mid-pregnant mammary gland were examined in serum-supplemented media. ER and PR were present in mixed cultures, and ER was functional as judged by induction of PR (Haslam & Levely, 1985). However, in epithelial cell cultures, no E induced PR were demonstrated until epithelial cells were cultured on collagen I coated surface, with fibroblast conditioned media, or on a layer of dead or live

fibroblasts. When cultured in those conditions PR were E inducible, and the basal level of PR was significantly increased. Thus, the substratum laid down by fibroblasts and/or soluble factors present in conditioned media appear to be important for the maintenance and synthesis of PR.

In the present studies, ER and PR were examined in epithelial and mixed cells cultured on collagen surface in serum-free media. In this culture condition, ER and PR were present in epithelial and mixed cultures of mid-pregnant mice, however, these cell cultures were less sensitive to E regulation of PR compared to nulliparous cell cultures. This result is different from the results obtained from in vivo studies and from in vitro studies in serum-supplemented conditions. Thus, it is possible that some factor is missing in serum-free conditions that is required for E regulation of PR in mammary epithelial cells of the pregnant stage. Another possibility is that under these culture conditions (i.e. collagen I culture substratum and absence of serum) that the cells are expressing a more differentiated phenotype and resemble more closely the lactational state. In this regard, it has been previously shown in vivo during lactation, that the mammary gland is refractory to estrogenic stimulation of cell proliferation and PR regulation (Shyamala & Haslam, 1980).

Summary:

In this study, by culturing epithelial and fibroblasts separately and culturing them together, the influence of epithelial cell and fibroblast interaction on specific hormone- and growth factor-mediated effects has been demonstrated. Specifically, E+R5020 stimulated proliferation of nulliparous fibroblasts only in presence of epithelial cells. Furthermore, by comparing cells from nulliparous and mid-pregnant mice, differences in hormonal regulation of cells at different developmental stages have also been identified. The PR level in nulliparous

epithelial cells could be regulated by E or H+Prl+I or IGF-I, whereas the regulatory effect of these hormones and growth factors on PR level in mid-pregnant epithelial cells was less pronounced. P directly stimulated proliferation of nulliparous epithelial cells only. In epithelial cells from mid-pregnant mice, no direct cell proliferative effect of P was demonstrated. In contrast to the effects of steroid hormones, the proliferative effects of the growth factors, EGF and IGF-I, on cells of nulliparous and mid-pregnant mice were similar. In view of these findings, it is possible that this culture system will help us to identify the mechanisms underlying cell-cell interactions and their relation to hormone- and growth factor regulated events. Ultimately this may allow us to identify changes in cell-cell interactions which occur in breast cancer and influence tumor growth and invasion.

Summary and Conclusions

In this study the effects of E and P on mammary growth and the relative effects on epithelial and stromal cells have been investigated in vivo. A serum-free monolayer culture system has been established for culturing mammary epithelial cells, fibroblasts and the mixed cells containing both epithelial cells and fibroblasts. The direct effects of E and P on epithelial cells and fibroblasts have been examined in vitro in these serum-free monolayer cell cultures. The influence of epithelial cell and fibroblast interactions on the effects of E and P has been examined in epithelial cell or fibroblast cultures and in the mixed cell cultures where both epithelial cells and fibroblasts are present.

In in vivo studies I found that:

1. The proliferative effects of E on nulliparous epithelial cells in vivo are likely mediated systemically, indicating that E may not be a direct mediator for epithelial cell proliferation.
2. The proliferative effects of P on nulliparous epithelial cells are likely a direct effect.
3. E+P synergistically stimulated nulliparous epithelial cell proliferation, and this synergistic effect is likely due to an E induced increase in PR in epithelial cells.
4. The proliferative effects of E or P on nulliparous stromal cells in vivo is likely mediated systemically, indicating that E or P may not be the direct mediators for stromal cell proliferation.

Correspondingly in vitro:

1. E did not stimulate epithelial cells to proliferate. Since in vitro studies examine direct effect of hormones, this result supports the in vivo observation that E may not be the direct mediator for epithelial cell proliferation.

2. In agreement with in vivo findings, P stimulated the proliferation of nulliparous epithelial cells. Thus, a direct effect of P on mammary epithelial cell proliferation is further supported.

3. E+P did not show synergistic cell proliferative effect in nulliparous epithelial cells. One possible explanation of this lack of a synergistic effect is the reduced induction of PR by E in cells in serum-free media. In addition, some component of the extracellular microenvironment which exists in vivo may be necessary for the biologic effect of E-induced PR, and may not be present in vitro.

4. E or P did not stimulate fibroblasts to proliferate. This result also supports the in vivo findings and suggests that E or P may not be the direct mediators for stromal cell proliferation in vivo. EGF or IGF-I stimulated epithelial cell proliferation, however, EGF and IGF-I failed to stimulate fibroblast cultures. These results indicate that EGF or IGF-I may not be the in vivo mediators of E or progestin on stromal cell proliferation. Alternatively, there might be other factors required for the E or P responses in cell culture.

From the in vitro studies I also observed that epithelial-stromal cell interactions influence hormonal or growth factor responsiveness. For example, E+P stimulated fibroblasts in nulliparous mixed cell cultures, but not in fibroblast enriched cell cultures, therefore the presence of epithelial cells is necessary for this hormone regulated response.

I also found that the developmental state of the gland (nulliparous vs. pregnant) can modulate hormonal responsiveness. The proliferation of pregnancy-derived epithelial cells and fibroblasts was only stimulated by growth factors, and failed to be stimulated by steroids. This did not appear to be due to the lack of ER or PR. The lack of responsiveness to E and P suggests that increased differentiation results in a loss of responsiveness to E+P.

In conclusion, this study has advanced our understanding of the growth regulation of mammary epithelial cells and fibroblasts by E and P, and increased our knowledge about the growth control of mammary cells at different developmental stages. By separately culturing epithelial cells and fibroblasts which coexist in mammary ducts in vivo, the differential effects of E, P and growth factors EGF and IGF-I on epithelial cells and fibroblasts have been identified. The influence of epithelial cell-fibroblast interactions on E, P and growth factor-mediated effects has been demonstrated by comparing the effect of E, P and growth factors on mixed cell cultures containing both epithelial cells and fibroblasts to epithelial or fibroblast enriched cell cultures. The changes in mammary growth control at different developmental stages have been revealed by comparing the effect of E, P and EGF and IGF-I on mammary cells from nulliparous and mid-pregnant mice.

This study opened an avenue for future studies toward discovering the effects of hormones and growth factors on other types of mammary cells, such as adipocytes, and the influence of interactions of different cell types on hormone and growth factor mediated events. In addition, the findings herein on growth regulation of normal mammary cells are of value by providing reference for identifying abnormal changes in regulation of E, P and growth factors and cell-cell interactions in hormone and growth factor mediated effects in breast cancer. Finally, since breast tumors increase with age and have their highest incidence in menopause, an understanding of the changes in growth control by hormones and growth factors on mammary cells at different developmental stages might be helpful in the further development of endocrine strategies for breast cancer treatment and prevention.

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