THE ROLE OF POLYPEPTIDE GROWTH FACTORS IN THE PROLIFERATION OF EPITHELIAL CELLS DERIVED FROM HUMAN MAMMARY GLAND

by

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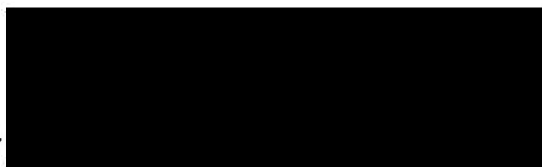

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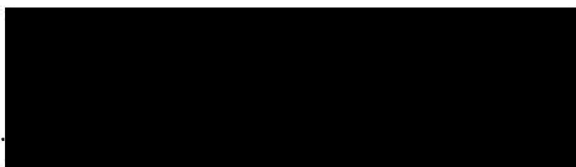

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ABSTRACT

Polypeptide growth factors play major roles in cell proliferation in culture and *in vivo*. Normal cells require more than one growth factor for proliferation *in vitro*. However, recent findings have shown that some normal cells, especially epithelial cells, produce growth factors to which they themselves respond and thus, have decreased requirement for the exogenous growth factors to maintain proliferation *in vitro*. On the other hand, many types of tumor cells over-produce growth factors and their receptors, which is believed to lead to a growth advantage, a cardinal feature of malignant cells. Therefore, autocrine activity of growth factors is of importance in controlling both normal and malignant proliferation.

The studies presented in this thesis have concentrated on human mammary epithelial cells (HMECs). Both their responsiveness to and production of growth factors which may be involved in growth regulation of these cells have been investigated. Differences in these aspects between normal and tumor-derived mammary epithelial cells have been addressed. In initial work, the role of basic fibroblast growth factor (bFGF) in proliferation of mammary epithelial cells and mammary tumorigenesis was investigated. bFGF was produced by all strains of normal HMECs tested while only one out of four tumor cell lines produced bFGF at a similar level to that in normal cells. Thus, expression/overexpression of bFGF does not appear to be a common mechanism in mammary tumor development.

Normal HMECs proliferated in a medium lacking epidermal growth factor (EGF) or FGF-like growth factors when plated at high density, although it has been shown that these cells absolutely require EGF for growth at clonal density. These results suggest that normal HMECs are capable of making some autocrine growth factor(s) which may stimulate their own growth. EGF, but not bFGF stimulated growth of these cells,

indicating that bFGF is unlikely to be a primary autocrine growth factor for these cells. The EGF-independent growth of normal HMECs was inhibited by treatment with heparin or by antibody-mediated EGF receptor blockade, highly suggesting amphiregulin (AR), a heparin-regulated EGF family member, as an autocrine growth mediator. This hypothesis is further supported by the fact that both AR mRNA and protein are produced in high abundance by normal HMECs. Another EGF family member, transforming growth factor alpha (TGF-α), is also produced by normal HMECs. High levels of AR and TGF-α mRNA expression in EGF-independent cultures of normal HMECs were maintained by activation of the EGF receptor by autocrine-acting ligands. These results indicated that AR and TGF-α may act in concert in controlling proliferation of normal HMECs in an autocrine fashion. In contrast to the normal cells, all mammary tumor derived cell lines tested had low to undetectable levels of AR mRNA. The expression of AR mRNA in certain tumor cells was greatly elevated by phorbol ester (PMA) whereas in normal HMECs, PMA decreased AR mRNA levels. Furthermore, a transcript of an AR-related gene was detected in all tumor cell lines tested but not in normal HMECs, raising the possibility that this gene product may be involved in mammary tumorigenesis and/or may be used as a marker for mammary transformation.

INTRODUCTION

A. Development of Human Mammary Gland

Mammary gland is perhaps the only organ that is not fully developed at birth (Russo and Russo, 1987). Although immaturity at birth is common in most systems, no other organs present such dramatic changes in size, shape, and function as does the mammary gland during post-natal growth and development.

Mammary gland development can be divided into four stages: prepuberty, puberty, pregnancy, and lactation (Russo and Russo, 1987). Development during prepuberty does little more than keep pace with the general growth of the body. During this time, the gland is composed mainly of ductal trees lined by epithelial cells and surrounded by connective tissue. At puberty, these ducts begin to grow and subdivide rapidly, forming club-shaped terminal end buds. The terminal end buds give rise to new branches and small ductules or alveolar buds. Clustered alveolar buds around a terminal duct form a lobule. During pregnancy, the mammary gland attains its maximum development. The early stage of pregnancy is characterized by growth, consisting of proliferation of the distal elements of the ductal tree. The lobules are further enlarged and increased in number. In the late stage of pregnancy, the alveolar cells of the ductal trees begin to secrete milk, an event that marks terminal differentiation. The mammary gland continues secreting milk until weaning occurs when accumulation of milk in the ducts and cytoplasm of alveolar cells inhibits further milk synthesis (Salazar and Tobon, 1974; Russo and Russo, 1987). The alveolar buds undergo atrophy and regeneration of the connective tissue occurs together with renewed proliferation of epithelial cells at terminal end of ductal trees. Finally, mammary involution begins as a consequence of decreased ovarian function. Although there is no rapid proliferation of epithelial ductal structure during this stage, mammary involution is important because of the high incidence rate of breast cancer during this

period (Leis, 1978).

Almost all mammary neoplasms are derived from ductal epithelial cells. About 70-80 percent of invasive primary breast neoplasms belong to the form called infiltrating ductal carcinoma which is derived from mammary epithelial cells and is localized at the terminal end of the ductal tree (Reichert *et al.*, 1988). The tumor is composed of primitive tubules, glandular formations and sheets of malignant epithelial cells that infiltrate a collagenized stroma.

B. Growth Control in Normal and Malignant Mammary Epithelium

Endocrine mechanisms

The ovarian steroid hormones, especially estrogen, are believed to be the most critical elements for the growth and development of the mammary gland as well as mammary neoplasms. Many studies have demonstrated that estrogen plays a key role in mammary growth *in vivo.* Estrogen has two prominent effects: 1) stimulation of growth of ductal epithelium and 2) increase in progesterone receptor concentration (for review see Leung, 1982). This latter effect may be the basis for the observed synergistic action of estrogen and progesterone on lobuloalveolar development (Nandi, 1958). The question of whether the mitogenic effects of estrogen on epithelial cells are direct or indirect has not been resolved. Numerous studies have shown that, instead of directly acting on epithelial cells and stimulating the proliferation, estrogen effects are mediated by multiple endocrine hormones, local mediators, and perhaps stromal-epithelial interaction. Bresciani showed that injection of estrogen in ovariectomized adult mice caused stimulation of DNA synthesis in quiescent ductal end epithelium and effected ductal elongation (Bresciani, 1968). This result was not obtained when similar experiments were performed in animals whose pituitary glands as well as ovaries had been removed (Nandi, 1958; Lieberman *et al.*, 1978). Such experiments suggested that estrogen does not act directly on mammary epithelium, but indirectly, through endocrine-acting substances.

Other studies have indicated that the indirect action of estrogen might be mediated through some locally active factors. Application of estrogen to one nipple area of the mammary glands of monkeys, rabbits, and guinea pigs stimulated epithelial growth at the site of application, but not in contralateral untreated glands (Chamberlin *et al.*, 1941; Nelson, 1941). Experiments in which estrogen-containing pellets were implanted in the developing mammary gland of mice support a local, but not a systemic-acting mechanism for estrogen because only proliferation of local end ductal epithelium was observed (Daniel *et al.*, 1987). *in vitro* studies with normal mammary epithelial cell cultures have shown that estrogen induced epithelial proliferation could only be detected when epithelial cells were cocultured with mammary stromal cells (McGrath, 1983; Haslem and Levely, 1985). These results are consistent with the notion of an indirect and local mechanism of estrogen action involving a close communication between stromal and epithelial components.

Studies using *in vitro* organ culture and epithelial cell culture have begun to shed light on the question of the nature of local factors mediating estrogen action. Insulin, prolactin, aldosterone, and hydrocortisone, along with an extract of estrogen-progesterone-primed gland, could induce *in vitro* lobuloalveolar development of an organ culture which had been "primed" with estrogen and progesterone *in vivo* (Ichinose and Nandi, 1966). Extracts of the estrogen-progesterone pretreated mammary glands contained an epidermal growth factor (EGF) receptor binding component and both EGF and transforming growth factor alpha (TGF-α) were able to substitute for the activity supplied by the glandular extract (Vonderhaar, 1984). Other studies have demonstrated that EGF and TGF-α both promoted local lobuloalveolar development *in vivo* when slow-release pellets were implanted into the mammary gland (Vonderhaar, 1987; Vonderhaar, 1988). *In vitro* studies on cell cultures of normal mammary epithelial cells also revealed the dependence of cell proliferation on the presence of factors such as EGF, insulin and hydrocortisone in the culture medium (Hammond *et al.*, 1984; Stampfer and Bartley,

1987). Taken together, these results imply that the interaction of multiple hormones is required to stimulate the growth of normal mammary gland. The mitogenic effect of estrogen is most likely to be mediated by some locally active factors which are secreted by stomal cells or possibly mammary epithelial cells themselves. Polypeptide growth factors are likely candidates to act as estrogen mediators.

Estrogen regulation of the growth of malignant mammary epithelial cells is basically a "modified remnant" of the normal regulatory mechanisms for mammary epithelial proliferation and differentiation (Lippman and Dickson, 1989). Estrogen has been shown to exert a considerable number of influences, both *in vivo* and *in vitro*, which may alter mammary tumor progression. Direct effects of estrogen on growth regulation include induction of enzymes, such as thymidine kinase (Kasid *et al.*, 1986), dihydrofolate reductase (Cowan *et al.*, 1982) and collagenolytic enzymes (Butler *et al.*, 1979), and induction of other proteins thought to be involved in mediating mitogenic, metastatic, and differentiated status. Indirect effects of estrogen on growth regulation are through modulating the production of growth mediators secreted by tumor cells themselves or by nearby tissues. Estrogen inducible endocrine-acting mitogens for mammary tumor cells, named estromedins, might be secreted by pituitary gland or other organs (Eidne *et al.*, 1985; Sirbasku, 1978; Sirbasku *et al.*, 1984). Studies conducted on MCF-7 cells, a well-characterized mammary tumor cell line, have revealed that estrogen treated MCF-7 cells produce increased amounts of TGF-α which is a mitogen for both anchorage-dependent or independent growth of these cells *in vitro*. In addition to its direct and indirect roles, estrogen may also act by allowing mammary tumor cells to overcome growth-inhibitory agents in their environment or by synergy with other stimulatory agents (Soto and Sonnenschein, 1987).

Dickson and Lippman have proposed a model describing possible roles of estrogen and other related factors in the growth control of mammary tumors (Dickson and

Lippman, 1987). In this model, they presented three hypotheses. Firstly, mammary tumor cells may be subject to direct endocrine influences of estrogen and other hormones. Estrogen may directly interact with these cells through its receptor and alter the expression of genes which are involved in the growth. Secondly, part of this alteration in gene expression induced by estrogen results in alterations in secretion of specific proteins. Some of these secreted proteins serve as autocrine growth factors which stimulate or inhibit the growth of these tumor cells. Some of the secreted proteins serve as paracrine growth factors which cause proliferation of stromal cells and neovascularization. Thirdly, some mammary tumors, characterized by loss of estrogenic control of growth, constitutively secrete an increased level of autocrine and paracrine growth factors without the necessity for estrogen activity. In section B and C, I will review evidence and results from many studies that support these hypotheses. Some of the results may also reflect the growth regulation mechanisms for normal mammary epithelial cells.

Autocrine and paracrine mechanisms

A group of polypeptide growth factors play major roles in autocrine and paracrine cell growth in culture and probably also *in vivo* (Goustin *et al.*, 1986). These growth factors presumably diffuse short-range within intercellular spaces and act locally. They modulate cell proliferation through binding to their specific cell surface receptors. Cells may produce a growth factor to which they respond (autocrine activity) or they may produce growth factors which modulate the growth of adjacent cells (paracrine activity). Until recently, it was believed that non-transformed cells required more than one exogenously acting growth factor for proliferation *in vitro* (Goustin *et al.*, 1986). Although loss of requirement for specific growth factors due to endogenously autologous growth factor synthesis is a common finding in many types of tumor cells (Kaplan *et al.*, 1982; Moses *et al.*, 1978), a number of studies have shown that normal cells, especially epithelial cells, are capable of autonomous growth indicating that normal cells may also produce growth

factors to which they themselves respond (Shipley *et al.*, 1989; Cook *et al.*, 1991a; Valverius *et al.*, 1989; Cook *et al.*, 1991b).

Three sets of observations support an autocrine/paracrine mechanism for growth regulation of human mammary epithelium. Firstly, various growth factors have been shown to modulate the growth of mammary epithelial cells in culture including EGF/TGF-α, insulin-like growth factor-1 (IGF-1), transforming growth factor-ß (TGF-ß) and basic fibroblast growth factor (bFGF) (Lippman and Dickson, 1989). EGF/TGF-α was also indicated as a mitogen for ductal epithelium *in vivo* (see section A). Secondly, both normal and tumor cells are found to synthesize specific growth factors and some of these growth factors are also found in the medium conditioned by these mammary cells (Dickson and Lippman, 1987). Conditioned medium prepared from a mammary tumor cell line was capable of stimulating DNA synthesis and increasing cell number of the same cells (Dickson *et al.*, 1986b; Vignon *et al.*, 1983). The same conditioned medium given by continuous infusion *in vivo* to the nude mouse stimulated tumorigenesis by this cell line (Dickson *et al.*, 1986a). Thirdly, mammary stromal cells appears to produce factors which promote the growth and tumor formation of malignant mammary epithelium (Camps *et al.*, 1990; McGrath, 1983).

C. Specific growth factors

Insulin and IGFs

The insulin family of growth factors is composed of a complex group of cross-reacting ligands and receptors. Mature insulin is composed of two polypeptide chains that are disulfide-linked. It is primarily synthesized in the islets of Langerhams in the pancreas. In contrast, the single polypeptide chain (7.5 kD) of IGF-1 and IGF-2 are synthesized in many tissues (Lippman and Dickson, 1989). Each ligand appears to have a species of receptor to which it preferentially binds, although at high ligand concentration cross-reactivity with other receptors in the same family is seen (Massague and Czech,

1982). The effect of this cross-reactivity was demonstrated by the fact that supraphysiological concentrations of insulin can replace IGF-1 requirement in defined medium by binding and stimulating the IGF-1 receptor (Shipley *et al.*, 1989; Van Wyk *et al.*, 1975).

IGF-1 (or supraphysiological concentrations of insulin) is required for the growth of normal mammary epithelial cells in defined medium (Hammond *et al.*, 1984). It was reported that IGF-1 also stimulates proliferation of some tumor-derived mammary epithelial cells in culture (Huff *et al.*, 1986). IGF-1 mRNA was detected in a majority of human mammary tumors (Yee *et al.*, 1989). Furthermore, an IGF-1 related species has been detected using radioimmunoassay in media conditioned by mammary tumor cell lines (Huff *et al.*, 1986). Recent studies have shown that an antibody blocking the IGF-1 receptor inhibits the growth of some mammary tumor cells both *in vitro* and *in vivo* (Artega *et al.*, 1988; Rohlik *et al.*, 1987), indicating a possible autocrine role for IGF-1.

IGF-2 has been shown to be a potent mitogen for a number of mammary tumor cell lines and its transcript was detectable in some mammary tumors (Lippman and Dickson, 1989). Although IGF-2 has been postulated to be a growth regulator during fetal development, it has no defined function in the adult. A number of studies have demonstrated that at least some of the biological activities of IGF-2 are mediated by the IGF-1 receptor (Kiess *et al.*, 1987; Roth, 1988; Cullen *et al.*, 1990). It has been suggested that the IGF-1 receptor plays an important role in mediating the mitogenic effects of both types of IGFs in mammary epithelial cells (Cullen *et al.*, 1990).

Platelet-derived growth factor (PDGF)

PDGF is a heterodimeric protein of approximately 30 kD which is found in high concentrations in platelets. PDGF is a potent mitogen produced by many transformed mesenchymal cells. It is thought to function in a paracrine mode to stimulate angiogenesis, stromal proliferation, and chemotaxis (Ross *et al.*, 1986). Many mammary tumor-derived

cell lines secrete a PDGF-related activity which can be detected by a "competency" assay using 3T3 fibroblasts (Rozengurt, 1986) and by immunoprecipitation with PDGF antiserum (Bronzert *et al.*, 1987). Transcripts of both PDGF A and B chains are observed in these cells. The significance of the production of PDGF growth factors by mammary tumor cells is not fully understood. Because the PDGF receptor, a ligand-inducible tyrosine kinase, has not been detected in any of the mammary tumor cell lines (Lippman and Dickson, 1989), it is unlikely that PDGF would act as an autocrine growth factor for these cells. It has been proposed that PDGF could act in a paracrine fashion in promoting the growth of mammary fibroblasts and stimulating fibroblasts to produce epithelial growth factors (Bronzert *et al.*, 1987). Thus, PDGF may play an important role in epithelial-stromal interaction during the process of normal and neoplastic growth.

TGF-ß

TGF-ß is now recognized as a multifunctional regulator of cell growth and differentiation in both normal and transformed cells (Roberts and Sporn, 1988). It is a 25 kD disulfide-linked homodimeric molecule (Derynck *et al.*, 1985) described initially as a factor which could stimulate the growth of normal fibroblasts in soft agar (Moses *et al.*, 1981; Roberts *et al.*, 1981). It has been shown that TGF-ß inhibits the growth of normal mammary epithelial cells *in vitro* (Stampfer *et al.*, 1989) while it induces these cells to synthesize milk fat globule antigen (Lippman and Dickson, 1989). *in vivo* studies in neonatal mouse implicated TGF-ß as a potent inhibitory factor for the mammary ductal development (Lippman and Dickson, 1989). TGF-ß has the same inhibitory activity on mammary tumor-derived cells grown in culture. All tumor cell lines reported express TGF-ß mRNA (Knabbe *et al.*, 1987; Wang and Hsu, 1986; Derynck *et al.*, 1987) and a TGF-ß-related activity was identified in medium conditioned by some mammary tumor cell lines using a receptor binding assay, immunoprecipitation with an antibody specific for TGF-ß, and a fibroblast transformation assay (Knabbe *et al.*, 1987; Derynck *et al.*,

1985). The production of TGF-ß by MCF-7 cells has been reported to be down-regulated by growth stimulators, estrogen and insulin, whereas it is up-regulated by growth-inhibitory antiestrogens and glucocorticoids (Knabbe *et al.*, 1987). Although the role of TGF-ß in normal and neoplastic states is yet to be elucidated, these studies suggest that TGF-ß production may be correlated with the growth state of the cells and thus, TGF-ß may play a role, along with other hormones and growth factors, in the delicately balanced process of mammary development.

Fibroblast growth factor (FGF) family

Seven genes have been found to encode proteins that have closely related structures and have the property of binding to the sulfated polysaccharide, heparin (Burgess and Maciag, 1989). The FGF family includes: basic FGF (bFGF) (Abraham *et al.*, 1986); acidic FGF (aFGF) (Gimenez-Gallego *et al.*, 1987); *hst*/Kaposi FGF (*hst*/KFGF) (Bovi *et al.*, 1987; Yoshida *et al.*, 1987); the *int*-2 locus (*int*-2) (Dickson and Peters, 1987); FGF-5 (Zhan *et al.*, 1988); FGF-6 (Marics *et al.*, 1989); and keratinocyte growth factor (KGF) (Finch *et al.*, 1989). FGFs have been shown to have a broad spectrum of biological effects on the growth and differentiation of mesodermally and neuroectodermally-derived cells in culture and *in vivo* (Burgess and Maciag, 1989).

aFGF and bFGF were the first members of this family to be discovered and are the most well-characterized. The AUG-initiated primary translation products of these genes are 155 amino acids in length (18 kD). In addition to the 18 kD form, higher molecular weight forms of bFGF have been reported in normal, tumor and transfected cells (Root and Shipley, 1991; Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). These higher molecular forms are believed to arise from translational initiation at CUG codons 5' to the AUG (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). The primary translation products of aFGF and bFGF do not contain a hydrophobic signal sequence. It is not clear how these factors are externalized from cells to interact with their cell surface receptors

and binding molecules. It was proposed that they either could be release by leakage from damaged cells or secreted by an uncharacterized mechanism (Thomas, 1987). Among other members in the family, *int*-2 also does not contain a typical amino-terminal leader sequence. In contrast, protein products of other members (*hst*/KFGF, FGF-5, FGF-6, and KGF) all have a classical hydrophobic signal sequence and would be expected to be secreted from cells by a conventional signal peptide-dependent mechanism (Thomas, 1987).

Four genes have now been identified that encode receptors for the FGF family of ligands (FGFR 1-4) (Ruta *et al.*, 1989; Dionne *et al.*, 1990; Bottaro *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991). The FGF receptors share a common structure of single membrane spanning polypeptides with 2 or 3 immunoglobin-like domains in their extracellular region, a transmembrane domain and an intracellular tyrosine kinase region. FGFs bind to these receptor with high affinity (Burgess and Maciag, 1989; Moscatelli, 1987; Bottaro *et al.*, 1990) and ligand binding specificity varies between different receptors.

aFGF and bFGF are mitogenic for most, if not all, non-terminally differentiated cells of both embryonic mesodermal and neuroectodermal origin (Thomas, 1987). Recent studies have demonstrated that FGFs are also mitogenic for ectodermally-derived cells such as human epidermal keratinocytes (Shipley *et al.*, 1989) suggesting that FGFs may be important in the regulation of epithelial cell growth and differentiation. Both aFGF and bFGF are expressed in a variety of cells including normal and tumor-derived cells (Root and Shipley, 1990; Lobb *et al.*, 1986; Moscatelli *et al.*, 1986; Presta *et al.*, 1986; Schweigerer *et al.*, 1987). Their strong mitogenic activities on vascular endothelial cells makes them potent inducers of blood vessel growth *in vivo*, a process termed angiogenesis (Folkman and Klagsbrun, 1987). FGFs have also been shown to induce the expression in endothelial cells of proteolytic enzymes such as plasminogen activator and collagenase

(Thomas, 1987). In addition to their function in the normal growth and development, FGFs have been implicated as potential autocrine growth factors involved in neoplastic growth, and their activities for endothelial cells may account for tumor angiogenesis and metastasis (Thomas, 1987).

It is not clear whether FGFs play a role in the growth control of normal and malignant mammary epithelial cells. bFGF has been shown to stimulate the growth of cultured normal mouse mammary epithelial cells (Levay-young *et al.*, 1989). bFGF has also been demonstrated to be mitogenic for the human mammary tumor cell lines, MCF-7 and T47D cell (Karey and Sirbasku, 1988) and cultured epithelial cells derived from both normal and malignant breast biopsies (Takahashi *et al.*, 1989). In a recent study, Gomm and coworkers have localized bFGF only in the myoepithelial cells of the normal human mammary gland by immunohistochemical staining (Gomm *et al.*, 1991). Whether this myoepithelial derived bFGF could act on adjacent epithelial cells and stimulate their proliferation remains to be determined. It is possible that the other members in the FGF family, most of which were isolated from human tumors and are transforming *in vitro*, may play a role in mammary malignancy. Theillet *et al.* (Theillet *et al.*, 1989) has found that the *hst* and *int*-2 oncogenes are coamplified in 17% of human mammary tumors and in situ hybridization showed them to be present in the tumor cells themselves.

EGF family

Five members of the EGF family of growth factors are found in human cells/tissues. These include EGF, TGF-α and newly isolated amphiregulin (AR), Cripto and heparin-binding EGF-like growth factor (HB-EGF) (Mroczkowski and Carpenter, 1988; Derynck, 1988; Plowman *et al.*, 1990a; Cook *et al.*, 1991b; Higashiyama *et al.*, 1991; Ciccodicola *et al.*, 1989). The EGF-like domain of these ligands is characterized by 6 cysteine residues that are spaced over a 35-40 amino acid region of the molecules. The interaction of these cysteine residues forms 3 disulfide bonds and three loop regions

characteristic of all of these growth factors. However, the overall conservation of amino acid sequence is limited (Mroczkowski and Carpenter, 1988; Derynck, 1988; Plowman *et al.*, 1990a; Higashiyama *et al.*, 1991; Ciccodicola *et al.*, 1989). EGF, TGF-α, AR and HB-EGF are synthesized as membrane-bound precursor molecules and the mature forms of these growth factors are proteolytically cleaved from the extracellular regions of these precursors (Mroczkowski and Carpenter, 1988; Derynck, 1988; Plowman *et al.*, 1990a; Higashiyama *et al.*, 1991). Cripto mRNA does not encode a hydrophobic secretory signal sequence nor a hydrophobic sequence indicative of a transmembrane domain and is apparently not secreted (Ciccodicola *et al.*, 1989). The mature form of TGF-α is a 50 amino acid unglycosylated molecule that resembles mature EGF in its structure, EGF receptor binding and biological activity (Derynck, 1988). The mature forms of AR and HB-EGF are larger, contain glycosylations and a highly hydrophilic domain in their N-terminal region (Plowman *et al.*, 1990a; Higashiyama *et al.*, 1991). Both of these molecules bind immobilized heparin (Cook *et al.*, 1991b; Higashiyama *et al.*, 1991) and the biological activity of AR has been shown to be negatively regulated by binding to soluble heparin and other sulfated polysaccharides (Cook *et al.*, 1991b; Cook *et al.*, 1992b). AR and HB-EGF both bind to EGF receptor, however, a number of discrepancies in the ability of these molecules to fully compete for ^{125}I-EGF binding have been noted (Higashiyama *et al.*, 1991; Shoyab *et al.*, 1989).

The EGF receptor family is structurally characterized by a single polypeptide having an extracellular ligand-binding domain, a short hydrophobic transmembrane region and a single intracellular domain with tyrosine kinase activity and sites for autophosphorylation. Besides the well-characterized EGF receptor type 1 (EGF-R1) which binds EGF and TGF-α, there are two genes encoding proteins that have homology to the EGF-R1. The human EGF receptor 2 (HER2 or EGF-R2) is the human homologue of the rat c-*erb*-B2 and is found to be amplified as well as overexpressed in many human

adenocarcinomas (Semba *et al.*, 1985; King *et al.*, 1985). Ligand(s) for the EGF-R2 have not been completely described, however, a heparin-binding glycoprotein(s) has been suggested as a ligand for this receptor (Lupu *et al.*, 1990; Yarden and Peles, 1991). Another EGF-R, *erb*-B3 or EGF-R3, was recently isolated (Plowman *et al.*, 1990b; Kraus *et al.*, 1989) and no ligand has been identified for the EGF-R3.

EGF and TGF-α are the most studied growth factors in the EGF family. Studies on the role of EGF/TGF-α in the growth and development of mammary epithelial cells have shown that these growth factors induce lobuloalveolar development *in vivo* as well as stimulate the proliferation of normal mammary epithelial cells (HMECs) in culture (Vonderhaar, 1984; Valverius *et al.*, 1989). Normal HMECs coexpress high levels of both TGF-α and EGF-R1 mRNA and protein (Zajchowski *et al.*, 1988; Bates *et al.*, 1990; Valverius *et al.*, 1989), and the levels of TGF-α mRNA expression appeared to be dependent upon EGF receptor occupancy (Bates *et al.*, 1990). These results suggest an autocrine role for TGF-α/EGF-R pathway in controlling the normal growth of HMECs *in vitro*, although it has not been directly demonstrated.

Like many other malignant human tissues/cells, tumor-derived mammary epithelial cells produce TGF-α. TGF-α mRNA has been detected in MCF-7 and other tumor cell lines as well as in mammary tumors (Bates *et al.*, 1988; Zajchowski *et al.*, 1988). An increased level of TGF-α mRMA in MCF-7 cells was noted when cells were treated with estrogen (Bates *et al.*, 1988). A 30 kD species of TGF-α-like molecule was identified in the medium conditioned by mammary tumor cells (Bates *et al.*, 1988). It is not yet clear whether this protein is related to the TGF-α precursor protein, a product of alternative mRNA splicing or a product of a novel TGF-α-related gene. The potential role of TGF-α in mammary tumor growth has been studied. It was noted that antibodies directed against either TGF-α or its receptor (EGF-R1) cause both anchorage-dependent or -independent growth suppression of MCF-7 *in vitro* (Bates *et al.*, 1988) indicating that TGF-α is a likely autocrine growth factor in mammary tumors.

The role of AR in the growth regulation of normal and malignant human mammary epithelial cells remains to be elucidated. AR was originally isolated from a phorbol ester-treated mammary tumor cell line, MCF-7 (Shoyab *et al.*, 1988). The mitogenic activity of AR appears to be cell type specific. AR stimulates the growth of normal human keratinocyte (Cook *et al.*, 1991b), fibroblasts and some tumor cell lines, but inhibits the proliferation of some other tumor cell lines (Shoyab *et al.*, 1988). A more recent study on human ovarian cells has suggested that the responsiveness of cells to AR may depend upon the amount of AR to which the cells are exposed (Johnson *et al.*, 1991). AR is expressed in variety of normal tissues (Plowman *et al.*, 1990a) and cells cultured *in vitro* including human keratinocytes and mammary epithelial cells (Cook *et al.*, 1991b). The level of AR mRNA expressed by normal HMECs appears to be higher than that by mammary tumor cells suggesting an important role of AR in normal epithelial growth. On the other hand, AR mRNA expression is markedly elevated in hyperproliferative and neoplastic human epithelium derived from other tissues such as psoriatic lesions, colon carcinomas and stomach carcinomas (Ciardiello *et al.*, 1991; Cook *et al.*, 1992a).

D. Thesis rationale

The purpose of these investigations was to understand the mechanisms by which polypeptide growth factors control proliferation in normal human mammary epithelial cells and how these mechanisms may be altered in the process of malignant transformation. The document is composed of three manuscripts, one of which has been published, one in press and one in preparation. In these studies, I have focused on the responsiveness of human mammary epithelial cells in culture to growth factors and their ability to express growth factors which may be involved in the growth regulation in these cell. The difference in these aspects between normal and malignant cells has also been investigated.

The first manuscript describes the role of bFGF in the growth of both normal and tumor-derived mammary epithelial cells. bFGF has been implicated in other human

tumors as an autocrine growth factor as well as an angiogenetic factor which is important in development of solid tumors (see Section C). bFGF is also a potent mitogen for epithelial cells derived from human skin. However, it is not clear whether bFGF plays a role in the growth control of human mammary epithelial cells. In this paper, I have described the expression of bFGF at both transcriptional and translational level in different strains of normal HMECs and in a number of mammary tumor-derived cell lines. The mitogenic activity of bFGF on these cells was also addressed. Finally, whether bFGF expression/overexpression or increased sensitivity to bFGF could play a role in the development of human mammary tumors was discussed.

The role of AR in controlling the autonomous growth of normal HMECs in culture is described in the second manuscript. In the first part of my project described above, I found that normal HMECs, when plated at high density, proliferate in medium lacking EGF-like or FGF-like growth factors. This observation supports the notion that normal human epithelial cells can proliferate autonomously via the production of and response to growth factors they themselves produce. AR was hypothesized as an autocrine growth factor responsible for the autonomous growth of normal HMECs because of the following: 1) AR mRNA appeared to be expressed in these cells (Cook *et al.*, 1991b); and 2) AR is an autocrine growth factor for epithelial cells derived from human skin (Cook *et al.*, 1991b). The studies presented in the second manuscript were designed to test this hypothesis. Several strains of normal HMECs were examined for AR mRNA expression and the presence of AR protein in the conditioned medium was determined. The effect of heparin, an inhibitor for AR mitogenic activity (see Introduction), and a blocking antibody specific for the EGF-R1 were studied. The possible interaction between two EGF-like growth factors, AR and TGF-α, and their roles in regulation of normal growth was discussed.

In the third manuscript, I described how AR, as well as TGF-α, mRNA expression is regulated by EGF-R occupancy, and investigated whether AR and/or TGF-

α induce AR and TGF-α message in normal HMECs. This part of the work was an attempt to understand how AR and TGF-α may, in a coordinated fashion, regulate the growth as well as their own expression. I have also examined AR mRNA expressed in mammary tumor cell lines and the discrepancies in levels and regulation of expression between normal and tumor cells were discussed in an initial effort to understand the underlying mechanisms of regulation of this gene and its function in tumor cells.

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Expression of Multiple Species of Basic Fibroblast Growth Factor mRNA and Protein in Normal and Tumor-derived Mammary Epithelial Cells in Culture

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Running Title: **bFGF in Mammary Epithelial Cells**

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Abstract

We examined the expression of the basic fibroblast growth factor (bFGF) gene in cultured normal and tumor-derived human mammary epithelial cells at both the transcriptional and translational level. Northern blot analysis revealed three bFGF mRNA transcripts of 7.5, 4.4 and 2.2 kb in all four strains (donors) of normal cells (HMECs) we examined, and in the immortal mammary cell line, HBL-100. Of the four mammary tumor-derived cell lines we examined (MCF-7, BT474, T47D and Hs578T), only the Hs578T cells produced detectable levels of bFGF mRNA. Western blot analysis of cell lysates using an anti-bFGF monoclonal antibody revealed corresponding results. bFGF protein was detected in normal HMEC strains 161 and 48 (other normal strains not tested), HBL-100 cells and in Hs578T cells, but not in the other tumor cell lines. In each case, three distinct molecular weight species of bFGF protein were detected which migrated in SDS-PAGE at 18, 24, and 27 kDa. We also investigated the ability of bFGF to stimulate the proliferation of normal and tumor-derived mammary epithelial cells. Addition of bFGF to serum-free cultures of these cells had no effect on the proliferation of HMECs under a variety of conditions, and was weakly mitogenic for Hs578T cells. Our results indicate that normal HMECs produce bFGF mRNA and protein(s) while only some mammary tumor-derived cells express this gene. Thus, our results do not support a general role for expression/over-expression of bFGF in the development of mammary tumors. However, bFGF could play a role in the normal development and homeostasis of the mammary gland.

Introduction

Numerous studies on the growth regulation of human mammary carcinomas have focused on steroidal hormones (especially estrogen) because, in about one third of clinical cases, breast cancer growth is dependent upon estrogenic hormones (1) and can be inhibited by antiestrogenic antagonists (2). Recently, the influence of polypeptide growth factors on cancer cell proliferation has received much attention. It has been suggested that estrogen-regulated tumor growth could be correlated with changes in expression of combinations of growth inhibitors and stimulators (for review, see ref.1). The possible involvement of secreted growth factors in growth regulation of breast cancer was supported by the finding that crude conditioned medium from human breast cancer cell lines was capable of stimulating the proliferation of other breast cancer cells (3), and by the finding that the initial growth rate of breast cancer cells in culture was proportional to the number of cells plated (4). The production of autostimulatory growth factors by breast cancer cells was additionally supported by the identification of several growth factors in conditioned media from cultured human breast cancer cells. Transcripts for these growth factors were also detected in these cells as well as in primary tumors (5). Among these growth factors, transforming growth factor type alpha (TGF-α) and insulin-like growth factor-1 (IGF-1) function as autocrine stimulatory growth factors in promoting the growth of breast cancer cells *in vitro* (5,6). A platelet-derived growth factor (PDGF)-like molecule has also been detected in extracts of breast cancer cells and media (7) and may have a paracrine role in mammary tumor development (3,7). A growth inhibitory, transforming growth factor type-beta (TGF-β)-related activity was found to be secreted by some breast cancer cells (8).

In contrast to the growth factors discussed above, It is not known if fibroblast growth factors (FGFs) play a role in the growth control of mammary carcinomas. Seven

genes have been found to encode proteins which have closely related structures (9-17), and are considered to be members of the FGF gene family. One of these genes, *int*-2, is an integration site of mouse mammary tumor virus (9). The human homolog of *int*-2 locus was found to be amplified in infiltrating ductal breast cancers (18). Two other FGF homologs, *hst* (11)/K-FGF (10) and FGF-5 (12), were both isolated from human tumors. Some studies have indicated basic FGF (bFGF) involvement in tumor cell proliferation and tumor development (19-22). bFGF has been identified and purified from human tumor cells such as hepatoma cells (20) and rhabdomyosarcoma cells (21). It was suggested that bFGF acts as an autocrine stimulator for these tumor cells, and as a potent angiogenic agent that fosters the growth of new blood vessels into the tumors (23). bFGF produced by tumor cells has also been reported to stimulate plasminogen activator and collagenase production which may contribute to angiogenesis and metastasis of tumors (20). On the other hand, high levels of bFGF gene expression have also been found in normal human cells such as human dermal fibroblasts (24,25).

In both normal and tumor cells, several species of bFGF mRNA transcripts have been reported. 7.0 kb and 3.7 kb transcripts were identified in cultured human hepatoma and rhabdomyosarcoma cells (21,26). In addition, two smaller transcripts of 2.2 and 1.6 kb were identified in normal human dermal fibroblasts (24). Numerous forms of bFGF proteins have been isolated from a variety of cells and tissues. One of them was 154 amino acids in length (27) which was identical to the sequence predicted from a bFGF cDNA clone (13). Several other lower molecular forms of bFGF have been identified which have undergone truncation at the N-terminus (28,29). More recently, higher molecular forms of bFGF proteins have been reported in normal, tumor and transfected cells (25,30,31). In hepatoma cells, Florkiewicz and Sommer detected four species of bFGF proteins which migrated in SDS-PAGE at 17.8, 22.5, 23.1 and 24.2 kDa (30). Root and Shipley identified at least three species of 18, 23 and 26.6 kDa in human dermal fibroblasts (25). Because

there are no in frame ATG codons upstream of the putative start codon which initiates the 154 amino acid form, it was suggested that some unusual start codons, such as CTG, were utilized to initiate the synthesis of high molecular forms of the molecule (30,31) (see also Discussion).

It has been shown that the bFGF gene expression in normal human fibroblasts increases after treatment of the cells with serum (24,25,32,33). The level of both mRNA (24,32,33) and proteins (25) increases dramatically within 4 hours of serum treatment. The serum factor(s) responsible for this induction has not been identified.

The purpose of this study was to examine normal and malignant mammary epithelial cells for bFGF gene expression and to determine effect of bFGF on the proliferation of these cells in order to determine if bFGF expression, overexpression or increased sensitivity to bFGF could play a role in the development of human mammary tumors.

Results

We examined the expression of bFGF mRNA in cultured normal human mammary epithelial cells and malignant mammary epithelial cells. Poly A^+ mRNA was isolated from cells in culture at 80-90% confluence and subjected to northern blot analysis as described in the Materials and Methods. As shown in Figure 1, all four strains of normal HMECs (HMEC 161, HMEC 172, HMEC 184 and HMEC 48) grown in complete HMEC medium expressed three bFGF mRNA transcripts of 7.5, 4.4 and 2.2 kb (Fig. 1, Lanes A-D). HBL-100, an immortal cell line grown in MCDB 170 supplemented with 5% FBS, also expressed these transcripts (Fig. 1, Lane E). Of four mammary tumor cell lines grown in serum-containing medium (Hs578T, BT474, MCF-7 and T47D), only Hs578T cells (Fig. 1, Lane G) expressed detectable bFGF message (Fig. 1, Lanes G,I,J and K).

Because serum has been shown to have a regulatory effect on bFGF gene expression (24,33), we minimized the differences in the growth conditions for HBL-100, Hs578T and normal HMEC by eliminating serum in the culture media in order to compare the level of bFGF gene expression in these cells. Initially, we tried to grow both cell lines (HBL-100 and Hs578T) in the medium for normal HMECs. HBL-100 grew very well in this medium even in the absence of EGF and BPE. Therefore, poly A^+ RNA isolated from HBL-100 cells grown in complete HMEC medium lacking EGF and BPE was analyzed for bFGF mRNA and compared with RNA from the same cells grown in serum-containing medium (Fig. 1, Lanes E and F). Unlike HBL-100 cells, Hs578T cells did not grow when placed directly in complete HMEC medium. We then tried to adapt this cell line to grow in serum-free medium. Hs578T cells were grown in medium MCDB 202a supplemented with 5% serum. In addition, EGF, Insulin, hydrocortisone, ethanolamine, phosphoethanolamine and BPE were added to the medium at the same concentrations as for complete HMEC medium. The serum concentration in the medium was reduced sequentially during passage of cells until a population of cells which was able to grow in medium without serum was

obtained. For ease of description we call this serum-free medium "complete MCDB 202a medium". During this serum reduction process the cells grew continuously at approximately the same rate and did not appear to undergo a crisis period. Thus, we do not believe that the serum-free cell strain that we obtained arose from a minor subpopulation in the original cell line. Poly A^+ mRNA isolated from Hs578T cells grown in complete MCDB 202a medium was included in the northern blot analysis (Fig. 1, Lane H). After analysis with the bFGF probe, the blot was subsequently probed with a constitutively expressed gene, 1B15, to determine the relative amount of RNA loaded on each lane. The total amount of the three bFGF transcripts expressed in the different cell types was then determined by densitometric analysis (Fig. 2). Among all types of human mammary epithelial cells tested, HBL-100 cells appeared to express the highest level of bFGF transcripts. Inclusion of serum in the culture medium did not significantly increase the amount of the transcripts in these cells. The level of bFGF transcripts in four normal HMEC strains was variable as shown in Figure 2. Hs578T cells grown in serum-free medium (complete MCDB 202a medium) had a much lower level of bFGF message compared to the Hs578T cells grown in serum-containing medium. When these serum-free cultures of cells were treated with serum, the levels of bFGF mRNAs were similar to those detected in the parental cell line growing in serum-containing medium (data not shown). Three tumor cell lines tested (MCF-7, BT474 and T47D) did not express any detectable bFGF transcripts (Fig. 1, 2), although intact RNA was present as verified by the 1B15 probe (Fig. 1, lower panel).

To find out whether bFGF mRNA expressed in human mammary epithelial cells is translated into proteins, we performed western blot analysis of cell lysates using a monoclonal anti-bFGF antibody. The results of these experiments directly correlated with the results obtained at the transcriptional level. bFGF proteins were identified in the cell lysates of normal HMEC strains 161 and 48 (other strains not tested), in the immortal cell line HBL-100 and in the tumor cell line Hs578T. In each case, three different molecular

weight species of bFGF protein were identified which migrated in SDS-PAGE with apparent mass of 18, 23 and 27 kDa (Fig. 3, Lane A-D). bFGF proteins were not detected in the mammary tumor cell lines MCF-7 or T47D (Fig. 3, Lane H, I). The human recombinant bFGF (154 amino acids form) loaded at different quantities (Fig. 3, Lane E,F,G and J,K,L) served as positive controls as well as standards for estimating approximate amount of bFGF proteins expressed in various cell types. The same three species of bFGF protein were also identified in the same cell types by a polyclonal anti-bFGF antibody. The ability of the polyclonal antibody to detect these bFGF species was blocked by preincubating the antibody with the decapeptide to which the antibody was generated (data not shown).

We then examined whether bFGF had any effect on the proliferation of normal HMECs or tumor cells *in vitro*. Normal HMEC strain 161 and Hs578T tumor cells which were selected to grow in serum-free medium were chosen for the growth experiments. Cells were plated at density of 5 x $10^3/cm^2$ in appropriate complete medium and, after 24 hours, the plates were washed with buffer saline solution A and the medium was replaced with basal medium supplemented with hydrocortisone, ethanolamine, and phosphoethanolamine (control). Various growth factors were then added to each well as indicated in the legends for Figures 4 and 5. The results in Figure 4 show a representative experiment for HMEC 161 cells. Cells in control medium survived during the assay period but did not grow. The addition of 5 μg/ml insulin to the control medium resulted in almost 3 population doublings. Addition of 5 ng/ml EGF had a little effect on cell growth by itself, whereas the activity of EGF was synergistic with insulin. Adding BPE together with EGF and insulin (complete HMEC medium) did not significantly increase the cell number compared to the growth obtained with EGF and insulin. Neither basic nor acidic FGF had a significant stimulatory effect on the proliferation of these cells either in the absence or presence of insulin.

A similar growth experiment was done with Hs578T cells (Fig. 5). In contrast with

the normal cells, Hs578T cells underwent two population doublings in the control medium without any added growth factors. Adding insulin, EGF or bFGF stimulated proliferation by one more population doubling. The combination of EGF and insulin or bFGF and insulin resulted in a slight increase in cell number when compared to the growth obtained with any of these agents alone. The combination of EGF, insulin and BPE resulted in greater cell numbers than EGF and insulin alone (Fig. 5).

Discussion

We have demonstrated that the bFGF gene is expressed to varing degrees in cultured normal human mammary epithelial cells, in an immortal mammary cell line, HBL-100, and in a mammary tumor cell line, Hs578T. In three other mammary tumor cell lines examined (BT474, MCF-7 and T47D), we did not detect expression of the bFGF gene. The results remained consistent at the transcriptional and translational level. Addition of exogenous bFGF (5 ng/ml) to serum-free cultures of normal HMECs had no effect on their proliferation, whereas it was a weak mitogen for Hs578T cells.

Three different sizes of bFGF transcripts were found in the mammary epithelial cells which expressed the gene. They were similar (7.5, 4.4 and 2.2 kb) to those found in human dermal fibroblasts (24,32,33) and other cell types (21,26,34,35). It has been suggested that size heterogeneity of bFGF transcripts could be due to any one or combination of the following: i) utilization of multiple polyadenylation sites (13,24,36); ii) transcription initiation at multiple sites; and iii) differential post-transcriptional RNA processing (24). We were unable to detect the 1.6 kb transcript which was reported in addition to three larger transcripts in human dermal fibroblasts (24). It is possible that our probe, which is complementary to 530 bp of the 3' noncoding region of the bFGF gene, was less efficient at recognizing transcripts terminated at the nearest polyadenylation site (36). This site lies only 97 bp from the 3' end of the antisense transcripts used to probe our blots. Thus, only 97 bp of the longest synthetic probes would overlap with the small transcript.

Multiple forms of bFGF proteins were identified by a monoclonal anti-bFGF antibody in two strains of normal mammary cells as well as in HBL-100 cells and Hs578T cells, indicating that at least some of the bFGF messages were translated. The open reading frame of the bFGF cDNAs that have been sequenced thus far indicate that the primary translation product of this gene beginning from the first ATG codon would be 155 amino

acids in length (about 18 kDa). At least two higher molecular mass forms of bFGF proteins, in addition to the 18 kDa molecule, have been identified in human hepatoma cells (30) and human dermal fibroblasts (25). *in vitro* transcription/translation and mutagenesis studies of a human hepatoma cDNA revealed that alternative CTG codons can be utilized to initiate the higher molecular weight species of bFGF (30,31). It has been shown that these high molecular weight proteins synthesized by transfected COS-1 cells were mitogenic for mouse 3T3 cells (30). Our results demonstrated the presence of at least three forms of bFGF proteins in human mammary epithelial cells. The two higher molecular mass forms (23 and 27 kDa) appear to be slightly larger than those previously reported (30,31). This could be due to the use of a different gel system or different molecular weight markers in our studies.

It has been reported that human mammary tumor cells produce several growth factors, and that the expression of some of these factors is under the regulation of estrogen (for reviews, see ref. 1). These growth factors could act via autocrine and/or paracrine pathways to stimulate the growth and development of tumors. Early studies showed that cultured mammary tumor cells produced TGF-α, IGF-1, TGF-β and PDGF (5-8). TGF-α secreted by MCF-7 cells was able to stimulate the same cells growing in soft agar indicating an autocrine role of this factor (6). Recent studies showed that normal mammary cells grown in culture actually expressed high levels of TGF-α mRNA (37,38). It was suggested that an autocrine loop for TGF-α could also exist in normal proliferating mammary epithelial cells. In our current study, we demonstrated that normal HMECs expressed significant amounts of bFGF transcripts while only one tumor cell line, Hs578T, expressed the gene. When Hs578T cells were grown in serum-containing medium they had higher amounts of bFGF message compared to the normal HMECs grown in serum-free medium. However, when the Hs578T cells were grown in medium lacking serum and supplemented with same additives as for normal cells, there was a reduction in the steady state levels of bFGF mRNA. Apparently, serum in the medium is responsible for the higher bFGF levels rather than the cell's tumorigenic nature. Our results indicate that

there is no direct relationship between bFGF expression/overexpression and human mammary tumorigenicity. In addition, bFGF expression does not seem to correlate with estrogen receptor levels in the tumor cells tested. Of the tumor cell lines lacking estrogen receptor (Hs578T and BT474), only Hs578T expressed the bFGF gene. bFGF expression was not detected in either of the cell lines which express the estrogen receptor (MCF-7 and T47D).

It has been proposed that cells could become malignant by somehow altering signal transduction after the ligand-receptor binding, rather than by increasing the production of growth factors and/or their receptors (38,39). That is: these cells could become hypersensitive to normal ligand-receptor interactions. This theory might explain our results on the responsiveness of Hs578T cells to exogenous bFGF in serum-free culture medium. Although Hs578T cells do not produce greater amounts of bFGF than normal HMECs, they responded to bFGF by increasing in cell number while normal HMECs did not respond to bFGF (Fig. 5). Thus, oversensitivity of Hs578T tumor cells to bFGF could be responsible for the autonomous growth of these cells in the absence of growth factors in serum-free cultures. Alternatively, normal HMECs may not express cell surface receptors for bFGF while tumor cells do.

By comparing the expression of the bFGF gene in normal and tumor-derived human mammary epithelial cells grown in culture, we have shown that normal HMECs produce bFGF mRNAs and proteins while only some mammary tumor-derived cells express the gene. Our results indicate that expression/overexpression of the bFGF gene is unlikely to be a common mechanism in the development of mammary tumors. However, some increase in sensitivity to bFGF was noted in Hs578T mammary tumor cells which also produced bFGF. Therefore, bFGF could be involved in some but not all mammary tumors. In addition, bFGF could play a role in the normal development and homeostasis of the mammary gland.

Materials and Methods

Cell culture

Normal human mammary epithelial cells (HMEC) strain 161,172,184 and 48 were generously supplied by Dr. M. Stampfer (Lawrence Berkeley Laboratories, Berkeley, CA). The HMECs used in the experiments reported here were between passage 8-11. HBL-100, an immortal cell line established from normal mammary epithelial cells, and four human mammary tumor cell lines (MCF-7, T47D, BT474 and Hs578T) were all purchased from the American Type Culture Collection (Rockville, MD). MCDB 170 was used as a basal medium for the growth of normal HMECs and MCDB 202a for T47D, BT474 and Hs578T cells. The two basal media were prepared in our laboratory using methods previously described (40,41). Human recombinant bFGF was provided by Dr. J. Abraham (California Biotechnology Inc., Mountain View, CA). Human recombinant acidic fibroblast growth factor (aFGF) was a gift from Dr. K. Thomas (Merck Institute, Rahway, NJ). Human recombinant epidermal growth factor (EGF) was purchased from Amgen Biologicals (Thousand Oaks, CA). Insulin, hydrocortisone, ethanolamine and phosphoethanolamine were all purchased from Sigma Chemical Co. (St.Louis, MO). Bovine pituitary extract (BPE) was obtained from Clonetics Corp. (San Diego, CA). The methods for preparation of the growth factors and other additives were described previously (42). Stock cultures of normal HMECs were maintained in medium MCDB 170 supplemented with 0.4% BPE, 5 ng/ml EGF, 5 μg/ml insulin, 1.4 x 10^{-6} M hydrocortisone, 1 x 10^{-4} M ethanolamine and 1 x 10^{-4} M phosphoethanolamine (40). This medium is referred to in the text as complete HMEC medium. HBL-100 stock cultures were grown in MCDB 170 supplemented with 5% fetal bovine serum (FBS). Three tumor cell lines (T47D, BT474 and Hs578T) were grown in MCDB 202a supplemented with 5% FBS. MCF-7 cells were grown in DMEM (43) supplemented with 5% FBS. A population of Hs578T cells capable of proliferating in serum-free medium were selected by stepwise reduction of the serum supplementation in

the 202a culture medium in the presence of the additives for complete HMEC medium. Once selected, they were maintained in serum-free medium MCDB 202a supplemented with same additives. In the experiments for northern and western blot analyses, the cells were plated at 5 x $10^3/cm^2$ in 100 mm culture plates (Corning) in the same media utilized for stock cultures, or in serum-free medium as indicated in the Figure Legends. The cells were allowed to grow for 5 to 12 days with the medium replenished every other day. Poly A^+ RNA or cell lysates were collected when cells in cultures reached 80-90% confluence.

Northern blot analysis

Poly A^+ RNA was isolated from cells as described previously (24). Briefly, cells were lysed in the culture plate by the addition of a lysis buffer containing 0.1 M NaCl, 10 mM Tris, 2.0 mM sodium ethylenediaminetetraacetate, and 1% sodium dodecylsulfate (pH 7.4). Cell lysates were scraped from the plates with a rubber spatula. DNA was sheared by passing the lysates through a 21 gauge needle 6 times. Cell lysates were then incubated with proteinase K (100 μg/ml) at 37°C for 30 min and poly A^+ RNA was purified by oligo(dT)-cellulose chromatography.

To prepare northern blots, RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to Nytran membranes (Schleicher and Schuell, Inc., Keene,NH) in 10 x SSC by capillary transfer. The membrane was then briefly air dried and baked at 80°C for 30 min.

A single-stranded, ^{32}P-labeled cRNA probe specific for bFGF was used for hybridization. The probe was synthesized as described (44) from a template of a human bFGF cDNA, pGb530. This vector contains 530 bp of 3' noncoding sequence, immediately 3' to the protein coding sequence for bFGF (13), which was cloned in an expression vector (pGEM4Z) with the antisense orientation under the control of the Sp6 promotor. A cRNA probe encoding a constitutive gene, cyclophilin (45), was synthesized from the plasmid p1B15 in the same way and was used to estimate loading and transfer efficiencies of RNA samples. 1B15 mRNA has been reported to be constitutively expressed in human cells in a

wide variety of culture conditions (24,33).

Prehybridization of blots was performed in a hybridization mixture (50% formamide, 5x SSC, 0.1% SDS, 10x Denhart's solution, 50 μg/ml polyadenosine, and 250 μg/ml Herring sperm DNA) for 3 hours at 60° C. The blots were then hybridized with 3-5 x 10^6 cpm/ml of bFGF probe in fresh hybridization mixture for 18-20 hours at 60° C. After hybridization, the blots were briefly rinsed with 5x SSC three times at room temperature and washed in 300 ml of a washing buffer (0.1x SSC, 5 mM EDTA and 0.1% SDS) for 2 hours at 65°C with two changes of the washing buffer. Hybridization was visualized by exposure to film (Kodak X-Omat AR) for 70 hours. After the radioactivity on the blot decayed, the same blot was subsequently hybridized with the 1B15 cRNA probe under same conditions. The autoradiographic signal obtained from the bFGF probes was then quantified using a scanning densitometer and normalized to the values obtained from the 1B15 probe to adjust for variability in the loading and transfer of the RNA samples.

Western blot analysis

Cell lysates were collected as described (25,30). Briefly, cells were lysed in a cold lysis buffer (1% NP-40, 50 mM Tris, pH 7.5, 400 mM NaCl, 1 mM MgCl and 1 μg/ml Leupeptin) and scraped from the culture plates. Nuclei and cell debris were removed by centrifugation at 15,000 x g for 10 min. at 4°C. The clarified cell lysate was then incubated with heparin-acrylic beads (Sigma) for 2 hours at 4°C, with rocking. The proteins binding to the beads were eluted by boiling the beads for 5 min. in 2x SDS/PAGE sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 200 mM dithiothreotol). The samples were then analyzed by 15% SDS/PAGE. The amount of lysate loaded into each lane was the equivalent of three plates of cells (5-9 million cells/plate). The separated proteins were then blotted to nitrocellulose using a dry blotter (LKB). The western blots were probed with a monoclonal anti-bFGF antibody raised against human recombinant bFGF (a gift from Dr. Charles Hart, Zymogenetics Inc. Seattle, WA). The secondary antibody used for

color development was alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, Madison, WI). In some experiments rabbit anti-bFGF antisera were used. This antisera (a gift from Dr.Eric Smith, Children's Hospital Med. Cntr. Cincinnati, OH) was raised against a synthetic decapeptide representing amino acids 24-33 of bFGF which is 154 amino acids in length.

Growth assays

Normal HMECs were removed from stock culture flasks using 0.025% trypsin/0.01% EDTA and resuspended in buffer saline solution A (46) containing 0.5% FBS. The cells were centrifuged at 180 x g for 5 min. and the pellet was resuspended in complete HMEC medium. The cell number was determined, appropriate dilutions made and 4.5×10^4 cells were inoculated into each well (9 cm^2) of a six-well plate (Corning) in 2 ml of complete HMEC medium. After 24 hours, the cells were washed twice with 2 ml/well of solution A and experimental media and growth factors were added as indicated in the figures. The cells were then allowed to grow for 7 days with the media changed every other day. Cell numbers were determined at the end of the assay by trypsinizing cells from each well, resuspending in 1 ml of solution A with 0.5% FBS and counting in a hemocytometer. The growth assays for the tumor cell line Hs578T were essentially the same except that the basal medium used was MCDB 202a instead of MCDB 170, and the assay time was 5 days. Data shown in Figure 4 and 5 represent mean ± standard deviation of triplicates for each condition.

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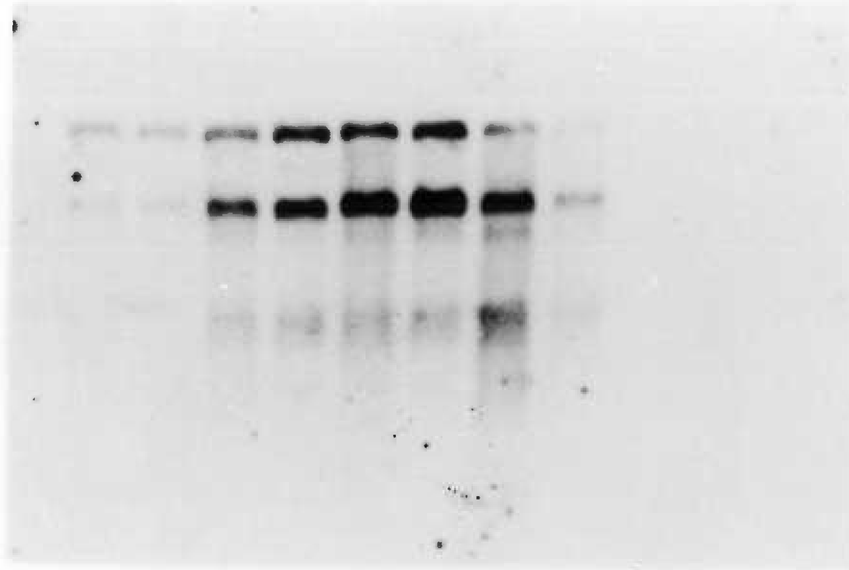
Figures and Legends

Figure 1. Northern blot analysis of poly A^+ mRNA from human mammary epithelial cells.

Normal and malignant mammary cells were grown in culture medium as indicated below. Poly A^+ mRNA was collected from different cell types and subjected to northern blot analysis (5 μg/lane) as described in "Materials and Methods". After hybridization with bFGF cRNA probe (upper panel), the same blot was reprobed with cyclophilin (1B15) cRNA (lower panel). Migration of standard RNA marker (BRL) is indicated by kilobases (kb). Lane A-D: Normal human mammary epithelial cells, strain 161 (A), 172 (B), 184 (C) and 48 (D), grown in complete HMEC medium; Lane E-F: Immortal cell line HBL-100 grown in MCDB 170 supplemented with 5% FBS (E), or in complete HMEC medium lacking EGF and BPE (F); Lane G-H: Hs578T tumor cell line grown in MCDB 202a supplemented with 5% calf serum (G), or in complete MCDB 202a medium (H); Lane I-K: Tumor cell lines BT474 (I), MCF-7 (J) and T47D (K) grown in serum-containing medium as described in "Materials and Methods".


A B C D E F G H I J K
Kb
—9.5
—7.5
—4.4
—2.4
—1.4

1B15

Figure 2. Densitometric analysis of bFGF mRNA expression in human mammary epithelial cells.

The autoradiographic signals obtained from the bFGF probe were quantified using a scanning densitometer. Data represents total density of the three major bands in each cell type shown in Figure 1 and is normalized to the value obtained from 1B15 autoradiographic signals. The expression of bFGF mRNA in various cell types was presented as a percentage of the maximal autoradiographic signal obtained. S+: serum; S-: serum-free.

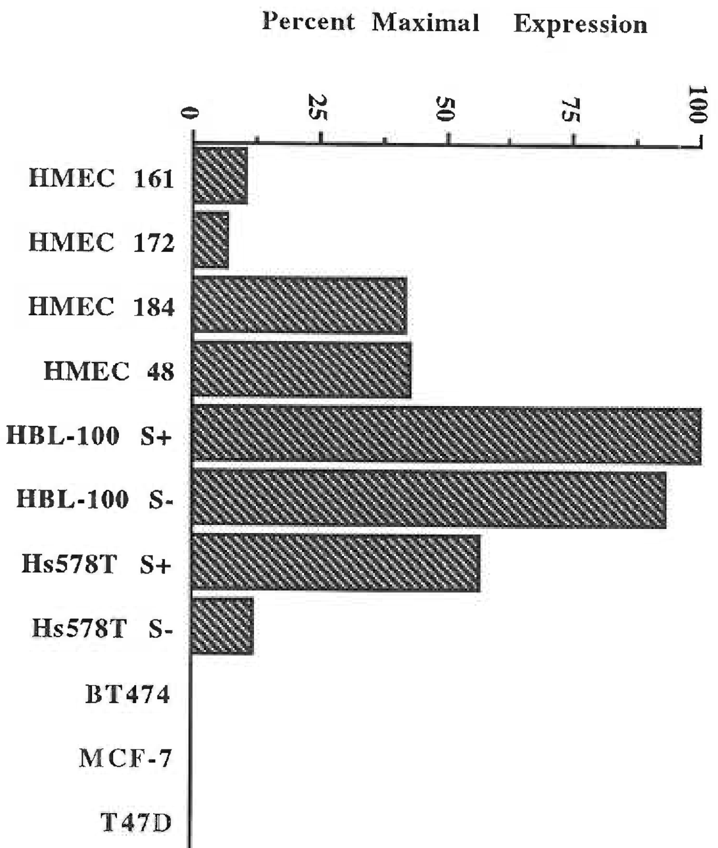
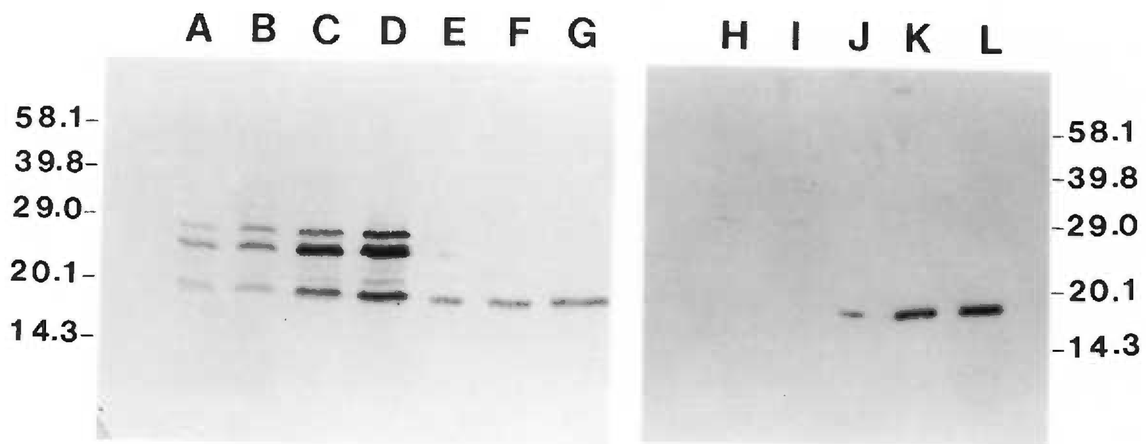

Percent Maximal Expression
0
25
50
75
100
HMEC 161
HMEC 172
HMEC 184
HMEC 48
HBL-100 S+
HBL-100 S-
Hs578T S+
Hs578T S-
BT474
MCF-7
T47D

Figure 3. Western blot analysis of bFGF proteins from human mammary epithelial cells.

Normal and malignant human mammary epithelial cells were grown in culture medium as indicated below. Cell lysates were collected and incubated with heparin-acrylic beads. Proteins eluted from the beads were detected with monoclonal anti-bFGF antibodies using western blot analysis as described in "Materials and Methods". Lane A-B: Normal HMECs, strain 161 (A) and strain 48 (B), grown in complete HMEC medium; Lane C: HBL-100 cell line grown in serum-containing medium; Lane D,H,I: Tumor cell lines Hs578T (D), MCF-7 (H) and T47D (I) grown in serum-containing medium; Lane E,F,G,J,K,and L: Recombinant human bFGF at 2 ng/lane (E,J), 5 ng/lane (F,K), 10 ng/lane (G,L). Molecular weights are given in kDa.



A B C D E F G
H I J K L
58.1-
39.8-
29.0-
20.1-
14.3-
-58.1
-39.8
-29.0
-20.1
-14.3

Figure 4. Effect of growth factors on the proliferation of normal human mammary epithelial cells strain 161.

Normal HMEC 161 were plated at the density of 5 x 10^3/cm^2 (4.5 x 10^4 cells/well) in complete HMEC medium. The cells were washed on the following day and switched to MCDB 170 supplemented with hydrocortisone, ethanolamine and phosphoethanolamine (control). Insulin (5 μg/ml), EGF (5 ng/ml), bFGF (5 ng/ml) or aFGF (5 ng/ml) were then added as indicated. Some cultures received completed medium containing BPE (0.4% v/v). The cells were allowed to grow under these experimental conditions and the cell number in each well was determined at the end of the assay. Each data point represents mean ± standard deviation of triplicate cultures of the same condition. The arrow indicates the plating density. I: Insulin, E: EGF.

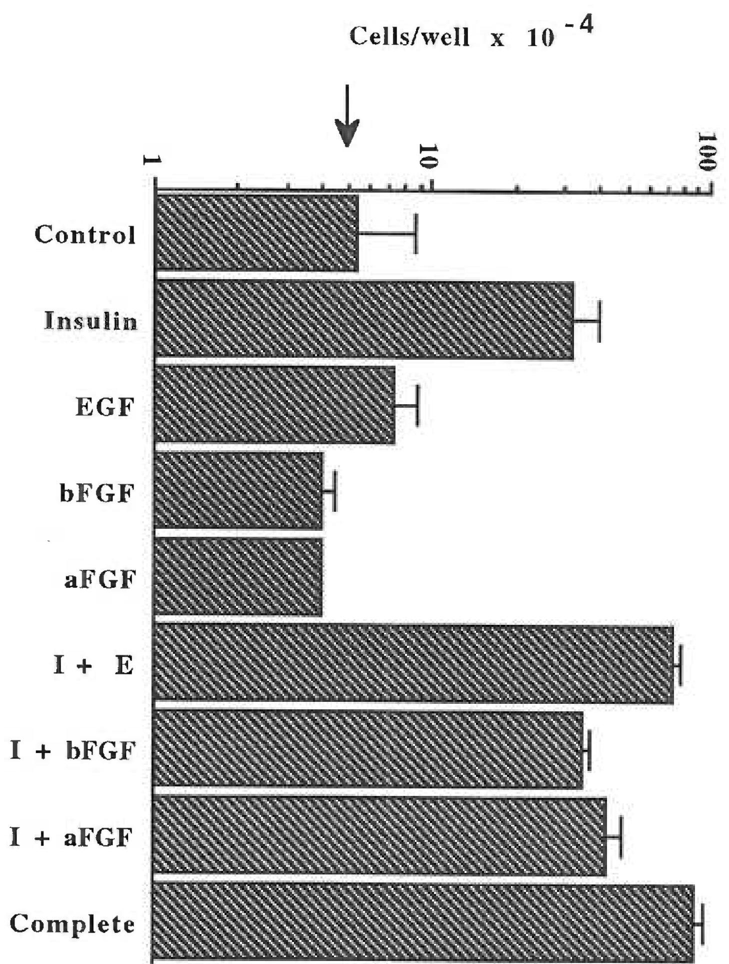
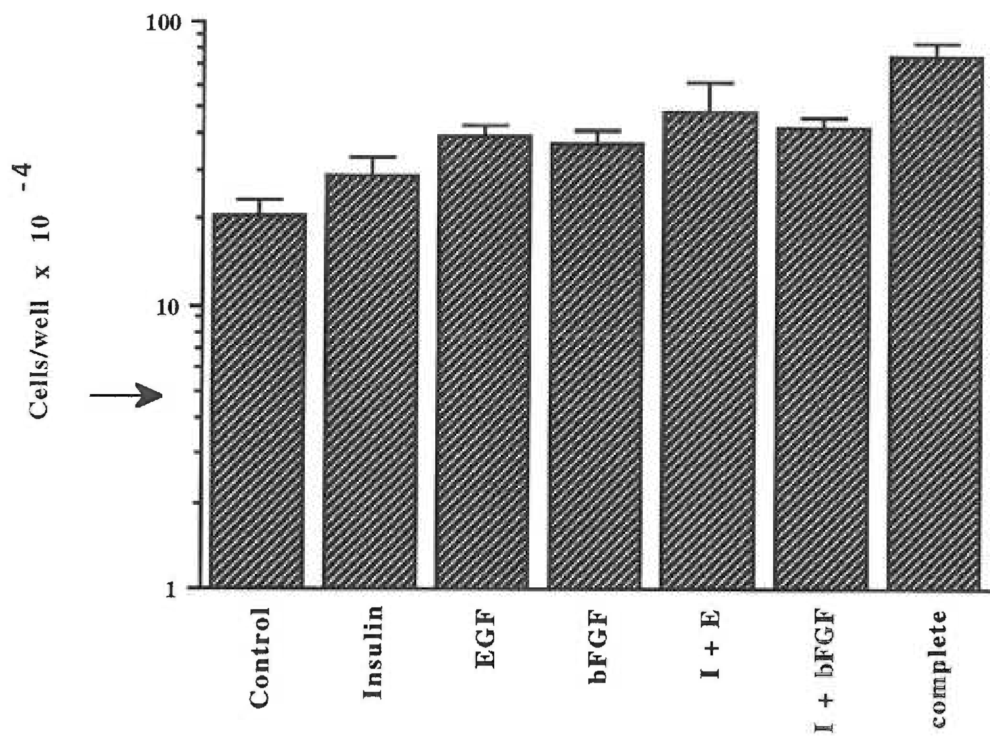

Cells/well x 10 -4
1
10
100
Control
Insulin
EGF
bFGF
aFGF
I + E
I + bFGF
I + aFGF
Complete

Figure 5. Effect of growth factors on the proliferation of Hs578T cells.

Hs578T cells were plated at the density of 5 x $10^3/cm^2$ in 202a complete medium. On the next day the cells were washed and switched to experimental conditions as described in the legend for Figure 4. Incubation, medium changes and cell number determination were performed as described in "Materials and Methods". I: Insulin, E: EGF.



Cells/well x 10 -4
100
10
1
Control
Insulin
EGF
bFGF
I + E
I + bFGF
complete

Heparin Inhibition of Autonomous Growth Implicates Amphiregulin as an Autocrine Growth Factor for Normal Human Mammary Epithelial Cells

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Running Head: **Amphiregulin in normal mammary epithelial cells**

5 figures; 1 table

Abstract

Normal human mammary epithelial cells (HMECs) proliferate in a serum-free defined growth medium in the absence of epidermal growth factor (Li and Shipley, 1991). Amphiregulin (AR) is a heparin-regulated, EGF-like growth factor. Our observation that one strain of HMECs produce AR mRNA (Cook *et al.*, 1991a) stimulated us to determine whether AR expression was a common phenomenon in HMECs and whether AR could act as an autocrine growth factor to support the EGF-independent growth of these cells. In this study, we detected high levels of AR expression in four separate HMEC strains while one immortal mammary cell line (HBL-100) and six mammary tumor-derived cell lines had low to undetectable levels of AR. The EGF-independent growth of HMECs was blocked by the addition of heparin or a monoclonal anti-EGF receptor antibody to the culture medium, implicating AR as an autocrine growth mediator. This hypothesis is further supported by the fact that medium conditioned by HMECs contains secreted AR protein. A mammary tumor-derived cell line, Hs578T, which proliferates in an EGF-independent manner does not express detectable levels of AR and is not growth inhibited by heparin. Examination of the same cell types for expression of transforming growth factor type-alpha (TGF-α) mRNA revealed coordinate expression of AR and TGF-α in these cells. These data suggest that both AR and TGF-α mRNA are produced in much greater abundance by normal HMECs than in tumor-derived cells in culture, and that AR is an important autostimulatory factor for the growth of normal HMECs.

Introduction

The excretory and ductal epithelial portions of the breast arise as downgrowths of the ectoderm (primitive epidermis) during development. The interaction of multiple hormones, including estrogen, is required to stimulate the proliferation of epithelial cells during the development of the normal mammary gland. Numerous studies suggest that estrogen does not act directly on mammary epithelium, but indirectly, through endocrine-acting substances or local (paracrine) mediators (polypeptide growth factors) secreted by mammary stromal cells (Lippman and Dickson, 1989). A number of well-characterized growth factors, such as epidermal growth factor (EGF) and the insulin-like growth factors (IGFs), have been shown to stimulate the proliferation and modify the differentiation of normal and malignant rodent and human mammary epithelial cells (Salomon *et al.*, 1989; Salomon *et al.*, 1986). EGF or transforming growth factor alpha (TGF-α) is able to promote local lobulo-alveolar development of mouse mammary glands in organ culture and *in vivo* (Tonelli and Soroff, 1980; Vonderhaar, 1987). In addition, EGF is required for the low density growth of normal human mammary epithelial cells in culture (Valverius *et al.*, 1989). Several growth factors, such as IGF-1, TGF-α and TGF-ß, have been found in the conditioned medium from cultured human mammary tumor-derived cell lines implicating the involvement of growth factors in mammary tumors (Huff *et al.*, 1986; Bates *et al.*, 1988; Knabbe *et al.*, 1987).

Recent studies have demonstrated that normal human epithelial cells cultured *in vitro* not only respond to exogenously added growth factors, but also produce growth factors to which they themselves respond (Coffey *et al.*, 1987; Shipley *et al.*, 1989; Bates *et al.*, 1990; Cook *et al.*, 1990; Cook *et al.*, 1991b; Cook *et al.*, 1991a). Amphiregulin (AR), a member of the EGF family of growth factors, is one of the factors which has been shown to be synthesized by human keratinocytes and to stimulate proliferation of these cells in an

autonomous fashion (Cook *et al.*, 1991a). AR was originally isolated from medium conditioned by phorbol ester treated human mammary carcinoma cells (MCF-7) (Shoyab *et al.*, 1988). AR was named because of its ability to stimulate the growth of some cells and inhibit the proliferation of several human carcinoma cell lines in culture (Shoyab *et al.*, 1988). Among the growth factors in the EGF family, AR is the only one whose mitogenic activity has been demonstrated to be inhibited by interaction with heparin, a sulfated glycosaminoglycan (Cook *et al.*, 1991a). AR competes with EGF for cell surface binding and may exert at least some of its biological effects through the EGF receptor (Shoyab *et al.*, 1989). This AR-mediated competition of cell surface EGF receptor occupancy is also inhibited by heparin (Cook *et al.*, 1991a). Cloning and sequencing of the AR gene revealed that AR is synthesized as a 252-amino-acid membrane bound precursor which is processed to non-membrane bound mature forms of 84 and 76 amino acids (Plowman *et al.*, 1990b). The primary structure of AR possesses a highly positively charged, Lys-Arg rich N-terminal hydrophilic region adjacent to a C-terminal EGF-like domain. It has been hypothesized that this hydrophilic domain could confer heparin-regulation of biological activity to this molecule (Cook *et al.*, 1991a).

Like human keratinocytes, cultured normal human mammary epithelial cells (HMECs) require EGF for growth at clonal density (Valverius *et al.*, 1989), but are capable of proliferation without exogenously added EGF-like growth factors when the cells are plated at moderate to high densities (Li and Shipley, 1991; Valverius *et al.*, 1989). These data suggest that maintenance of rapid growth in the HMECs *in vitro* occurs via an autocrine pathway. TGF-α has been suggested as a candidate autocrine factor for HMECs based on the finding that normal HMECs express both TGF-α and EGF receptor (Bates *et al.*, 1990; Salomon *et al.*, 1989). However, it has not been clearly demonstrated that TGF-α is a mediator of autocrine growth in these cells.

In the work described here, we demonstrate that normal HMECs produce AR

mRNA and protein. These cells do not require EGF for growth at high density and this autonomous growth is inhibited by heparin and monoclonal anti-EGF receptor antibody. Thus, our data strongly suggest that AR is an important autocrine growth factor for normal HMECs. In contrast, we find that mammary tumor-derived cells produce very little or no AR when compared to the normal cells and the autocrine growth of at least one tumor cell line does not appear to be dependent on AR production.

Materials and Methods

Cell culture

Normal human mammary epithelial cells (HMECs) were generously provided by Dr. M. Stampfer (Lawrence Berkeley Laboratories, Berkeley, CA). The HMECs used in the experiments reported here were from passages 8-10. HBL-100, an immortal cell line established from normal mammary epithelial cells, and four human mammary tumor cell lines (MCF-7, T47-D, Hs578T and ZR-75-1) were purchased from the American Type Culture Collection (Rockville, MD). Two mammary tumor cell lines (BT474 and SKBR-3) were supplied by Dr. G. M. Clinton (Oregon Health Sciences University, Portland, OR). Human recombinant epidermal growth factor (EGF) was purchased from Amgen Biologicals (Thousand Oaks, CA). Human recombinant acidic and basic fibroblast growth factors (FGF) was generously provided by Drs. K. Thomas (Merck Institute, Rahway, NJ) and J. Abraham (California Biotechnology, Inc., Mountain View, CA), respectively. Heparin (from porcine intestinal mucosa), insulin, hydrocortisone, ethanolamine, and phosphoethanolamine were all purchased from Sigma Chemical Co. (St. Louis, MO). Bovine pituitary extract (BPE) was obtained from Clonetics Corp. (San Diego, CA). The methods for preparation of the growth factors and other additives were described previously (Shipley *et al.*, 1989). Stock cultures of normal HMECs were maintained in complete HMEC medium which is MCDB 170 supplemented with 0.4% BPE, 5 ng/ml EGF, 5 μg/ml insulin, 1.4 x 10^{-6} M hydrocortisone, 1 x 10^{-4} M ethanolamine, and 1 x 10^{-4} M phosphoethanolamine (Hammond *et al.*, 1984; Li and Shipley, 1991). Tumor cell lines (MCF-7, SKBR-3 and ZR-75-1) were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 5-10% fetal bovine serum (FBS). Other tumor cell lines (BT474, T47D and Hs578T) were maintained in medium MCDB 202a (McKeehan and Ham, 1977; Hammond *et al.*, 1984) supplemented with 5% FBS. A

population of Hs578T cells capable of proliferating in serum-free medium (Li and Shipley, 1991) was maintained in MCDB 202a supplemented with the same additives as for the normal HMECs (complete MCDB 202a medium). In the experiments for Northern blot analysis, the cells were plated at 5 x $10^3/cm^2$ in 100-mm culture plates (Corning) in the media indicated in the figure legends. The cells were allowed to grow for 5-12 days with the medium replenished every other day and poly A^+ RNA was isolated when cells in the cultures reached 80% - 90% confluence.

Northern blot analysis

Poly A^+ RNA was isolated from cells as described previously (Schwab *et al.*, 1983; Sternfeld *et al.*, 1988) and subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH) in 10x SSC by capillary transfer.

Single stranded, ^{32}P-labeled cRNA probes specific for AR or TGF-α were used for hybridization. The AR probe was synthesized as described (Melton *et al.*, 1984) from 200 ng of linearized pTZ/AR-2 plasmid (Cook *et al.*, 1991a) which contains cDNA encoding the entire AR coding region. TGF-α cRNA probe was synthesized from a cDNA insert in plasmid SP65 (Coffey *et al.*, 1987). The cDNA insert corresponds to the complete TGF-α coding sequence (Derynck *et al.*, 1984). Northern blots were hybridized with 5 x 10^6 cpm/ml of cRNA probe for 16 hours at 60°C in a hybridization mixture (50% formamide, 5x SSC, 0.1% SDS, 10x Denhardt's solution, 50 μg/ml polyadenosine, and 250 μg/ml herring sperm DNA). After hybridization, the blots were briefly rinsed three times with 5x SSC at room temperature and washed in 300 ml of a washing buffer (0.1x SSC, 5mM EDTA, and 0.1% SDS) for 2 hours at 65°C with two changes of the washing buffer. Transcripts hybridized to the probe were visualized by exposure to Kodak X-Omat AR film. An identical method was used to probe the same blot for the expression of the constitutively expressed cyclophillin (1B15) gene product (Danielson *et al.*, 1988), in order to visualize any variability in the loading and transfer of the RNA samples.

Growth assays

Normal HMECs were removed from stock culture flasks using 0.025% trypsin/0.01% EDTA and resuspended in buffer saline solution A (Shipley and Ham, 1981) containing 0.5% FBS. The cells were centrifuged at 180 x g for 5 min., the pellet was resuspended in complete HMEC medium, and then 4.5 x 10^4 cells were inoculated into each well (9 cm^2) of a six-well plate (Corning) in 2 ml of complete HMEC medium. After 24 hours, the medium was removed, the cells were washed twice with 2 ml/well of solution A and experimental media and factors were added as indicated in the legend to the figures. In the assay where the mouse anti-human EGF receptor monoclonal antibody, clone LA1 (Upstate Biotechnology, Inc., Lake Placid, NY), was used, the cells were incubated with 33nM of the antibody at room temperature for 5 min. prior to the addition of growth factors. The cells were then allowed to grow for 7 days with the media changed every other day. Cell numbers were determined at the end of the assay by trypsinizing cells from each well, resuspending in 1 ml of solution A with 0.5% FBS and counting in a hemocytometer. The growth assays for the tumor cell line Hs578T were essentially the same except that the basal medium used was MCDB 202a instead of MCDB 170, and the assay duration was 5 days.

Immunoprecipitation

Immunoprecipitation of the EGF receptor was performed as described (Lin *et al.*, 1990). Briefly, normal HMECs grown in the absence of EGF and A431 cells grown in DMEM plus 5% serum were collected and lysed in TG buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 1% Aprotinin) for 5 min. The cell lysates were centrifuged for 5 min to remove insoluble material. The supernatant was incubated for 2 hours with antibody (monoclonal anti-EGF receptor antibody, or polyclonal anti-EGF receptor antibody P3 kindly provided by Dr. G. M. Clinton at Oregon Health Sciences University) absorbed to protein A-Sepharose and then the beads were washed 3 times with TG buffer.

The immune complex was then incubated in a kinase reaction mixture (20 mM HEPES, pH 7.4, 2 mM DTT, 0.5% NP-40, 0.2 mM $MgCl_2$, 25 μM sodium vanadate, 1 mM ATP, and 10 μCi [γ-^{32}P]ATP, total volume 100 μl) at 34°C for 5 min. The reaction was stopped with cold M-RIPA buffer (Akiyama *et al.*, 1988). The beads/immune complexes were washed 3 times with M-RIPA to remove unincorporated label. The proteins were removed from the beads by boiling in Laemmli sample buffer for 2 min and fractionated by SDS polyacrylamide gel electrophoresis. Labeled proteins were visualized by autoradiography.

AR specific ELISA

For collecting conditioned medium, HMECs were plated at a density of 5 x $10^3/cm^2$ in complete HMEC medium and switched to medium lacking EGF the next day after extensive washing with solution A. Two days later, fresh medium was added and collected thereafter at 24 to 48 h intervals while cells were rapidly growing. Conditioned medium was stored at -20°C until use for detection of AR immunoreactivity. Mouse monoclonal antibody AR1 was raised against a peptide (amino acids 144-184) spanning the EGF-like domain of mature AR (Plowman *et al.*, 1990b) and was used for ELISA at 100 ng/well. AR containing samples (conditioned medium) were bound to the bottom of 96-well Falcon tissue culture plates in 0.1 ml 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate) by incubation at 37°C for 2 hours. The plates were subsequently processed as described (Cook *et al.*, 1991a). The optical density at A_{450} was determined for each sample on a microplate reader at the end of assay and the concentration of AR in the sample was calculated from a standard curve generated with purified 18 kD AR.

Results

Expression of amphiregulin in cultured human mammary epithelial cells

In a previous study, we found that amphiregulin mRNA was expressed at high levels in one normal HMECs strain while certain human mammary tumor cell lines expressed much lower levels of the gene (Cook *et al.*, 1991a). To determine whether this elevated expression of AR in normal cells is a general phenomenon, we examined four different strains (donors) of normal HMECs, one immortal mammary cell line and six human mammary tumor-derived cell lines. As shown in fig. 1, northern blot analysis of AR mRNA in normal HMECs and mammary tumor-derived cell lines revealed striking differences in the expression of this gene. All four strains of normal HMECs (HMEC 171, HMEC 172, HMEC 184 and HMEC 84) expressed a prominent AR mRNA transcript of 1.5 kb (fig. 1A). The abundance of this transcript is similar to that in normal human keratinocytes (fig. 1B, lanes b, c, d). Relatively minor hybridization to several other higher molecular weight transcripts was also detected in these cells. On the other hand, all mammary tumor-derived cell lines as well as the immortal cell line (HBL-100) (fig. 1B, lanes e-k) had greatly reduced levels of expression of this gene compared to that in the normal cells (fig. 1B, lanes c, d). No hybridization to this 1.5 kb transcript was detected in mRNA derived from the Hs578T (fig. 1B, lane f), or SKBR-3 (fig. 1B lane i) mammary tumor cell lines, even after longer exposure. AR mRNA was not detected in human fibroblasts (fig. 1B, lane a) as previously reported (Cook *et al.*, 1991a). Treatment of the tumor cell line MCF-7 with phorbol 12-myristate 13-acetate (PMA) for 24 hours greatly increased the level of AR gene expression in these cells (fig. 1B, lane l), confirming a previous report (Plowman *et al.*, 1990b). The levels of AR mRNA in PMA treated MCF-7 cells was similar to the levels of the gene expressed in normal HMECs.

Another EGF-like polypeptide growth factor, TGF-α, has also been reported to be

expressed in both normal and tumor-derived human mammary epithelial cells (Zajchowski *et al.*, 1988; Bates *et al.*, 1990; Valverius *et al.*, 1989; Bates *et al.*, 1988). Interestingly, when we examined the expression of TGF-α in several of the cell strains in which we detected AR, we found that the pattern of expression of TGF-α was similar. Normal HMECs (fig. 2, lane a-d) expressed much higher levels of TGF-α mRNA than the immortal cell line (HBL-100) or the tumor-derived cell lines (fig. 2, lane e-k). Hs578T, a tumor cell line which had no detectable AR mRNA as shown in fig. 1B, did not express detectable levels of TGF-α mRNA (fig. 2 lane g and h).

Effect of heparin on the EGF-independent growth of normal HMECs

Previous studies (Cook *et al.*, 1991a) have shown that cultured human keratinocytes produce high levels of AR mRNA and protein, and that AR acts as an autocrine mitogenic factor for these cells. In this study, we utilized a culture system that supports the EGF-independent growth of normal HMECs (Li and Shipley, 1991) to determine whether AR could be acting as an autocrine factor to support the EGF-independent proliferation of these cells. We plated HMECs at a density of $5 \times 10^3/cm^2$ in complete medium. The following day the cells were washed with buffered saline, the medium was replaced with basal medium supplemented with hydrocortisone and insulin, and various growth factors were added as indicated in the legend to figure 3. Heparin (10 μg/ml) was added to identical cultures. As we have previously demonstrated, cultured normal HMECs proliferated in the absence of EGF. As shown in fig. 3A, heparin completely blocked the EGF-independent growth of these cells, indicating that autocrine AR activity contributes to their proliferation. The addition of exogenous EGF or acidic FGF overcame heparin inhibition of growth, while basic FGF and BPE were not as effective. A similar experiment was performed on a mammary tumor-derived cell line, Hs578T, which can be grown in serum-free medium (Li and Shipley, 1991) and contains undetectable levels of AR mRNA (fig. 1B, lane f). As with the normal cells, the Hs578T cells proliferated in the absence of EGF. However, unlike the normal HMECs, the

EGF-independent growth of Hs578T cells was not blocked by the addition of heparin to the culture medium (fig. 3B).

Effect of an anti-human EGF receptor antibody on the EGF-independent growth of HMECs

It has been shown that AR competes with EGF for binding to the type-1 human EGF receptor (EGF-R) (Shoyab *et al.*, 1989; Cook *et al.*, 1991a). This observation led us to determine whether an anti-human EGF-R monoclonal antibody (mAb LA1) which blocks EGF/TGF-α-mediated mitogenicity in keratinocyte cultures (Cook *et al.*, 1991b) could block the EGF-independent proliferation of normal HMECs at high density. When 33 nM of mAb LA1 was added to the culture medium, EGF-independent growth was completely inhibited ($0.8 \pm 0.2 \times 10^4$ cells/well compared to $14.1 \pm 3.4 \times 10^4$ cells/well in the untreated control cultures). In fact, fewer cells were recovered at the end of the assay than were plated in the antibody-treated cultures. However, the cells were still capable of responding to EGF in the presence of the antibody, as increasing the level of EGF to 33 nM resulted in significant proliferation (fig. 4).

To determine whether mAb LA1 reacts only with a single receptor species on HMECs, we performed immunoprecipitation of HMEC lysates using this antibody and a previously characterized EGF-R antibody (P3). Analysis of the data shown in figure 5 demonstrates that the mAb LA1 only interacted with a single protein in normal HMECs with kinase activity. Molecular weight of this mAb LA1-precipitated protein was approximately 170 Kd. The mAb recognized a kinase of similar size on A431 cells (data not shown). A polyclonal antibody P3, raised against a 14 amino acid peptide corresponding to residues 1059-1072 at the carboxyl terminus of the EGF-R, precipitated a protein of identical molecular weight in both HMECs (fig. 5) and A431 cells (data not shown).

The inhibitory effect of mAb LA1 was specific for EGF-R-mediated mitogenic response. The antibody completely blocked the mitogenic effect of EGF and TGF-α in

normal human fibroblasts, but had no effect on the mitogenic activity of bFGF (data not shown). These results demonstrate that the inhibitory effect of the antibody is specific for ligands known to bind the EGF receptor.

Collectively, these results indicate that the EGF-independent proliferation of normal HMECs is dependent upon freely functional EGF receptors.

Detection of AR immunoreactivity in medium conditioned by normal HMECs

To further test the assumption that AR is an autocrine growth factor for HMECs, we determined the concentration of AR protein in the conditioned medium of HMECs by measuring AR immunoreactivity in an AR-specific ELISA (Cook *et al.*, 1991a). The cells were plated in complete medium, washed with saline solution and grown in medium lacking EGF. The medium conditioned by these cells was collected at intervals of 24 and 48 hours while the cells were rapidly growing. A monoclonal antibody raised against the carboxy-terminal EGF-like region (amino acid residues 144 to 184) of mature AR was used in the ELISA. The antibody is highly specific for AR and does not crossreact with other growth factors (EGF or TGF-α) in the EGF family (Cook *et al.*, 1991a). AR was detected in the HMEC conditioned medium at a concentration range of 0.141 ng/ml to 0.523 ng/ml (Table 1) as the cells proliferated and the density of the cultures increased. The results demonstrate that the normal HMECs synthesize AR protein and secrete this factor into the culture medium.

Discussion

Until recently, it was believed that normal cells do not produce growth factors to which they themself respond, and, thus, require exogenous (paracrine) growth factors for proliferation, while transformed cells produce and respond to mitogenic factors in an autonomous fashion. However, studies on the growth and maintenance of normal human epithelial cells in culture have clearly revealed that both normal human keratinocytes and mammary epithelial cells are capable of proliferation in the absence of exogenously added growth factors from the EGF or FGF families (Shipley *et al.*, 1989; Li and Shipley, 1991; Cook *et al.*, 1991b; Cook *et al.*, 1991a; Valverius *et al.*, 1989). Therefore, generalized theories which claim that only transformed cells produce and respond to autocrine growth factors are not applicable to some epithelial cell types.

Investigations into potential autocrine growth factors for human epithelial cells have implicated TGF-α as an active agent (Coffey *et al.*, 1987; Bates *et al.*, 1990). TGF-α and EGF-R mRNA were both detected in normal HMEC, although at relatively higher levels compared to several mammary tumor-derived cell lines (fig. 2, Zajchowski *et al.*, 1988). Furthermore, increased expression of TGF-α in normal HMECs appeared to be associated with the proliferation state of the cells. Although TGF-α was expressed at high levels in rapid growing HMECs, it was not detected in nonproliferating organoids from which the HMECs were derived (Bates *et al.*, 1990). It has also been shown that blocking the EGF receptor on the cell surface of HMECs with an antibody reduced the expression of TGF-α and inhibited cell growth, indicating the existence of an autocrine pathway mediated by the EGF-R (Bates *et al.*, 1990). However, no data to date have been able to directly demonstrate that TGF-α is an autocrine growth factor for normal HMECs.

Although normal HMECs produce both TGF-α and AR, we propose that AR is the primary autocrine mediator responsible for the EGF-independent growth of normal HMECs in culture based on the following findings: 1) normal HMECs in culture express

high levels of AR mRNA; 2) AR protein is detected in the medium conditioned by normal HMECs proliferating in the EGF-independent mode; 3) addition of heparin to the culture medium results in complete inhibition of EGF-independent cell growth. This is not a nonspecific effect of heparin toxicity in these cultures, because the addition of EGF or aFGF overcame the heparin inhibition of EGF-independent growth. To date, AR is the only known EGF-like growth factor whose mitogenic activity can be inhibited by heparin (Cook *et al.*, 1991a). Another EGF family member that we have not yet examined is heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama *et al.*, 1991). HB-EGF binds the EGF receptor and binds to immobilized heparin. Therefore, the possibility that HB-EGF may be involved in the autocrine growth of normal HMECs can not be ruled out at this time. However, there are no published data to date demonstrating that the biological activity of HB-EGF can be inhibited by heparin; 4) the EGF-independent growth of HMECs was completely abolished in the presence of the monoclonal antibody LA1, supporting the existence of an autostimulatory pathway for EGF-R-binding ligands. We do not believe that TGF-α is the primary mitogen responsible for the EGF-independent growth of HMECs because the mitogenic activity of TGF-α is not blocked by heparin (Cook *et al.*, 1991b). However, TGF-α could still be acting in concert with AR by regulating the expression of AR or by directly mediating signal transduction events in order to stimulate the autocrine growth of these cells.

The results of the current study suggest that two EGF-like factors (AR and TGF-α) are coordinately regulated in HMECs. When we compared the expression of AR and TGF-α mRNA in normal HMECs to that in mammary tumor-derived cells, we found that normal cells expressed much higher levels of both growth factor mRNAs than tumor cells. In the case of the Hs578T tumor cell line, neither AR nor TGF-α mRNA could be detected. The growth of this cell line was not affected by the addition of heparin (fig. 3B) or mAb LA1 (data not shown) to the culture medium. These results are curious as it is generally assumed that tumor cells would produce higher levels of growth factor mRNAs

than their normal counterparts. It has been reported that AR can act as a growth inhibitor for some human tumor-derived cell lines (Shoyab *et al.*, 1988). Thus, it may be the case that the mammary tumor cell lines do not express elevated levels of AR by virtue of the fact that AR, acting as a growth inhibitor, would impede the proliferation of these cells. On the other hand, it has been suggested that AR may elicit some of its biological activity via a receptor different from the EGF-R (Shoyab *et al.*, 1989; Cook *et al.*, 1991a; Plowman *et al.*, 1990a). Thus, differential expression of multiple receptors for AR by each cell type may be one of the mechanisms which determines how cells respond to AR.

We, as well as others, have demonstrated that treatment of several tumor cell lines with phorbol 12-myristate 13-acetate (PMA) increases AR mRNA expression in these cells (Plowman *et al.*, 1990b; fig. 1). In contrast, PMA significantly decreased AR mRNA expression in normal HMECs (data not shown), suggesting expression of the AR gene in normal and malignant cells may be differently regulated.

In summary, we have demonstrated that normal HMECs grown in culture produce both AR and TGF-α at levels much higher than those produced by several mammary tumor cell lines. Normal HMECs proliferate in culture autonomously when plated at high density and the autocrine growth of these cells is inhibited by heparin or an anti-EGF-R antibody. Therefore, we propose that AR is an important autostimulatory factor required for EGF-independent growth of normal mammary epithelial cells. Future investigations will be required to determine the role of AR in normal growth, development and differentiation as well as the pathogenesis of tumors and other diseases.

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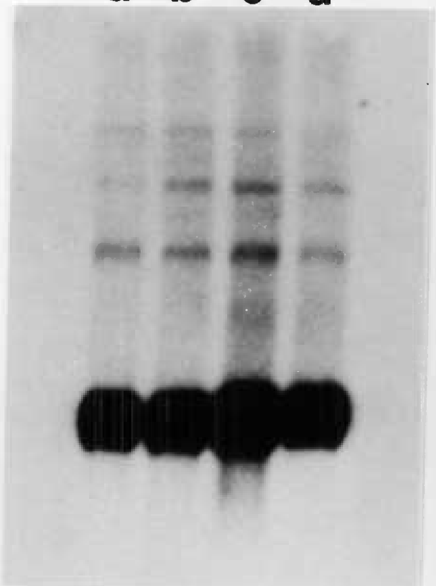
Figures and legends

Figure 1. Expression of amphiregulin mRNA in human mammary epithelial cells

Northern blot analysis of poly A^+ mRNA was performed as described in Materials and Methods. Poly A^+ mRNA was isolated from the following sources: Panel A, Lanes a-d: Normal HMEC strain 161 (a), 172 (b), 184 (c) and 48 (d) grown in complete HMEC medium; Panel B, Lane a: normal human fibroblasts grown in MCDB 202a supplemented with 5 ng/ml EGF, 1 μg/ml insulin and 100 μg/ml BSA. Lane b: normal human keratinocytes (strain 8) grown in standard medium (Cook *et al.*, 1991b) plus 10 ng/ml insulin-like growth factor type-1; Lanes c-d: normal HMEC strain 161 (c) and 48 (d) grown in complete HMEC medium; Lane e: immortal mammary cell line HBL-100 grown in MCDB 170 supplemented with 5% FBS; Lanes f-j: human mammary tumor cell lines Hs578T (f), BT474 (g), T47D (h), SKBR-3 (i) and ZR-75-1 (j) grown in medium supplemented with 5-10% serum. Lane k: tumor cell line MCF-7 grown in DMEM plus 10% FBS. Lane l: MCF-7 grown in serum-containing medium and treated with 100 ng/ml PMA for 24 hours prior to the isolation of poly A^+ mRNA. Migration of standard RNA size markers is indicated in kilobases (Kb). The blot shown in panel B was subsequently reprobed with a ^{32}P-labeled cRNA probe corresponding to the constitutively expressed cyclophillin (1B15) gene product (lower panel).


A.
a
b
c
d

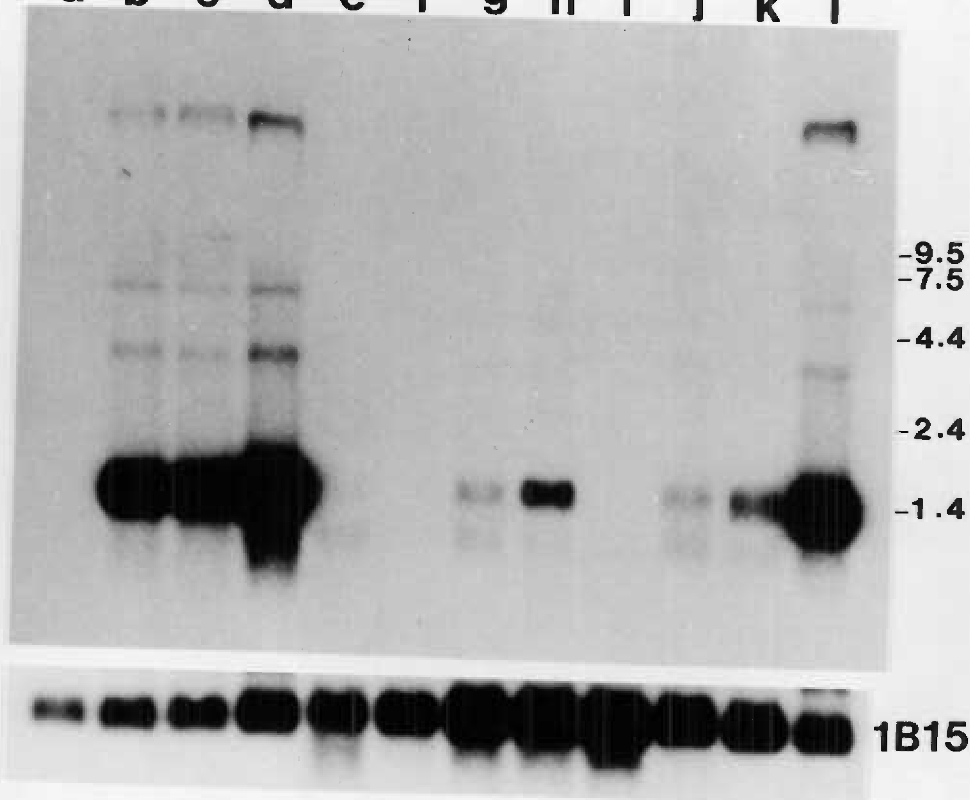

B.
a
b
c
d
e
f
g
h
i
j
k
l
-9.5
-7.5
-4.4
-2.4
-1.4
1B15

Figure 2. Expression of TGF-α mRNA in human mammary epithelial cells

Northern blot analysis of poly A^+ mRNA was performed as described in Materials and Methods. Poly A^+ mRNA was isolated from the following sources: Lanes a-d: normal HMEC strain 161 (a), 172 (b), 184 (c) and 48 (d) grown in complete HMEC medium; Lanes e-f: HBL-100 cells grown in MCDB 170 plus 5% FBS (e) or in complete HMEC medium lacking EGF and BPE (f); Lanes g-h: Hs578T tumor cell line grown in MCDB 202a plus 5% calf serum (g) or in complete MCDB 202a medium (h); Lanes i-k: tumor cell lines BT474 (i), MCF-7 (j), and T47D (k) grown in serum-containing medium, as described in Materials and Methods. The lower panel shows the same blot reprobed with cyclophilin (1B15) cRNA.

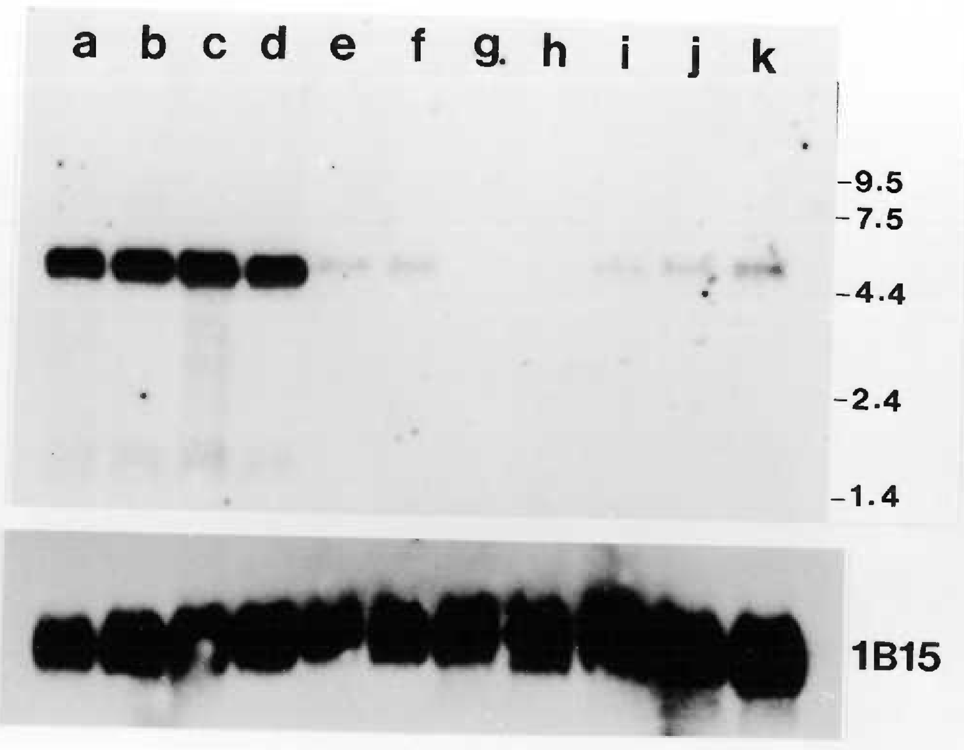
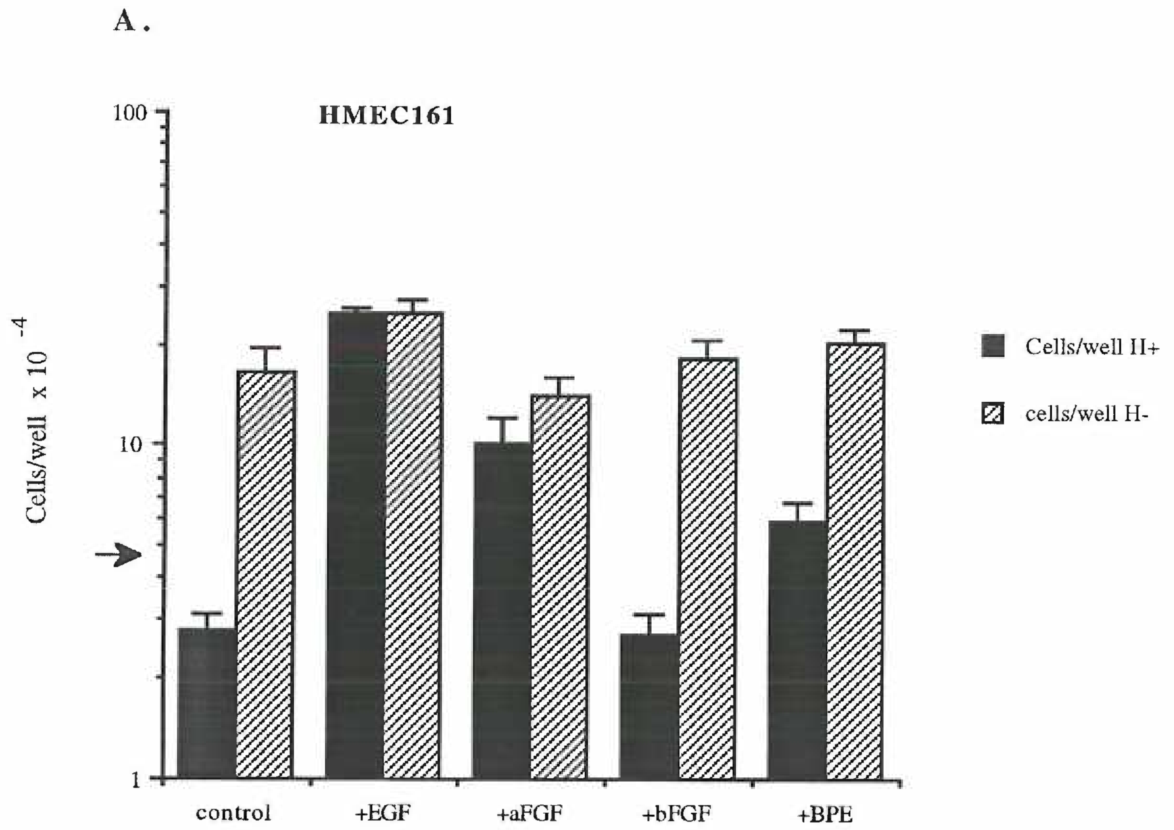

a b c d e f g h i j k
-9.5
-7.5
-4.4
-2.4
-1.4
1B15

Figure 3. Effect of heparin on the proliferation of normal and tumor-derived mammary epithelial cells

Panel A: normal HMECs (strain 161) were plated at a density of $5 \times 10^3/cm^2$ (4.5×10^4/well) in complete HMEC medium. The cells were washed on the following day and switched to MCDB 170 supplemented with 1.4×10^{-6} M hydrocortisone and 5 μg/ml insulin (control). EGF (5 ng/ml), aFGF (5 ng/ml), bFGF (5ng/ml) and BPE (0.4% v/v) were then added as indicated. The cells were allowed to grow in the absence (H-) or presence (H+) of heparin (10 μg/ml) for 6 days with the media changed every other day. The cell number in each well was determined at the end of assay as described in Materials and Methods. Each data point represents the mean $\pm$ standard deviation of triplicate cultures of the same condition. The arrow indicates the plating density.

Panel B: Hs578T mammary tumor cells were plated at a density of $5 \times 10^3/cm^2$ in 202a complete medium. On the next day the cells were washed and switched to experimental conditions as described in the legend for panel A. The cells were allowed to grow in the absence (H-) or presence (H+) of heparin (10 μg/ml) for 5 days. Medium changes and cell number determinations were performed as described in the legend for panel A.

A.



B.

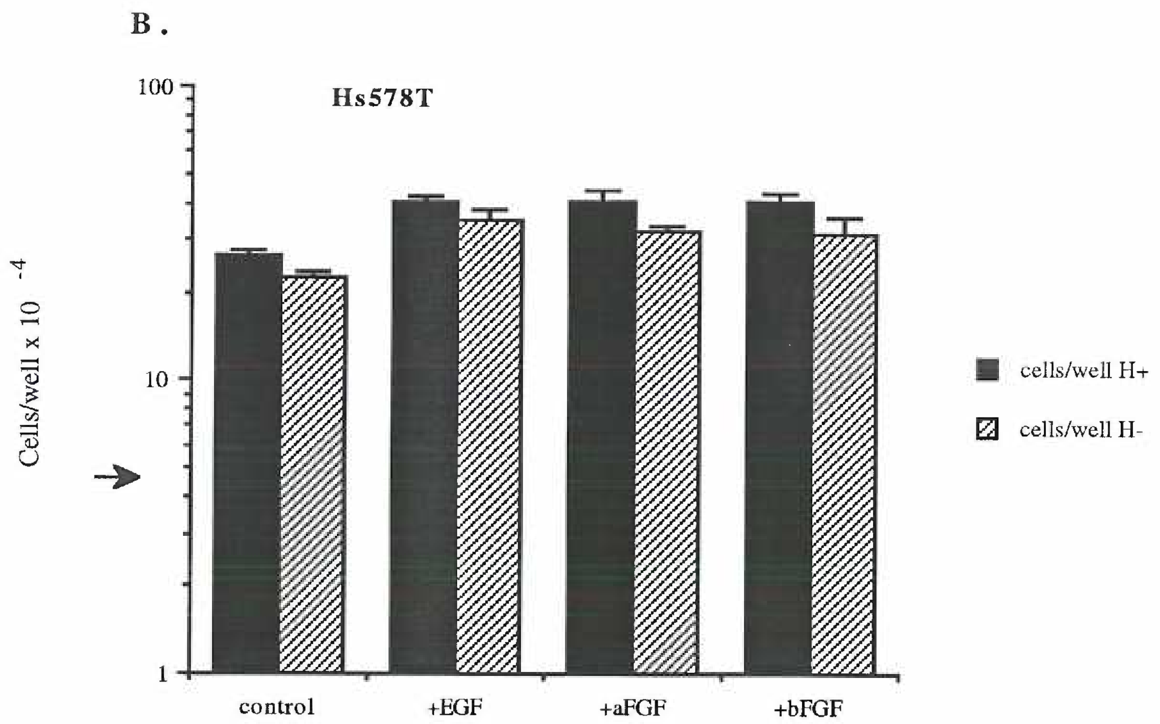
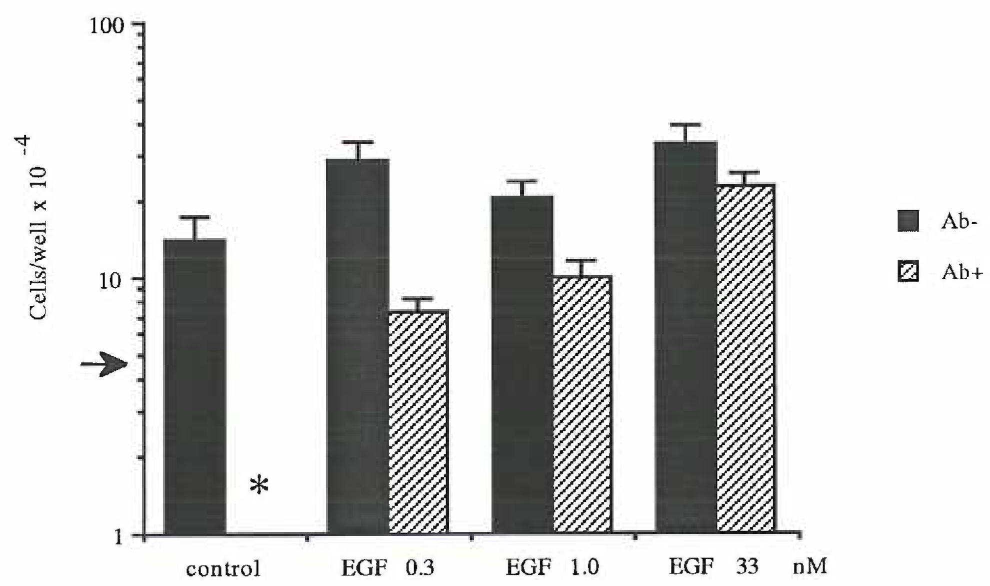


Figure 4. Effect of anti-human EGF receptor monoclonal antibody on the proliferation of normal mammary epithelial cells

Normal HMECs (strain 161) were plated and switched to the control medium as described in the legend to figure 3A. The cells were then allowed to grow in medium with different concentrations of EGF in the presence (Ab+) or absence (Ab-) of 33 nM mAb LA1 for 6 days. Medium changes and cell number determinations were performed as described in the legend for fig. 3A. * Cell number determined for control cells treated with antibody was $0.8 \pm 0.2 \times 10^4$ cells/well (not shown).



100
10
1
Cells/well x 10 -4
*
control
EGF 0.3
EGF 1.0
EGF 33
nM
Ab-
Ab+

Figure 5. Detection of EGF receptor in normal HMECs with anti-EGF receptor antibodies

Cell lysates were prepared from normal HMECs and immunoprecipitated with monoclonal anti-EGF-R antibody (LA1) or polyclonal anti-EGF-R antibody (P3). The protein in immune complex was labeled in a kinase reaction with [τ-^{32}P]ATP, fractionated by SDS polyacrylamide gel electrophoresis and visualized by autoradiography as described in Materials and Methods.

HMEC161

LA1 P3

Table 1. **Detection of amphiregulin in medium conditioned by normal HMECs**

Time of Collection of Conditioned Medium [1] (hours)	Amphiregulin [2] (ng / ml)
24	0.141 ± 0.001
48	0.293 ± 0.027
96	0.523 ± 0.020

[1] HMEC strain 161 was plated and grown in the medium lacking EGF as described in the Materials and Methods. Conditioned medium was collected and medium replaced at the hours indicated.

[2] Concentration of AR in conditioned medium was determined by ELISA as described in the Materials and Methods. Each data point represents the average of duplicate assays. For each assay, samples were serially diluted and the original AR concentration in the sample was calculated from the concentration determined in each diluted sample.

Expression and Regulation of mRNA

Coding for Amphiregulin in Human Mammary Epithelial Cells

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Running Title: **Amphiregulin in Mammary Epithelial Cells**

Abstract

Amphiregulin (AR) is a member in the epidermal growth factor (EGF) family. It is a bifunctional growth mediator for a variety of cell types in culture and its biological activity is inhibited by heparin. AR mRNA is expressed in normal human mammary epithelial cells (HMECs) while much lower levels of AR mRNA are found in mammary tumor-derived cell lines. AR has been shown to act as an autocrine growth factor for normal HMECs. In this study, we have examined the regulation of AR mRNA expression in both normal and tumor derived mammary epithelial cells. In normal HMECs, AR mRNA expression was not greatly decrease by cessation of cell growth associated with confluence while it was highly regulated by activation of the EGF receptor. A monoclonal antibody against the EGF receptor was used to show that endogenous ligands maintain high steady state levels of AR mRNA in these cells through binding to the EGF receptor. Treatment with phorbol ester (PMA) reduced the abundance of AR mRNA in normal HMECs while induced AR mRNA levels in tumor cells, indicating the mechanism(s) by which PMA regulates AR expression in normal HMECs may be different with that in mammary tumor cells. Moreover, a novel mRNA species was detected with an AR specific probe in all mammary tumor cell lines tested. Differential hybridization revealed that the transcript is a product of another gene with sequence homology to the 5' region of the AR cDNA. These results suggest that AR mRNA is differentially expressed and regulated in normal HMECs versus mammary tumor-derived cells. Therefore, AR and AR-related molecules may play different roles in growth regulations of normal or malignant cells.

Introduction

Amphiregulin (AR), a heparin-binding member of the epidermal growth factor (EGF) family, has been isolated from culture medium conditioned by both normal and malignant epithelial cells (Shoyab *et al.*, 1988; Cook *et al.*, 1991b). AR is synthesized as a 252 amino acid transmembrane precursor which is proteolytically processed to mature forms of 84 and 76 amino acids (Plowman *et al.*, 1990). The mature form(s) of AR has a domain which exhibits amino acid sequence homology to EGF. In addition, mature AR has a highly positively charged, Lys-Arg rich N-terminal hydrophilic region adjacent to a C-terminal EGF-like domain (Shoyab *et al.*, 1989). Another heparin-binding EGF-like growth factor, HB-EGF, which exhibits a high degree of sequence homology to AR has recently been described (Higashiyama *et al.*, 1991).

The mitogenic activity of AR appears to be cell-type specific. AR stimulates the growth of normal human keratinocytes (Cook *et al.*, 1991b), fibroblasts and some tumor cell lines (Shoyab *et al.*, 1988), but inhibits the proliferation of other tumor cell lines. A recent study on human ovarian cells has suggested that the responsiveness of cells to AR may depend upon the amount of AR to which the cells are exposed (Johnson *et al.*, 1991). AR competes with EGF for cell surface binding and may exert some of its biological effects through the EGF receptor type-1 (EGF-R) (Shoyab *et al.*, 1989). However, some studies have revealed that AR binds to EGF-R at lower affinity than EGF and that the cell-type specificities of AR mitogenic activity are different from other EGF-R ligands (Shoyab *et al.*, 1989; Shoyab *et al.*, 1988; Cook *et al.*, 1991b; Cook *et al.*, 1992b). These results suggest that AR may elicit some of its biological activity via some yet to be identified receptors.

The biological activity of AR is negatively regulated by binding to soluble heparin and other sulfated polysaccharides (Cook *et al.*, 1991b; Cook *et al.*, 1992b). Dextran

sulfates, heparin and heparan sulfate were potent inhibitors of AR mediated mitogenic activity in human keratinocytes and in a mouse fibroblast cell line (Cook *et al.*, 1992b). However, chondroitin sulfates B and C were only weakly inhibitory while hyaluronic acid and dextran were not inhibitory for AR activity (Cook *et al.*, 1992b). These results suggest that sulfation and polysaccharide structure are important in determining the ability of compounds to inhibit AR mitogenic activity. The ability of AR to compete for ^{125}I-EGF binding is abolished by heparin (Cook *et al.*, 1991b) suggesting that heparin inhibits AR mitogenic activity by keeping AR from binding to its receptor(s).

AR mRNA was detected in variety of normal human tissues such as ovary, placenta, colon, breast, pancreas, cardiac muscle, and testis (Plowman *et al.*, 1990). Cells cultured *in vitro* including human keratinocytes (Cook *et al.*, 1991b) and mammary epithelial cells (HMECs) (Li *et al.*, 1992) also express AR. Normal HMECs express high levels of AR mRNA and AR protein has been detected in the medium conditioned by these cells (Li *et al.*, 1992). Another member of the EGF family of growth factors, transforming growth factor type-alpha (TGF-α) is coordinately expressed with AR in these cells (Li *et al.*, 1992). Normal HMECs plated at high density in culture proliferate in medium lacking EGF-like growth factors (Li and Shipley, 1991; Li *et al.*, 1992; Valverius *et al.*, 1989) and, both AR and TGF-α have been implicated as autocrine growth factors responsible for the EGF-independent growth of these cells. Because the EGF-independent growth of normal HMECs is inhibited by heparin, we have suggested that AR may be the predominant autocrine factor controlling the growth of these cells (Li *et al.*, 1992).

AR has also been reported to be expressed in primary colon and stomach tumors (Ciardiello *et al.*, 1991; Cook *et al.*, 1992a) and in tumor cell lines including cells derived from ovarian (Johnson *et al.*, 1991), colon (Ciardiello *et al.*, 1991) and mammary tumors (Li *et al.*, 1992; Plowman *et al.*, 1990). In one study, the majority of the colon cancer cell lines express high levels of AR mRNA and more than 50% of primary colon carcinoma

while only 14.8% of normal colon mucosa express AR mRNA (Ciardiello *et al.*, 1991). In contrast, the expression levels of AR mRNA in tumor-derived mammary epithelial cells in culture is substantially lower than that in normal HMECs (Cook *et al.*, 1991b; Li *et al.*, 1992). Interestingly, TGF-α mRNA level is also lower in mammary tumor cell lines than in normal cells (Li *et al.*, 1992; Zajchowski *et al.*, 1988).

The purpose of this study was to identify factors that modulate AR mRNA expression in normal HMECs in an attempt to begin to understand how AR and TGF-α may, in a coordinated fashion, regulate the growth of normal HMECs. We have also examined AR mRNA expressed in mammary tumor cell lines in an initial effort to understand the underlying mechanisms of regulation of this gene and its function in tumor cells.

Materials and Methods

Media and supplements

Medium MCDB 170 and MCDB 202a were prepared from stock chemical components as previously described (Hammond *et al.*, 1984; McKeehan and Ham, 1977). Minimum essential medium (MEM) was purchased from Gibco Laboratories (Grand Island, NY). Human recombinant epidermal growth factor (EGF) was purchased from Amgen Biologicals (Thousand Oaks, CA). Human recombinant transforming growth factor alpha (TGF-α) was a generous gift from Dr. R. J. Coffey (Vanderbilt University, Nashville, TN). Heparin (from porcine intestinal mucosa), insulin, hydrocortisone, ethanolamine, and phosphoethanolamine were all purchased from Sigma Chemical Co. (St. Louis, MO). Bovine pituitary extract (BPE) was obtained from Clonetics Corp. (San Diego, CA). The methods for preparation of the growth factors and other additives were described previously (Shipley *et al.*, 1989). Mouse anti-human EGF receptor monoclonal antibody (mAb LA1) was purchased from Upstate Biotechnology, Inc., (Lake Placid, NY). Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co.) was dissolved in DMSO at concentration of 2 mg/ml, stored at -80°C and diluted in DMSO to 1,000x the final concentration before use.

Cell culture

Normal human mammary epithelial cells (HMECs) were generously provided by Dr. M. Stampfer (Lawrence Berkeley Laboratories, Berkeley, CA). The HMECs used in the experiments reported here were from passages 8-10. HBL-100, an immortal cell line established from normal mammary epithelial cells, and four human mammary tumor cell lines (MCF-7, T47-D, Hs578T and ZR-75-1) were purchased from the American Type Culture Collection (Rockville, MD). Two mammary tumor cell lines (BT474 and SKBR-3) were supplied by Dr. G. M. Clinton (Oregon Health Sciences University, Portland, OR).

Stock cultures of normal HMECs were maintained in complete HMEC medium which is MCDB 170 supplemented with 0.4% BPE, 5 ng/ml EGF, 5 μg/ml insulin, 1.4 x 10^{-6} M hydrocortisone, 1 x 10^{-4} M ethanolamine, and 1 x 10^{-4} M phosphoethanolamine (Hammond *et al.*, 1984; Li and Shipley, 1991). Tumor cell lines (MCF-7, SKBR-3 and ZR-75-1) were grown in MEM supplemented with 5-10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, Utah). Other tumor cell lines (BT474, T47D and Hs578T) were maintained in medium MCDB 202a supplemented with 5% FBS. In the experiments for northern blot analysis, normal HMECs were plated at 5 x 10^3/cm^2 in 100-mm culture plates in complete HMEC medium. After 24-48 hours, the medium was removed, the cells were washed twice with 10 ml of buffered saline solution A (Shipley and Ham, 1981) and fresh medium lacking EGF and BPE was added. The cells were allowed to grow with the medium replenished every other day until cells in the cultures reached 70-80% confluence (rapidly growth) or 100% (complete) confluence. Cells were then treated with growth factors or other reagents and poly A^+ RNA or total RNA was isolated after the length of time indicated in the figure legends. All tumor cell lines were plated and grown in the same media utilized for stock cultures and poly A^+ RNA was isolated when cells in culture reached 80-90% confluence.

Preparation of probes

Single stranded, ^{32}P-labeled cRNA probes specific for AR or TGF-α were used for hybridization with northern blots. A plasmid, pTZ/AR-2 (Cook *et al.*, 1991b), containing 880-bp EcoRI/Hind III restriction fragment encoding the entire AR coding region, was used or modified for use as templates for generating various AR probes (fig. 1). *in vitro* transcription reactions were carried out as described (Melton *et al.*, 1984) using EcoRI or BstEII linearized pTZ/AR-2 as a template to generate antisense AR^{1-880} and $AR^{213-880}$ probes respectively. To generate cRNA probes corresponding to various 5' AR regions, the pTZ/AR-2 was modified by restriction endonuclease digestion to eliminate

partial 3' sequence of the AR cDNA, and the vectors religated to form the following constructs (see fig. 1): pTZ/AR88 contains only a 88 bp EcoRI/NarI fragment of 5' AR region and served as a template for generating AR^{1-88} probe; pTZ/AR213 consists of a 213-bp EcoRI/BstEII fragment of 5' sequence and was used as a template for synthesizing AR^{1-213}, AR^{88-213} and $AR^{125-213}$ probes; pTZ/AR291 contains a 291-bp EcoRI/AvaI fragment of 5' sequence and was used for generating $AR^{231-291}$ probe.

TGF-α cRNA probe was synthesized from a cDNA insert in plasmid SP65 (Coffey R.J.Jr. *et al.*, 1987). The cDNA insert corresponds to the complete TGF-α coding sequence (Derynck *et al.*, 1984). A cRNA probe corresponding to a constitutive gene, cyclophilin (1B15), was synthesized from a 680-bp cDNA insert in plasmid SP65-1B15 (Danielson *et al.*, 1988).

Northern blot analysis

Poly A^+ RNA was isolated from cells as described previously (Schwab *et al.*, 1983; Sternfeld *et al.*, 1988). Total RNA was isolated using method described by Gough (Gough, 1988). Poly A^+ (2.5-5 μg) or total (20 μg) RNA was subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH) in 10x SSC by capillary transfer and cross-linked to the membrane using an UV Stratalinker (Stratagene, La Jolla, CA). Northern blots were hybridized with 1-5 x 10^6 cpm/ml of cRNA probe for 16-20 hours at 60°C or indicated temperature in a hybridization mixture (50% formamide, 5x SSC, 0.1% SDS, 10x Denhardt's solution, 50 μg/ml polyadenosine, and 250 μg/ml herring sperm DNA). After hybridization, the blots were briefly rinsed three times with 5x SSC at room temperature and washed in 300 ml of a washing buffer (0.1x SSC, 5 mM EDTA, and 0.1% SDS) for 2-4 hours at 65°C with two changes of the washing buffer. Transcripts hybridized to the probe were visualized by exposure to Kodak X-Omat AR film. Some blots were subsequently hybridized with the 1B15 cRNA probe in order to visualize any variability in the loading and transfer of the RNA samples. 1B15 mRNA, encoding the

cyclophilin protein, has been reported to be constitutively expressed in human cells in a wide variety of culture conditions (Danielson *et al.*, 1988; Sternfeld *et al.*, 1988; Cook *et al.*, 1990) and was used to estimate the relative expression of growth factor mRNA.

Southern blot analysis

High molecular weight genomic DNA was isolated from cultured cells using the method described (Blin and Stafford, 1976). DNA (15 μg) was digested, analyzed on 0.8% agarose gels and transferred to Nytran membrane in 10x SSC. The blots were hybridized at 42°C for 18-20 hours in a hybridization buffer (50% formamide, 5x SSC, 1% SDS, 5x Denhardt's solution, 250 μg/ml herring sperm DNA, 25 μg/ml polyadenosine and 25 mM Na_2HPO_4) with 5 x 10^6 cpm/ml of AR specific probe (AR^{1-213}). Filters were then washed in 0.1x SSC, 1% SDS at 60°C and exposed to Kodak X-Omat AR film.

Results

Expression and regulation of AR mRNA in normal HMECs

Based on our previous result that AR is an important growth factor responsible for the autonomous growth of normal HMECs (Li *et al.*, 1992), we hypothesized that AR gene expression could be linked to the growth state of the cells, i.e. high in rapidly growing cultures and low in confluent slowly dividing cultures. To test this hypothesis, we examined AR mRNA expression in normal HMECs grown to different cell densities in the absence of EGF. As shown in figure 2, cells examined 2 days after complete confluence had less AR mRNA compared to the cells that were rapid growing, although the difference is not as great as we anticipated. Cessation of rapid growth associated with confluence of the cells did not result in a drastic reduction in the steady-state level of AR mRNA in these cells. Similar results were obtained on mRNA accumulation of TGF-α in these cells grown at different densities (fig. 2).

To investigate whether EGF-R occupancy by AR or TGF-α could mediate the expression of AR mRNA, we tested the effect of a blocking antibody specific for the EGF-R (mAb LA1) on the expression of AR mRNA in normal HMECs. The antibody has been shown to block the mitogenic effect of EGF and TGF-α in human fibroblasts and to block the EGF-independent autonomous growth of normal HMECs (Li *et al.*, 1992). Treatment of EGF-independent, rapidly growing cells with mAb LA1 for 24 hours resulted in a striking reduction of AR mRNA level (fig. 3). The effect of the antibody was reversible if an excess amount of exogenous EGF-R ligand, TGF-α, was added together with the antibody (data not shown). Furthermore, treatment of confluent cells with soluble heparin, which inhibits the binding of AR to the EGF-R, greatly reduced AR mRNA level in these cells (fig. 4). AR mRNA down-regulation by heparin could also be reversed by including TGF-α in the medium (fig. 4). Interestingly, heparin had less effect on AR mRNA levels in rapidly growing cells (figure 3).

The same RNA was examined by northern blot analysis for expression of TGF-α mRNA. As demonstrated in the lower panels of figures 3 and 4, TGF-α mRNA expression in these cells parallels that of AR when cells are treated with mAb LA1, heparin and TGF-α. Collectively, these results suggest the importance of the EGF-R occupancy by endogenously produced ligands in maintaining high levels of both AR and TGF-α mRNA in normal HMECs.

To determine whether active phorbol esters, which up-regulate AR gene expression in mammary tumor-derived cells (Plowman *et al.*, 1990; Li *et al.*, 1992), have any effect on AR mRNA levels in normal HMECs, we treated these cells with phorbol 12-myristate 13-acetate (PMA). The AR mRNA level in these cells was significantly decreased by treatment with PMA for 24 hours (fig. 3). In contrast, PMA treatment did not result in a reduced level of TGF-α transcripts in these cells (fig. 3).

Expression of AR and a AR-related gene in tumor-derived HMECs

In our previous studies, we have demonstrated that immortal and tumor-derived mammary epithelial cell lines express significantly lower amounts of AR mRNA when compared with normal HMECs (Cook *et al.*, 1991b; Li *et al.*, 1992). In addition, we and others (Plowman *et al.*, 1990; Li *et al.*, 1992) have shown that, unlike in normal HMECs, PMA induces AR mRNA in tumor-derived mammary cell lines (Plowman *et al.*, 1990; Li *et al.*, 1992). In MCF-7 cells, PMA treatment resulted in increased accumulation of AR transcripts as well as secretion of AR protein (Li *et al.*, 1992; Shoyab *et al.*, 1988; Plowman *et al.*, 1990). Southern blot analysis of genomic DNA showed no evidence for amplification or gross rearrangements of the AR gene in MCF-7 cells. The restriction endonuclease patterns (EcoRI and Hind III) of DNA isolated from MCF-7 cells or normal HMECs is shown in fig. 5. These results indicate that the difference in the levels of AR transcripts between normal and tumor cells is not due to alterations in the AR gene.

During the course of our study, we found that all of the tumor cell lines examined expressed another mRNA species which hybridized to a cRNA probe encoding the full

length AR coding region (AR^{1-880}) (fig. 1). This mRNA species migrated slightly faster than the AR mRNA with an estimated size of 1 kb. To determine whether this mRNA species was a truncated form of the AR mRNA or a transcript from an AR-related gene, we probed similar blots both at higher stringency and with a truncated AR cRNA. Figure 6 a & b shows that at higher hybridization temperature (65°C), the AR^{1-880} probe no longer hybridizes to the lower molecular weight RNA species from the tumor cells while it still hybridizes to the AR mRNA. When the same experiment was performed with a probe that was truncated at 5' end of the coding sequence ($AR^{213-880}$ probe) (fig. 1), the smaller RNA species was not detected even at the lower hybridization temperature (fig. 6 c & d). On the other hand, a cRNA probe encoding 213 bp of the 5' coding region (AR^{1-213}) hybridizes strongly to the smaller mRNA species at 60°C but does not hybridize at 65°C (fig. 6 e & f). Thus, we believe that the lower molecular weight mRNA species present in the mammary tumor cell lines has homology with but is not identical to the 5' end of the AR transcript. We have called this transcript AR-like (ARL) for ease of description.

To further define the region of homology between AR and ARL mRNA, we probed northern blots with probes complementary to different regions within the 5' end (213 bp) of the AR transcript. The results shown in figure 7 a-d demonstrated that all three probes (AR^{1-88}, AR^{88-213} and $AR^{125-213}$) hybridized to ARL mRNA with different intensities, indicating that the homology between AR and ARL exists throughout the 213 bp 5' end of our AR construct. A probe complementary to $AR^{213-291}$ (fig. 1) failed to hybridize to ARL mRNA (fig. 7e) indicating that the homology between AR and ARL decreases at or about this point in the AR sequence.

Discussion

Polypeptide growth factors are present in a variety of tissues and are synthesized by various cell types grown in culture. It is believed that growth factors are involved in the control of growth and differentiation of the cells which produce them (autocrine activity) and/or adjacent cells (paracrine activity). Alteration in production of or responsiveness to these growth factors may result in pathological states including malignant transformation (Goustin *et al.*, 1986). Production of an autocrine growth factor has been thought to be a characteristic of transformed cells and a loss of growth control due to continuous autocrine stimulation of cell proliferation has been shown in some tumor cells (Bates *et al.*, 1988; Goustin *et al.*, 1986; Smith *et al.*, 1987). The ability to culture normal human epithelial cells under highly defined conditions has made it possible to study whether normal cells produce autocrine factors. A number of studies have clearly demonstrated that normal human epithelial cells (keratinocytes and mammary cells) proliferate in culture in an autocrine fashion (Shipley *et al.*, 1989; Cook *et al.*, 1991a; Cook *et al.*, 1991b; Li and Shipley, 1991; Li *et al.*, 1992; Valverius *et al.*, 1989). These studies suggest that autocrine growth factors play important role(s) in normal cellular proliferation. Thus, the regulation of production of these autocrine growth factors could be crucial in the process of controlling normal proliferation.

AR is an important growth factor which supports the EGF-independent growth of normal HMECs in culture, and this autocrine stimulatory activity of AR appears to require a functional EGF-R (Li *et al.*, 1992). In the present study, we have used an anti-EGF-R antibody to show that AR mRNA expression in normal HMECs is mediated by EGF-R occupancy. The effect of the antibody could be overcome by adding an excess amount of the EGF-R ligand, TGF-α, to the culture medium indicating that i) the antibody effect is specific and ii) TGF-α enhances the accumulation of AR mRNA by binding to the EGF-

R. EGF-R occupancy is also important for keeping the mRNA levels of TGF-α high. This result is in agreement with the studies reported previously by Bates *et al.* (Bates *et al.*, 1990). The auto-induction mechanism for TGF-α has been documented in normal human keratinocytes (Coffey R.J.Jr. *et al.*, 1987) as well as in other types of tissues and cells (Mead and Fausto, 1989; Mueller *et al.*, 1989).

Endogenous production of AR appears to regulate its own mRNA level and TGF-α mRNA level. This was demonstrated by experiments in which confluent cultures of HMECs in the absence of EGF were treated with heparin. In these cultures, the levels of AR and TGF-α mRNA were significantly decreased compared to untreated cultures. Because heparin inhibits the binding of AR to the EGF-R (Cook *et al.*, 1991b), these results suggest that AR protein, acting through the EGF-R (or other receptors), maintained high levels of AR/TGF-α mRNA in confluent HMECs. Unlike in the confluent cultures, heparin did not greatly decrease AR/TGF-α mRNA levels in rapidly growing HMECs. One explanation for this result could be that endogenous TGF-α levels decrease in confluent cells while AR levels do not decrease at confluency. Thus, in subconfluent cultures, TGF-α may maintain AR (and TGF-α) mRNA levels in the presence of heparin, whereas in confluent cultures, heparin inhibition of AR binding causes a reduction in AR (and TGF-α) mRNA levels. Further studies determining the TGF-α and AR protein concentration in the media conditioned by both rapidly growing and confluent cultures should help to clarify this issue.

Taken together, our results suggest that secretion of AR and TGF-α in normal HMEC, the binding of both factors to the EGF-R or other cell surface receptors (AR), and stimulation of cell proliferation, would be accompanied by a further induction in the levels of AR and TGF-α. This autoregulation mechanism has been suggested in other growth factor systems (Paulsson *et al.*, 1987; Warner *et al.*, 1987; Richmond *et al.*, 1988). The positive amplification of AR and TGF-α production may play a key role in the growth of normal HMECs and may reflect a mechanism for the development of mammary gland *in vivo*.

The effect of phorbol esters (PMA) on the expression of AR or TGF-α mRNA is different in normal HMECs than in tumor-derived cells. PMA greatly decreased the level of AR mRNA while it had no effect or slightly increased the levels of TGF-α mRNA. It has been shown that PMA inhibited the growth of normal HMECs in culture (Bates *et al.*, 1990). It could be that PMA exerts its inhibitory activity through down-regulating the production of AR. Our results showed that TGF-α mRNA accumulation in normal HMECs was at least not decreased by PMA, indicating different mechanisms for PMA-mediated regulation on the expression of AR or TGF-α.

In contrast with its activity on AR expression in normal HMECs, PMA greatly induced the expression of AR mRNA in certain mammary tumor-derived cell lines (Plowman *et al.*, 1990; Li *et al.*, 1992). Southern blot analysis suggests that there is no gross rearrangement of the gene in tumor-derived cells (fig. 5) (Plowman *et al.*, 1990). These results suggest that PMA-mediated regulation of AR mRNA expression is not due to alterations of the AR gene. A PMA responsive promotor in the 5' regulatory region of the AR gene has been identified (Plowman *et al.*, 1990). Close analysis of this promotor region revealed that it lacked the consensus PMA-responsive element sequence (Plowman *et al.*, 1990). Similar experiments could be conducted in normal HMECs to help elucidate the mechanism by which PMA regulates AR mRNA expression in these cells.

With the AR probe, we have identified another mRNA species (ARL) which appears to be preferentially expressed in mammary tumor-derived cell lines. ARL is not a truncated form of AR due to alternative splicing or polyadenylation because AR probes that hybridize to ARL under standard conditions do not hybridize to ARL under more stringent conditions while they still hybridize to AR. Thus, ARL is a product of another gene with sequence homology to AR. The region of homology is located at the 5' of the AR gene encoding the untranslated region, signal peptide and the part of N-terminal AR precursor. Further characterizations of the properties of ARL can be performed when sequence information becomes available. The fact that ARL appears to be specifically

expressed in mammary tumor cells implicates a role of ARL in mammary tumorigenesis and opens the possibility of using ARL as a marker for mammary tumors.

In summary, we have demonstrated in our present studies that the expression of AR mRNA in normal HMECs is highly dependent upon the occupancy of EGF-R and that AR itself and TGF-α both can regulate the level of AR mRNA by binding to the EGF-R. The expression of AR in tumor-derived HMECs is differently regulated by agent like PMA compared to their normal counterpart. Moreover, we reported, for the first time, that mammary tumor cells in culture express an AR-related gene which shares sequence homology to AR. Further studies will aim at understanding the molecular mechanisms for differential expression and regulation of the AR gene in normal and tumor-derived HMECs and the role of AR and its related gene in the growth and development of mammary tumors.

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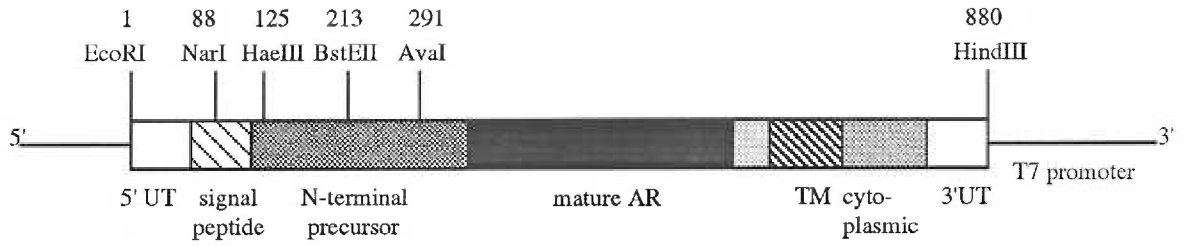
Figures and legends

Figure 1. Map of plasmid pTZ/AR-2 and various probes generated from this plasmid.

(a) The cDNA cloned into plasmid pTZ19 that encodes the entire AR coding region and adjacent 5' and 3' non-coding sequences is drawn to scale in a 5'-to-3' orientation. Shaded boxes represent protein domains. The restriction sites used for generating probes are shown with numbers indicating the position of these sites in the nucleotide sequence. The solid lines between 5' and 3' represent partial plasmid sequence and the position of a T7 promotor, where synthesis of probes is initiated, is indicated. (b) The length of various AR cRNA probes is represented by solid lines with the first and last nucleotides numbered. The corresponding names used in the text for the probes are listed on the left.

a.



b.

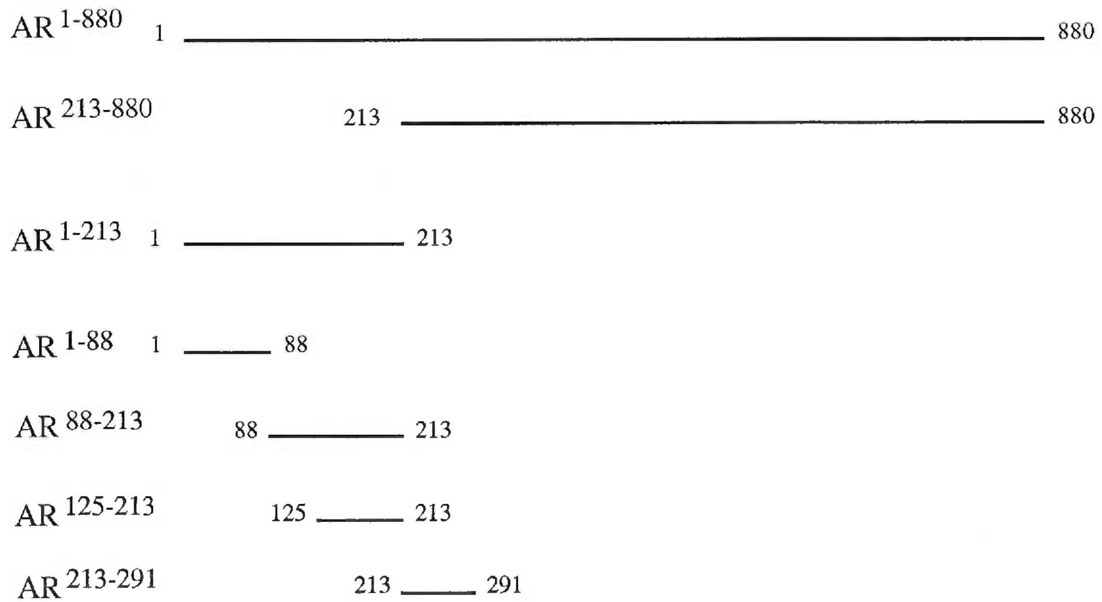


Figure 2. AR and TGF-α mRNA expression in growing and confluent normal HMECs.

Northern blot analysis was performed as described in the Materials and Methods. Normal HMECs were grown in the medium lacking EGF and total RNA was isolated when cells in culture reached the densities indicated. The blots were initially hybridized with an AR probe (AR^{1-880}) or a TGF-α probe (upper panels) and subsequentially hybridized with a 1B15 probe (lower panels).

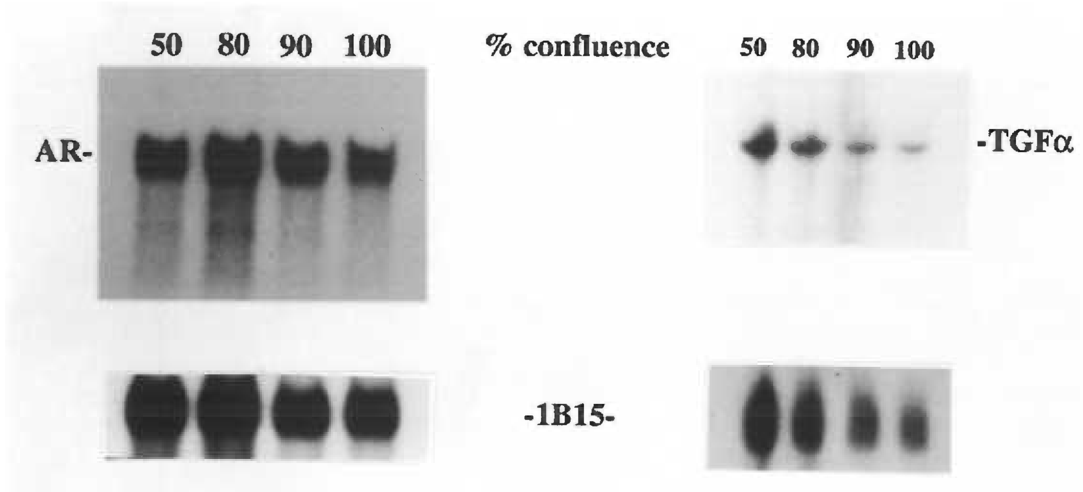

50 80 90 100
% confluence
50 80 90 100
AR-
-TGFα
-1B15-

Figure 3. Regulation of AR and TGF-α mRNA expression in subconfluent normal HMECs.

Northern blot analysis was performed as described in the Materials and Methods. Normal HMECs were grown in the medium lacking EGF until they reached 70% confluence. Prior to poly A^+ mRNA isolation, some cultures received no treatment (control), or 10 nM of the EGF-R blocking antibody (mAb LA1), or 10 μg/ml of heparin for 48 hours; or 100 ng/ml of phorbol ester (PMA) for 24 hours as indicated. The blot was hybridized with $AR^{1\text{-}880}$ (upper panel) and subsequentially with the TGF-α probe (bottom panel) and the 1B15 probe (middle).

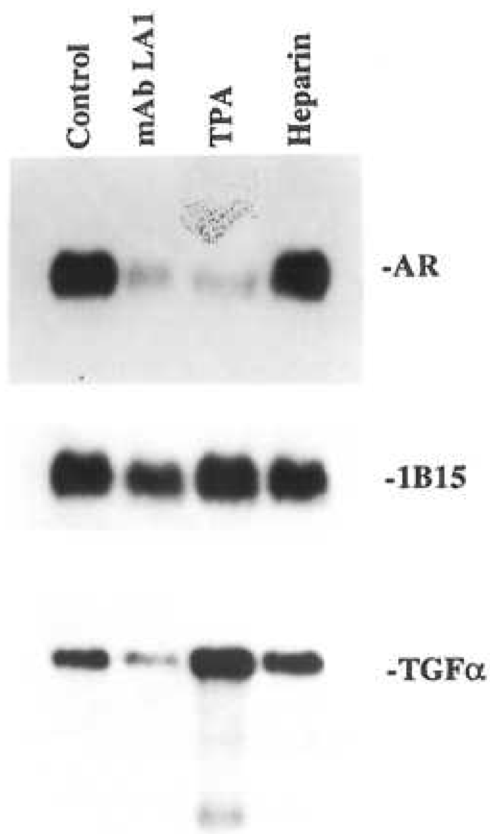

Control
mAb LA1
TPA
Heparin
-AR
-1B15
-TGFα

Figure 4. Regulation of AR and TGF-α mRNA expression in confluent normal HMECs.

Northern blot analysis was performed as described in the Materials and Methods. Normal HMECs were grown in the medium lacking EGF until they reached to complete confluence. Prior to poly A^+ mRNA isolation, some of the cultures received no treatment (control), or 10 μg/ml heparin (Hep), or Hep plus TGF-α (1 nM) for 24 hours. Duplicated blots were hybridized with $AR^{1\text{-}880}$ (upper panel) or with TGF-α probe (lower panel). Both blots were then hybridized with the 1B15 probe.

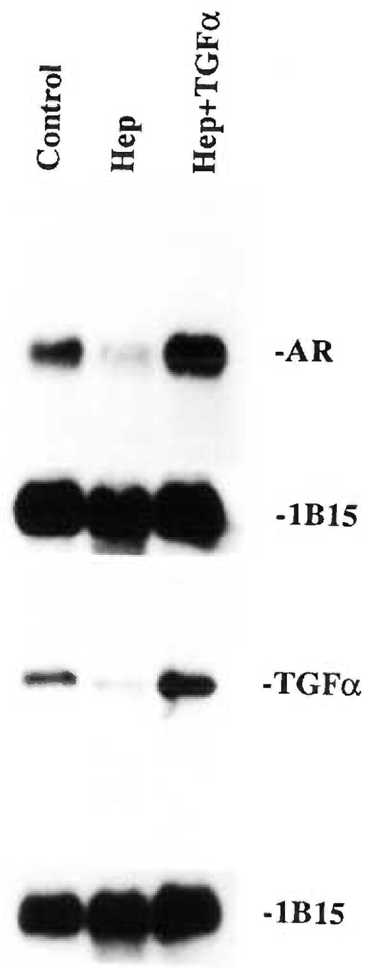

Control
Hep
Hep+TGFα
-AR
-1B15
-TGFα
-1B15

Figure 5. Southern blot analysis of genomic DNA from normal and tumor-derived mammary epithelial cells.

High molecular weight DNA (15 μg) was digested with EcoR1 or Hind III and the southern blot prepared as described in the Materials and Methods. The blot was hybridized with AR^{1-213} riboprobe and washed and autoradiography performed as described in the Materials and Methods. lane 1, HMEC 161; lane 2, MCF-7.

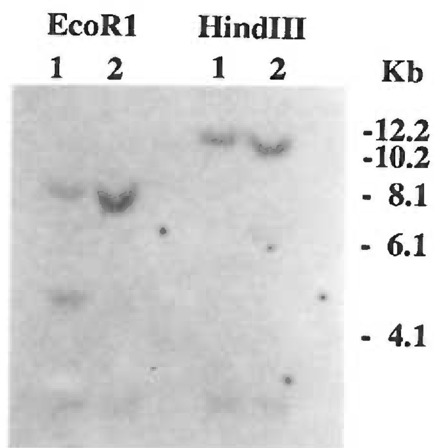
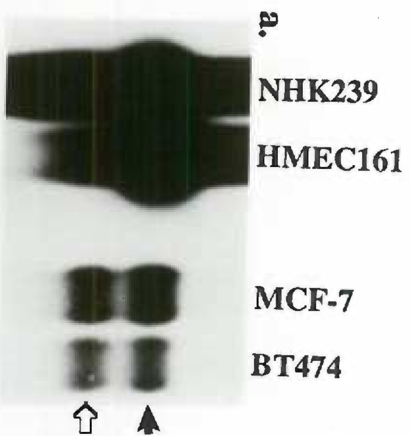

EcoR1
HindIII
1
2
1
2
Kb
-12.2
-10.2
- 8.1
- 6.1
- 4.1

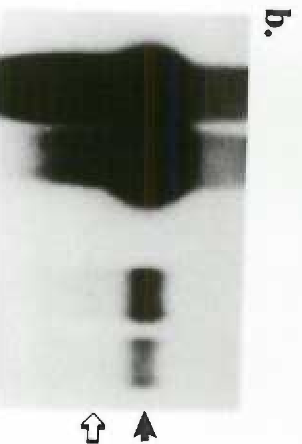
Figure 6. Expression of AR and ARL gene in normal and tumor-derived mammary epithelial cells.

Duplicates northern blots of poly A^+ mRNA from normal and malignant mammary epithelial cells were prepared as described in the Materials and Methods. Probes are described in figure 1. Panel a and b: Duplicated blots were hybridized with AR^{1-880}, the full length probe, at 60°C (panel a) or 65°C (panel b). Panel c and d: Duplicated blots were hybridized at 60°C with AR^{1-880} (panel c) or with $AR^{213-880}$, a truncated probe (panel d). Panel e and f: Duplicated blots were hybridized with AR^{1-213} at 60°C (panel e) or 65°C (panel f). The filled arrow indicates AR specific mRNA and the open arrow indicates the ARL gene transcript (see text).

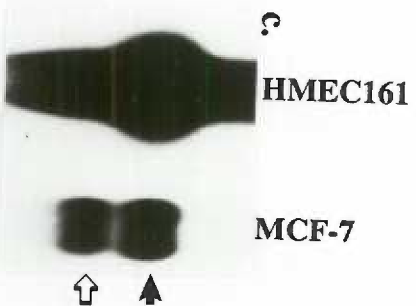
a.



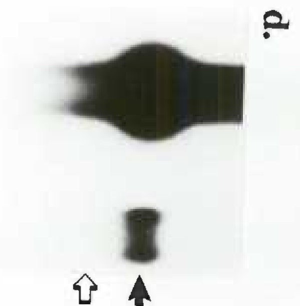
b.



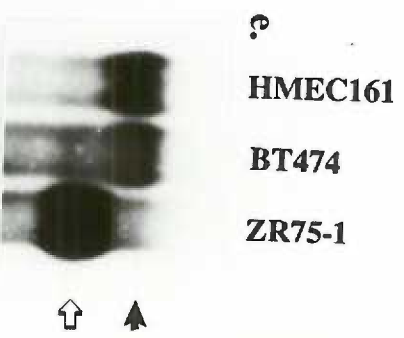
c.



d.



e.



f.

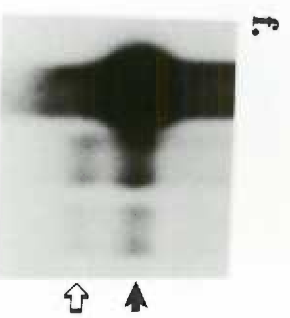
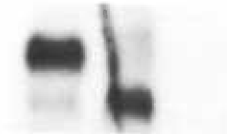


Figure 7. Localization of homology between AR and ARL mRNA.

Northern blot analysis was performed as described in the Materials and Mathods. Probes are described in figure 1. Northern blots with poly A^+ mRNA isolated from normal HMECs (lane 1) or MCF-7 cells (lane 2) were hybridized with (a) AR^{1-88} cRNA probe at 60°C; (b) AR^{1-88} at 65°C; (c) AR^{88-213} at 60°C; (d) $AR^{125-213}$ at 60°C; and (e) $AR^{213-291}$ at 60°C.

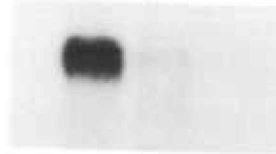
a. AR^{1-88} 60^0C

1 2



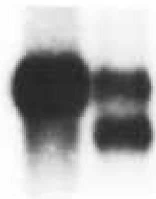
b. AR^{1-88} 65^0 C

1 2



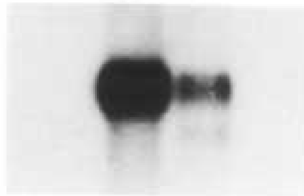
c. AR^{88-213}

1 2



d. $AR^{125-213}$

1 2



e. $AR^{213-291}$

1 2

DISCUSSION AND CONCLUSIONS

A. Summary of principal findings

bFGF expression in human mammary epithelial cells and its role in mammary tumor development

1. bFGF was expressed in all four strains of normal HMECs examined and in one immortal normal mammary cell line.
2. Of the four mammary tumor-derived cell lines examined, only the Hs578T cell line expressed bFGF.
3. Normal HMECs proliferated in the absence of EGF and bFGF had no effect on the proliferation of these cells.
4. Hs578T cells proliferated in an autocrine fashion and bFGF was a weak mitogen for these cells.

Role of amphiregulin in controlling proliferation of normal HMECs

1. The EGF-independent growth of normal HMECs was inhibited by heparin and by an EGF-R blocking antibody.
2. The autonomous growth of Hs578T cells (tumor) was not inhibited by heparin or by the EGF-R blocking antibody.
3. Normal HMECs expressed much higher levels of AR mRNA than tumor-derived mammary epithelial cells.
4. Medium conditioned by normal HMECs contains AR protein.
5. TGF-α mRNA was coordinately expressed with AR in normal and tumor-derived HMECs.

Expression and regulation of amphiregulin mRNA in human mammary epithelial cells

In normal HMECs

1. Cessation of growth associated with confluence of the cells did not result in drastic reduction in AR mRNA level.
2. Treatment with heparin greatly reduced AR mRNA levels in confluent cells and this effect was reversed by simultaneous treatment with TGF-α.
3. Treatment with an EGF-R blocking antibody caused a significant reduction in AR mRNA levels in rapidly growing cells and this effect was reversed by simultaneous treatment with TGF-α.
4. When expression of TGF-α mRNA was examined under the conditions described in 1-3 above, similar results were obtained.
5. TPA treatment decreased AR, but increased TGF-α, mRNA accumulation.

In tumor-derived cells

1. Much lower levels of AR and TGF-α mRNA are expressed compared to normal HMECs.
2. PMA treatment increased AR mRNA levels.
3. No amplification or rearrangement of the AR gene was detected.
4. A novel mRNA species was detected with AR probe in all tumor cell lines tested. This mRNA was identified as a transcript from an AR-related gene (ARL). The homology between AR and ARL was localized to the 5' (213 bp) AR cDNA.

B. Discussion

Polypeptide growth factors are major growth-regulatory molecules for cells in culture and probably also for cells *in vivo*. Until recently, normal cells were believed to require exogenous growth factors for proliferation in culture and generally, more than one growth factor was thought to be required (Goustin *et al.*, 1986). The loss of or decreased

requirement for specific growth factors has been proposed as a common occurrence in neoplastic transformation which may lead to a growth advantage, a fundamental feature of cancer cells (Kaplan *et al.*, 1982; Moses *et al.*, 1978). Production or over-production in the cancer cells of growth factors to which they respond is believed to be one of the mechanisms mediating the process of the malignancy. Recently, the ability to culture normal human cells under highly defined conditions has made it possible to study growth regulation in normal cells. Numerous studies have clearly demonstrated that normal human epithelial cells (keratinocytes and mammary cells) proliferate in culture in an autocrine fashion via the production of growth factors to which they themselves respond (Shipley *et al.*, 1989; Cook *et al.*, 1991a; Cook *et al.*, 1991b; Li and Shipley, 1991; Li *et al.*, 1992; Valverius *et al.*, 1989). These studies suggested that autocrine growth factors could play important role(s) in normal cellular proliferation and, thus, generalized theories which claim that only transformed cells produce and respond to autocrine growth factors are not applicable to some epithelial cell types.

I have demonstrated in the first manuscript that bFGF mRNA and protein were produced in all strains of normal HMECs tested while only one mammary tumor-derived cell line, Hs578T, produced this growth factor. Therefore, although bFGF has been reported to be involved in many human tumors, there appears to be no direct relationship between bFGF expression/overexpression and human mammary tumorigenicity. In addition, bFGF expression does not seem to correlate with estrogen receptor levels in the tumor cells tested. Of the tumor cell lines lacking estrogen receptor (Hs578T and BT474), only Hs578T expressed the bFGF gene. bFGF expression was not detected in either of the cell lines which express the estrogen receptor (MCF-7 and T47D).

Although Hs578T cells did not produce greater amounts of bFGF than normal HMECs, they responded to bFGF by increasing in cell number, whereas normal HMECs did not appear to respond to bFGF. Thus, oversensitivity of Hs578T tumor cells to bFGF

could be responsible for the autonomous growth of these cells in the absence of growth factors in serum-free cultures. This hypersensitivity of tumor cells to a normal ligand-receptor interaction could be the result of alterations in signal transduction pathways (Kaplan and Ozanne, 1983). Alternatively, normal HMECs may not express cell surface receptors for bFGF, whereas tumor cells do.

In the first manuscript, I have shown that normal HMECs proliferate in medium lacking EGF or FGF-like growth factors. This finding is in agreement with the results reported by Valverius and coworkers (Valverius *et al.*, 1989), and further supported the notion that normal cells (especially epithelial cells) are capable of proliferation in the absence of exogenously added growth factors. Since normal HMECs do not respond to bFGF for growth, it is unlikely that bFGF produced by these cells acts as an autocrine growth factor responsible for the EGF-independent growth of these cells in culture. However, bFGF produced by normal HMECs may act as a paracrine growth factor which may stimulate the growth of mammary stromal cells as well as vascular endothelial cells *in vivo*.

Studies presented in the second manuscript strongly suggested that AR is the autocrine growth factor responsible for the EGF-independent growth of normal HMECs in culture. Based on the data in this manuscript, I propose that an autocrine feedback loop involving AR/EGF-R activities apparently exists in high density HMECs cultures. In this loop, a substantial amount of AR is produced in normal HMECs. AR binds to EGF receptor on the cell surface of HMECs and elicits its mitogenic activity, thus, abrogating the requirement of HMECs for exogenous EGF-like growth factors. Factors which interrupt this pathway are shown to block autocrine growth of normal HMECs. For instance, heparin which keeps AR from binding to its receptor, and mAb LA1 which blocks ligand binding to the EGF receptor cause cessation of autocrine growth.

A number of studies on normal HMECs have implicated TGF-α, another member

in EGF family of growth factors, as an autocrine mediator for the proliferation of these cells (Bates *et al.*, 1990; Valverius *et al.*, 1989). TGF-α is produced in these cells and the expression of TGF-α appeared to be associated with the proliferation state of the cells (Bates *et al.*, 1990). The results presented in the second manuscript revealed that both AR and TGF-α were expressed at higher levels in normal HMECs than in mammary tumor-derived cells indicating both factors could be important in controlling proliferation in normal cells. We do not believe that TGF-α is the primary mitogen responsible for the EGF-independent growth of HMECs because the mitogenic activity of mature TGF-α is not blocked by heparin (Cook *et al.*, 1991a). However, TGF-α could still be acting in concert with AR by regulating the expression of AR or by directly mediating signal transduction events in order to stimulate the autocrine growth of these cells.

Because AR plays an important role in the proliferation of normal HMECs, elucidating mechanisms of how AR expression is regulated in these cells is crucial for understanding fundamental mechanisms of growth regulation in these cells. Results presented in my third manuscript have shown that activation of EGF receptor contributes substantially to the high steady-state level of AR mRNA in EGF-independent normal HMEC cultures because an EGF receptor blocking antibody greatly reduced AR mRNA level. In addition, AR mRNA levels in confluent cultures decreased with heparin treatment suggesting that AR protein acting through the EGF receptor (or unidentified receptor) maintains high levels of AR mRNA. When TGF-α was included in the medium with heparin, it reversed the inhibitory effect of heparin on AR mRNA expression, once again indicating that EGF receptor occupancy can maintain high levels of AR mRNA. Therefore, in normal HMECs, secretion of AR and TGF-α, the binding of both factors to the EGF-R or other cell surface receptors (AR), and stimulation of cell proliferation, would be accompanied by a further induction in the levels of AR and TGF-α production. This positive amplification of the production of AR and TGF-α may play a key role in the growth of normal HMECs and may reflect a mechanism for the development of mammary

gland *in vivo*.

I have demonstrated that mammary tumor-derived cells express much lower levels of AR and TGF-α than normal HMECs. The autonomous growth of one tumor cell line, Hs578T, was not affected by the addition of heparin to the culture medium. These results are curious as *in vivo* studies have shown that AR is over-expressed in tumors of the stomach and colon (Cook *et al.*, 1992). There is no ready explanation for these results. It has been reported that AR can act as a growth inhibitor for some human tumor-derived cell lines (Shoyab *et al.*, 1988). Thus, it may be the case that mammary tumor cell lines do not express elevated levels of AR by virtue of the fact that AR, acting as a growth inhibitor, would impede the proliferation of these cells. On the other hand, it has been suggested that AR may elicit some of its biological activity via a receptor different from the EGF-R (Shoyab *et al.*, 1989; Cook *et al.*, 1991b; Plowman *et al.*, 1990). Thus, differential expression of multiple receptors for AR by each cell type may be one of the mechanisms which determines how cells respond to AR.

In addition to AR, I have also identified with the AR cDNA another mRNA species (ARL) which was expressed in all mammary tumor-derived cell lines tested but was undetectable in normal HMECs. I have further determined that ARL is a product of another gene with sequence homology to AR. The most homologous sequences between AR and ARL exist in the 5' region of the AR cDNA. This region encodes the 5' untranslated region, the signal peptide and part of N-terminal AR precursor. The properties of ARL and its role in mammary tumorigenesis cannot be determined until the sequence of this RNA becomes available. The fact that ARL appears to be specifically expressed in mammary tumor cells makes it intriguing as a potential marker for mammary tumors.

In the second and the third manuscripts, I have shown that the expression of AR mRNA is differentially regulated by phorbol ester (PMA) in normal HMECs versus in

tumor-derived cells. PMA greatly induces the expression of AR mRNA in certain mammary tumor-derived cell lines while it decreases its expression in normal HMECs. Southern blot analysis suggested that there is no gross rearrangement of the AR gene in tumor-derived cells indicating that difference in the levels of AR mRNA between normal and tumor cells is not due to alterations in the AR gene. These results suggest that different mechanisms might be involved in the regulation of AR expression in normal or tumor-derived mammary cells.

Further studies will be required in order to understand mechanisms by which AR functions differently in normal or tumor-derived mammary epithelial cells. Such efforts will aim:

1. To determine the mitogenic activity of AR on both normal and tumor-derived HMECs.
2. To determine whether AR binds to receptors other than EGF-R and how these receptors are distributed in normal or tumor cells.
3. To determine the mechanism by which PMA regulates AR expression in normal and tumor cells.
4. To determine the sequence of ARL and characterize its function in tumor-derived mammary epithelial cells.

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APPENDIX

Expression of Multiple Species of Basic Fibroblast Growth Factor mRNA and Protein in Normal and Tumor-derived Mammary Epithelial Cells in Culture

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Abstract

We examined the expression of the basic fibroblast growth factor (bFGF) gene in cultured normal and tumor-derived human mammary epithelial cells at both the transcriptional and translational level. Northern blot analysis revealed three bFGF mRNA transcripts of 7.5, 4.4, and 2.2 kilobases in all four strains (donors) of normal cells (HMECs) we examined and in the immortal mammary cell line HBL-100. Of the four mammary tumor-derived cell lines we examined (MCF-7, BT-474, T-47D, and Hs578T), only the Hs578T cells produced detectable levels of bFGF mRNA. Western blot analysis of cell lysates using an anti-bFGF monoclonal antibody revealed corresponding results. bFGF protein was detected in normal HMEC strains 161 and 48 (other normal strains not tested), in HBL-100 cells, and in Hs578T cells, but not in the other tumor cell lines. In each case, three distinct molecular weight species of bFGF protein were detected which migrated in sodium dodecyl sulfate-polyacrylamide gel at 18, 24, and 27 kDa. We also investigated the ability of bFGF to stimulate the proliferation of normal and tumor-derived mammary epithelial cells. Addition of bFGF to serum-free cultures of these cells had no effect on the proliferation of HMECs under a variety of conditions and was weakly mitogenic for Hs578T cells. Our results indicate that normal HMECs produce bFGF mRNA and protein(s), whereas only some mammary tumor-derived cells express this gene. Thus, our results do not support a general role for expression/overexpression of bFGF in the development of mammary tumors. However, bFGF could play a role in the normal development and homeostasis of the mammary gland.

Introduction

Numerous studies on the growth regulation of human mammary carcinomas have focused on steroidal hormones (especially estrogen), because, in about one-third of clinical cases, breast cancer growth is dependent upon estrogenic hormones (1) and can be inhibited by antiestrogenic antagonists (2). Recently, the influence of polypeptide growth factors on cancer cell proliferation has received much attention. It has been suggested that estrogen-regulated tumor growth could be correlated with changes in expression of combinations of growth inhibitors and stimulators (for a review, see Ref. 1). The possible involvement of secreted growth factors in the growth regulation of breast cancer was supported by the finding that crude conditioned medium from human breast cancer cell lines was capable of stimulating the proliferation of other breast cancer cells (3) and by the finding that the initial growth rate of breast cancer cells in culture was proportional to the number of cells plated (4). The production of autostimulatory growth factors by breast cancer cells was additionally supported by the identification of several growth factors in conditioned media from cultured human breast cancer cells. Transcripts for these growth factors were also detected in these cells as well as in primary tumors (5). Among these growth factors, TGF-α[3] and insulin-like growth factor 1 function as autocrine stimulatory growth factors in promoting the growth of breast cancer cells *in vitro* (5, 6). A platelet-derived growth factor-like molecule has also been detected in extracts of breast cancer cells and media (7) and may have a paracrine role in mammary tumor development (3, 7). A growth-inhibitory, TGF-β-related activity was found to be secreted by some breast cancer cells (8).

In contrast to the growth factors discussed above, it is not known whether FGFs play a role in the growth control of mammary carcinomas. Seven genes have been found to encode proteins that have closely related structures (9–17) and are considered to be members of the FGF gene family. One of these genes, *int*-2, is an integration site of mouse mammary tumor virus (9). The human homologue of *int*-2 locus was found to be amplified in infiltrating ductal breast cancers (18). Two other FGF homologues, hst (11)/Kaposi FGF (10) and FGF-5 (12), were both isolated from human tumors. Some studies have indicated bFGF involvement in tumor cell proliferation and tumor development (19–22). bFGF has been identified and purified from human tumor cells such as hepatoma cells (20) and rhabdomyosarcoma cells (21). It was suggested that bFGF acts as an autocrine stimulator for these tumor cells and as a potent angiogenic agent that fosters the growth of new blood vessels into the tumors (23). bFGF produced by tumor cells has also been reported to stimulate plasminogen activator and collagenase production, which may contribute to angiogenesis and metastasis of tumors (20). On the other hand, high levels of bFGF gene expression have also been

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[3] The abbreviations used are: TGF-α and -β, transforming growth factor α and β; FGF, fibroblast growth factor; b-, basic; kb, kilobase(s); cDNA, complementary DNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; poly(A)$^+$, polyadenylated; HMEC, human mammary epithelial cell; FBS, fetal bovine serum; EGF, epidermal growth factor; BPE, bovine pituitary extract; bp, base pair(s); SSC, standard saline citrate.

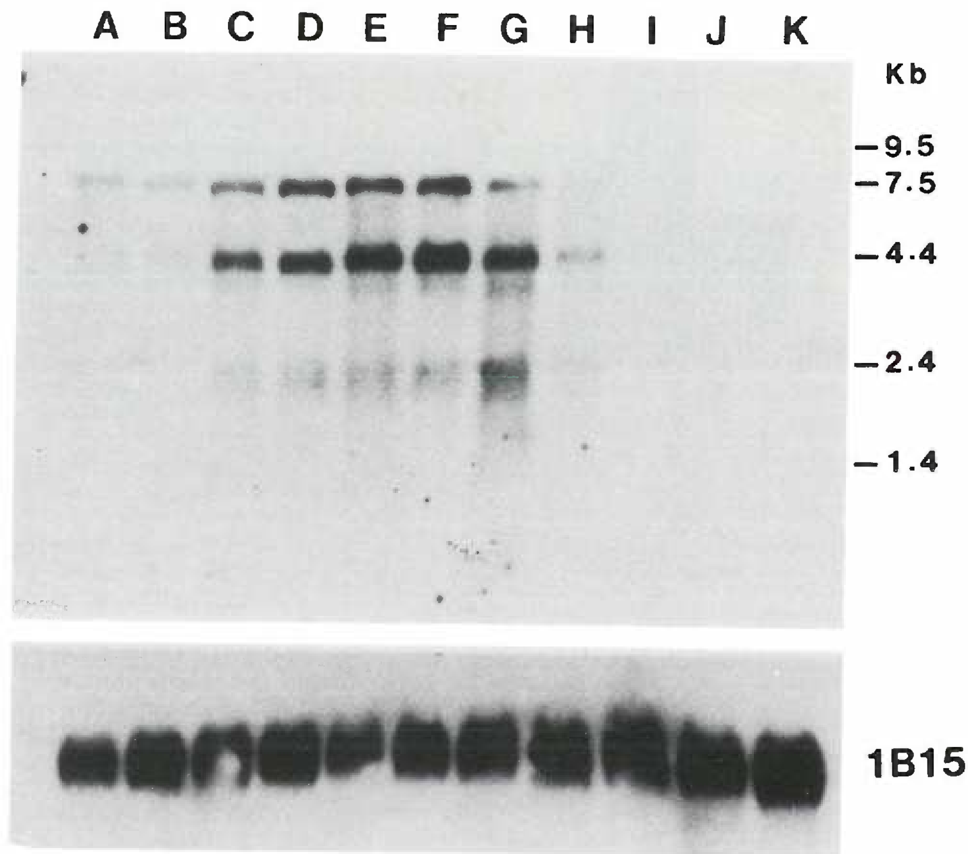

Fig. 1. Northern blot analysis of poly(A)+ mRNA from human mammary epithelial cells. Normal and malignant mammary cells were grown in culture medium, as indicated below. Poly(A)+ mRNA was collected from different cell types and subjected to Northern blot analysis (5 μg/lane), as described in "Materials and Methods." After hybridization with bFGF cRNA probe (*upper panel*), the same blot was reprobed with cyclophilin (*1B15*) cRNA (*lower panel*). Migration of standard RNA marker (BRL) is indicated in kilobases (*Kb*). *Lanes A–D*, normal human mammary epithelial cells, strain 161 (*A*), 172 (*B*), 184 (*C*), and 48 (*D*), grown in complete HMEC medium; *Lanes E–F*, immortal cell line HBL-100 grown in MCDB 170 supplemented with 5% FBS (*E*) or in complete HMEC medium lacking EGF and BPE (*F*); *Lanes G* and *H*, Hs578T tumor cell line grown in MCDB 202a supplemented with 5% calf serum (*G*) or in complete MCDB 202a medium (*H*); *Lanes I–K*, tumor cell lines BT474 (*I*), MCF-7 (*J*), and T47D (*K*) grown in serum-containing medium, as described in "Materials and Methods."

different molecular weight species of bFGF protein were identified which migrated in SDS-PAGE with apparent masses of 18, 23, and 27 kDa (Fig. 3, *Lanes A–D*). bFGF proteins were not detected in the other two mammary tumor cell lines, MCF-7 and T47D (Fig. 3, *Lanes H* and *I*). The human recombinant bFGF (154 amino acids form), loaded at different quantities (Fig. 3, *Lanes E, F, G* and *J, K, L*), served as positive controls as well as standards for estimating the approximate amount of bFGF proteins expressed in various cell types. The same three species of bFGF protein were also identified in the same cell types by a polyclonal anti-bFGF antibody. The ability of the polyclonal antibody to detect these bFGF species was blocked by preincubating the antibody with the decapeptide to which the antibody was generated (data not shown).

We then examined whether bFGF had any effect on the proliferation of normal HMECs or tumor cells *in vitro*. Normal HMEC strain 161 and Hs578T tumor cells, which were selected to grow in serum-free medium, were chosen for the growth experiments. Cells were plated at a density of 5×10^3/cm^2 in appropriate complete medium, and, after 24 h, the plates were washed with buffer saline solution A, and the medium was replaced with basal medium supplemented with hydrocortisone, ethanolamine, and phosphoethanolamine (control). Various growth factors were then added to each well, as indicated in the legends to Figs. 4 and 5. The results in Fig. 4 show a representative experiment for HMEC 161 cells. Cells in control medium survived during the assay period but did not grow. The addition of 5 μg/ml insulin to the control medium resulted in almost three population doublings. Addition of 5 ng/ml EGF had little effect on cell growth by itself, whereas the activity of EGF was synergistic with insulin. Adding BPE together with EGF and insulin (complete HMEC medium) did not significantly increase the cell number compared to the growth obtained with EGF and insulin. Neither basic nor acidic FGF had a significant stimulatory effect on the proliferation of these cells either in the absence or presence of insulin.

A similar growth experiment was performed with Hs578T cells (Fig. 5). In contrast with the normal cells, Hs578T cells underwent two population doublings in the control medium without any added growth factors. Adding insulin, EGF, or bFGF stimulated proliferation by one more population doubling. The combination of EGF and insulin or bFGF and insulin resulted in a slight increase in cell number when compared to the growth obtained with any of these agents alone. The combination of EGF, insulin, and BPE resulted in greater cell numbers than EGF and insulin alone (Fig. 5).

Discussion

We have demonstrated that the bFGF gene is expressed to varying degrees in cultured normal human mammary epithelial cells, in an immortal mammary cell line, HBL-100, and in a mammary tumor cell line, Hs578T. In three other mammary tumor cell lines examined (BT474, MCF-7, and T47D), we did not detect expression of the bFGF gene. The results remained consistent at the transcrip-

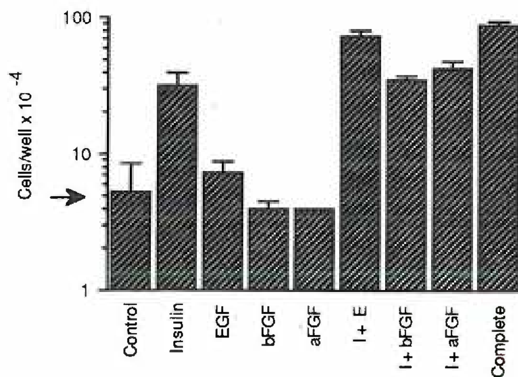

Fig. 4. Effect of growth factors on the proliferation of normal human mammary epithelial cell, strain 161. Normal HMEC 161 cells were plated at a density of $5 \times 10^3/cm^2$ (4.5×10^4 cells/well) in complete HMEC medium. The cells were washed on the following day and switched to MCDB 170 supplemented with hydrocortisone, ethanolamine, and phosphoethanolamine (control). Insulin (5 μg/ml), EGF (5 ng/ml), bFGF (5 ng/ml), or acidic FGF (*aFGF*) (5 ng/ml) were then added as indicated. Some cultures received complete medium containing BPE (0.4% v/v). The cells were allowed to grow under these experimental conditions, and the cell number in each well was determined at the end of the assay. Each *data point*, mean ± SD (*bars*) of triplicate cultures of the same condition. *Arrow*, plating density. *I*, insulin; *E*, EGF.

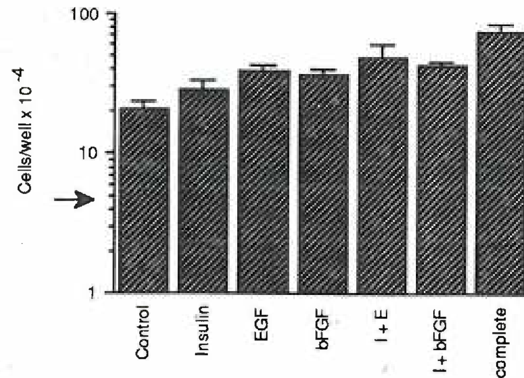

Fig. 5. Effect of growth factors on the proliferation of Hs578T cells. Hs578T cells were plated at a density of $5 \times 10^3/cm^2$ in MCDB 202a complete medium. On the next day, the cells were washed and switched to experimental conditions as described in the legend to Fig. 4. Incubation, medium changes, and cell number determination were performed as described in "Materials and Methods." *I*, insulin; *E*, EGF.

TGF-α, insulin-like growth factor 1, TGF-β, and platelet-derived growth factor (5–8). TGF-α secreted by MCF-7 cells was able to stimulate the same cells growing in soft agar, indicating an autocrine role of this factor (6). Recent studies showed that normal mammary cells grown in culture actually expressed high levels of TGF-α mRNA (35, 36). It was suggested that an autocrine loop for TGF-α could also exist in normal proliferating mammary epithelial cells. In our current study, we demonstrated that normal HMECs expressed significant amounts of bFGF transcripts, whereas only one tumor cell line, Hs578T, expressed the gene. When Hs578T cells were grown in serum-containing medium, they had slightly higher amounts of bFGF message compared to the normal HMECs grown in serum-free medium. However, when the Hs578T cells were grown in medium lacking serum and supplemented with the same additives as for normal cells, there was a reduction in the steady-state levels of bFGF messages. Apparently, serum in the medium is responsible for the higher bFGF levels rather than the tumorigenic nature of the cell. Our results indicate that there is no direct relationship between bFGF expression/overexpression and human mammary tumorigenicity. In addition, bFGF expression does not seem to correlate with estrogen receptor levels in the tumor cells tested. Of the tumor cell lines lacking estrogen receptor (Hs578T and BT474), only Hs578T expressed the bFGF gene. bFGF expression was not detected in either of the cell lines which express the estrogen receptor (MCF-7 and T47D).

It has been proposed that cells could become malignant by somehow altering signal transduction after the ligand-receptor binding, rather than by increasing the production of growth factors and/or their receptors (36, 37). That is, these cells could become hypersensitive to normal ligand-receptor interactions. This theory might explain our results on the responsiveness of Hs578T cells to exogenous bFGF in serum-free culture medium. Although Hs578T cells did not produce greater amounts of bFGF than normal HMECs, they responded to bFGF by increasing in cell number, whereas normal HMECs did not respond to bFGF (Fig. 5). Thus, oversensitivity of Hs578T tumor cells to bFGF could be responsible for the autonomous growth of these cells in the absence of growth factors in serum-free cultures. Alternatively, normal HMECs may not express cell surface receptors for bFGF, whereas tumor cells do.

By comparing the expression of the bFGF gene in normal and tumor-derived human mammary epithelial cells grown in culutre, we have shown that normal HMECs produce bFGF mRNAs and proteins, whereas only some mammary tumor-derived cells express the gene. Our results indicate that expression/overexpression of the bFGF gene is unlikely to be a common mechanism in the development of mammary tumors. However, some increase in sensitivity to bFGF was noted in Hs578T mammary tumor cells that also produced bFGF. Therefore, bFGF could be involved in some but not all mammary tumors. In addition, bFGF could play a role in the normal development and homeostasis of the mammary gland.

Materials and Methods

Cell Culture. Normal human mammary epithelial cells, strains 161, 172, 184, and 48, were generously supplied by Dr. M. Stampfer (Lawrence Berkeley Laboratories, Berkeley, CA). The HMECs used in the experiments reported here were from passages 8–11. HBL-100, an immortal cell line established from normal mammary epithelial cells, and four human mammary tumor cell lines (MCF-7, T47D, BT474, and Hs578T) were all purchased from the American Type Culture Collection (Rockville, MD). MCDB 170 was used as a basal medium for the growth of normal HMECs and MCDB 202a for T47D, BT474, and Hs578T cells. The two basal media were prepared in our laboratory using methods previously described (38, 39). Human recombinant bFGF was provided by Dr. J. Abraham (California Biotechnology, Inc., Mountain View, CA). Human recombinant acidic

MCDB 170, and the assay time was 5 days. Data shown in Figs. 4 and 5 represent mean ± SD of triplicate cultures for each condition.

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