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II. Oncogenes and Growth Factors

STIMULATORY AND INHIBITORY GROWTH FACTORS AND BREAST CANCER

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Summary—While steroid hormones act as endocrine effectors of growth and development of normal breast and of carcinogenesis and progression of malignant breast, recent evidence suggests that local hormonal effectors also exist. These are the growth regulatory growth factors. This article summarizes current status of our understanding of structure and function of growth factors secreted by the normal and malignant mammary epithelium. While growth inhibitory factors and their receptors generally suppress development of the transformed phenotype and promote differentiation, growth stimulatory factors and their receptors may be necessary for both normal proliferation and early stages of malignant progression of breast cancer. Overexpression of two receptors, c-*erbB*-2 and EGF receptor, have also been associated with poor prognosis in the clinical disease.

INTRODUCTION

Early observations of Beatson [1] made it clear that endocrine influences are important in breast cancer. In this review, we will consider emerging evidence that locally-acting growth factors may be important as well in growth control of both normal and malignant breast epithelium. The mechanisms regulating growth of normal and malignant mammary epithelial cells are not well understood, but it is known that hormonal influences are critical in the normal development of the mammary gland. The ovaries (under pituitary control) promote glandular growth and differentiation, while the pituitary itself directly controls lactation. Proliferation of normal mammary epithelium seems to require both estrogen and progesterone, but cellular mitoses occur predominantly in the luteal phase of the menstrual cycle, when progesterone is in highest abundance. This is in striking contrast to the endometrium (which is where cellular mitoses occur predominantly in the follicular phase, when estrogen is "unopposed" by progesterone) [2]. The pituitary and ovaries are also required for the development of breast cancer in women. The influences of the ovaries in breast cancer seem to be mediated by estrogen and progesterone (again under pituitary control) [3]. In rodent models of carcinogen induced breast cancer, it has been shown that both progesterone and estrogen seem to be able to support initial tumor formation and early tumor growth [4, 5]. Presumably the mechanism of interaction of estrogen and progesterone in normal and malignant breast is related to the requirement of estrogen to induce expression of progesterone receptor. However, other types of interactions have not been ruled out [6]. Current controversy also surrounds both progestin and estrogen components of the oral contraceptive as risk factors in developing breast cancer [7]. Estrogen and progesterone receptors have been localized to a luminal subpopulation of ductal and lobular epithelial cells in women and rodents. Receptors appear to be absent from terminal end bud epithelial cells. Thus, estrogen and progesterone receptors appear to be present in at least partially differentiated epithelium. It is not yet clear whether these steroid receptor positive cells are precursor to breast cancer, though circumstantial evidence suggests the possibility [8, 9]. In human breast cancer treatment, both antiestrogen and antiprogestin therapy have been successfully used as adjuvant to surgery [6].

Recent studies have begun to address mechanisms of action of estrogen and progesterone in

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promotion and growth of malignancy. Many studies are examining oncogenes and growth factors by their expression as well as by gene transfer in cells or the germ line of intact animals [10–13]. In hormone responsive breast cancer cells, growth stimulation by estrogen is accompanied by an increase in growth stimulatory transforming growth factor alpha (TGFα) production [14–16], whereas growth inhibition of hormone responsive breast cancer cell lines by an antiestrogen is paralleled by augmented secretion of growth inhibitory transforming growth factor beta (TGFβ) [17]. In hormone independent breast cancer cell lines, compared to hormone dependent breast cancer cells, both of these growth factors, as well as many other growth regulatory peptides, are constitutively produced [18–20]. These results are consistent with, but do not prove, a role for growth factors in the expression of a more malignant phenotype and escape from normal hormonal control. It is of special note that milk, the natural secretory product of the mammary epithelial cell, is an extraordinarily rich source of growth factors. The three factors upon which our laboratory has focussed, TGFα, MDGF-1 and TGFβ, are all found in high quantities in milk [21].

The transforming growth factors derive their name from their ability to reversibly induce the transformed phenotype (initially defined as the capacity for anchorage independent growth) in certain rodent fibroblasts [reviewed in 22]. They are polypeptides, which were initially found to be synthesized and secreted by a variety of retrovirally, chemically or oncogene-transformed human and rodent cell lines [13]. Two major classes of structurally and functionally distinct transforming growth factors are TGFα and TGFβ. TGFα and TGFα-like peptides consist of multiple species ranging from apparent molecular masses of 6–35 kDa [14], and compete with the structural homolog EGF for binding to the same receptor [23]. The TGFβ family consists of several related gene products, each forming 25 kDa dimeric species [24]. There appears to be a complex pattern of interactions of these species with the TGFβ receptor(s), which has been described as three different molecular weight species. TGFβ, and more recently TGFα, have been found in the urine and pleural and peritoneal effusions of cancer patients [25–27]. These growth factors have also been observed in some normal tissues [13, 27]. A more recently-described growth factor, mammary derived growth factor 1 (MDGF-1) has been found in human milk and in conditioned medium from human breast cancer cell lines [29–31]. This 62 kDa growth factor may also play a role in growth regulation of normal and malignant human mammary epithelium. It has been hypothesized that transformation of cells from normal to malignant may directly result from increased production of growth stimulatory factors or decreased production of growth inhibitory substances, or altered responsiveness to either or both of transforming groups of growth factors (reviewed in [13]). An important perspective to gain in understanding pathways of growth control in human neoplastic cells, is a knowledge of growth regulation of the normal cells from which the cancer was derived. To date, this area of investigation in epithelial cells has lagged behind in studies of the influence of growth factors in cancer due to the difficulties involved in culture of normal epithelial cells. However, the recent development of specialized serum-free culture conditions has facilitated the study of growth regulation in normal human keratinocytes [32], normal human bronchial epithelial cells [33] and normal human mammary epithelial cells [34, 35].

EGF AND TGFα IN PROLIFERATION AND CARCINOGENESIS OF THE MAMMARY GLAND

The growth factor EGF appears to be an important regulator both of the proliferation and differentiation of the mouse mammary gland *in vivo* and of mouse mammary explants *in vitro* [36, 37]. EGF is also a required supplement for the clonal growth, *in vitro*, of normal human mammary epithelial cells [38]. However, human breast cancer cells do not require exogenous EGF for continuous growth, although many breast cancer cell lines retain receptors and growth stimulatory responses to EGF [39, 40]. TGFα, a structural and functional homolog of EGF, can produce essentially the same biological effects in mouse mammary explants and cultured human and mouse mammary epithelial cell lines as EGF [36, 41], but its role in normal or malignant mammary development has not been fully defined. It is of interest that TGFα mRNA has been detected in mammary epithelium by *in situ* hybridization during the proliferative, lobuloalveolar development states of rodent and human pregnancy [42]. TGFα mRNA and protein and EGF receptor are detected *in vitro* in proliferating human

mammary epithelium, but are very low in resting organoids [43]. The TGFα acts as an autocrine growth factor in normal human mammary epithelial cells in mass culture; an anti-EGF receptor antibody reversibly inhibits proliferation [44]. Mouse salivary gland-derived EGF appears to be necessary for spontaneous mammary tumor formation in the mouse model [45] as well as for growth of the tumors once they are formed. EGF can also partially replace estrogen to promote limited tumor growth of a human breast cancer cell line (MCF-7) implanted in nude mice [46]. A new member of this growth factor family, termed amphiregulin [47], has also been discovered in a breast cancer cell line treated with a tumor promoter, but its role in normal and malignant proliferation remains to be determined. It appears to inhibit breast tumor cells, but not normal cells [48].

The growth factor TGFα has been directly implicated as a modulator of cellular transformation in a number of studies. Overexpression of TGFα following transfection of a human TGFα cDNA expression vector into the immortal, but non-tumorigenic, mouse mammary epithelial cell line NOG-8 led to its capacity for anchorage-independent growth [49]. Another study utilized the MCF-10, a newly-described, spontaneously immortalized breast epithelial cell line as recipient for the TGFα gene. This cell line, which is negative for estrogen and progesterone receptors, but contains a high level of EGF receptors, was also transformed by TGFα transfection [50]. In contrast TGFα transfection into MCF-7 cells which have low levels of EGF receptor does not confer a significant growth advantage *in vitro* or *in vivo* [51]. In two of three studies using rodent fibroblasts as recipients for human or rat TGFα cDNA, transformation to full tumorigenicity was also achieved [52, 53]. In contrast, in a third study, TGFα could induce proliferation but not full malignant progression tumorigenicity [54]. EGF can also act as a transformation-inducing agent (an oncogene) when transfected and overexpressed in rodent fibroblasts [55]. Some evidence also suggests that oncogene expression in breast cancer is associated with the level of secretion of TGFα. A direct correlation between the degree of TGFα production, *ras* oncogene expression and the degree of malignant transformation has been demonstrated in a recent study utilizing a glucocorticoid-inducible point-mutated c-Ha-*ras* construct transfected into immortal mouse mammary epithelial cells [56]. However, in this study, transformation of cells was observed with a more rapid time course than induction of TGFα synthesis, suggesting that production of the growth factor might be a secondary response to growth rather than an essential mediator of growth. The same group has also observed that in the MCF-10 cell line, v-ras^H-induced transformation is also accompanied by TGFα induction, and here it was possible to block the transformed phenotype with antisera to TGFα [50]. The relationships among oncogene expression, TGFα expression and TGFα function are probably dependent upon the cell type in question. In studies of human breast cancer biopsies, TGFα mRNA and protein were detected in 70% or more of the specimens [14, 57] and in approx. 30% of benign breast lesions [58]. Immunoreactive TGFα has been found in fibroadenomas and 25–50% of primary human mammary carcinomas [15, 59] and an EGF-related protein of 43 kDa has been recently isolated from breast cancer patient urine [60].

A very recent trio of studies has addressed the function of TGFα overexpressed in the mammary glands of transgenic mice. In one study, the gland was hyperproliferative, but exhibited delayed penetration of the epithelial ducts into the stromal fat pad [61]. Such a delayed penetration has also been observed with local mammary implants of EGF [62]. Two other TGFα transgenic mouse studies have also shown the mammary glands to be hyperproliferative, sometimes eventually resulting in mammary cancer after multiple pregnancies [63, 64].

THE *ras* AND *myc* ONCOGENES IN BREAST CANCER; INTERACTION WITH GROWTH FACTORS

In the classical studies of rodent fibroblasts transformed by Harvey, Kirsten or Maloney murine sarcoma viruses [22, 65–67], increased production of "sarcoma growth factor" (SGF) was demonstrated. SGF was later characterized as consisting of the two growth factors and TGFβ. Similarly, increased production of TGFα has been reported following transfection of MCF-7 human breast cancer cells with v-Ha-*ras* (the oncogene of Harvey sarcoma virus) or of mouse mammary epithelial cells by a point-mutated human c-Ha-*ras* gene [41, 56, 68]. The actual incidence of the point-mutations of the c-Ha-*ras* and c-Ki-*ras* proto-oncogenes in breast cancer appears to be low; they have been observed so far in only two hormone independent human breast cancer cell lines [69, 70].

However, the role of unmutated but overexpressed c-Ha-*ras* protooncogene in clinical cases of human breast cancer has not yet been fully clarified. One study indicates a positive correlation between expression of p21 *ras* protein and malignant progression of human breast cancer [71]. In another study, no such correlation could be observed, although malignant and dysplastic breast lesions did have elevated levels of p21 *ras* protein compared to normal tissues [72]. Neither or these studies addressed the state of mutational activation of the *ras* gene, but studies by several other groups indicate that *ras* activation along with some additional event(s) are necessary for neoplastic transformation (reviewed in [73]).

The protooncogene known as c-*myc* is also of interest in breast cancer. This protooncogene can confer immortality to fibroblasts [74] and alter fibroblast responsiveness to growth factors [75, 76]. In human primary breast cancer, c-*myc* amplification has been reported in 15–30% of the tumors and found to correlate with poor prognosis [77]. Expression of the c-*myc* protooncogene under control of the mammary lactation specific WAP-promoter in transgenic mice gave rise to mammary tumors in more than 80% of the animals after pregnancies [78]. *In vitro* studies have also introduced the c-*myc* gene into immortalized human mammary epithelial cells using an amphotropic retroviral vector. In immortalized mammary epithelial cells transfected either with c-*myc* or SV40T (but not v-ras^H) it was observed that the cells could be stimulated to grow in soft agar by either bFGF, aFGF, EGF or TGFα [79]. These data suggest that c-*myc* might function in early breast cancer lesions to allow growth factors to act aberrantly, by driving transformed growth.

THE EGF/TGFα RECEPTOR, c-*erb*B-2 AND c-*erb*B-3

The potential roles of TGFα or EGF in transformation may also involve alterations in the expression and function of their receptor, the EGF receptor. Clinical evidence for the role of increased expression of the EGF receptor, and its structurally related homolog c-*erb* B-2, in more aggressive and hormone unresponsive breast cancer has accumulated in recent years [80–83]. This is also supported by studies of *in vitro* cultured primary human breast cancer biopsies [84] and in established human breast cancer cell lines [40]. The EGF receptor expression (but not c-*erb* B-2 expression) appears to be inversely correlated with expression of the estrogen receptor [80]. In contrast to breast cellular transfections with mutated c-Ha-*ras* oncogene, which induces TGFα, transfection with the oncogenic counterpart of c-*erb* B-2 (or *neu*) does not induce TGFα [85]. In transfection studies on rodent fibroblasts, overexpression of the EGF receptor can predispose cells to expression of the transformed phenotype upon stimulation by EGF [86–90]. Likewise, transfection of rodent fibroblasts with c-*erb* B-2, structurally related to the EGF receptor but lacking EGF binding capacity, results in transformation [89, 90]. A new family member, c-*erb* B-3 has also been recently identified in breast cancer [91], however, its implications for breast cancer biology or prognosis are unknown at present.

Until recently, no ligands for c-*erb* B-2 has been identified. A 30 kDa TGFα-related species has been isolated from the conditioned medium of the MDA-MB-231 breast cancer cell line (and some other hormone-independent breast cancer cell lines [14, 17, 68, 92, 93]). When tested on cells containing EGF receptor, such as fibroblasts, normal mammary epithelial cells, and hormone dependent breast cancer cells, the purified growth factor is stimulatory. However, on cells expressing high levels of c-*erb* B-2, the growth factor is inhibitory [94]. Inhibition is associated with c-*erb* B-2 phosphorylation on tyrosine residues. The TGFα-like molecule is capable of displacing monoclonal antibody 4D5 from its epitope in c-*erb* B-2, and appears to be the first candidate ligand for c-*erb* B-2 receptor [94]. The 30 kDa species had the peculiar property of binding heparin sepharose, and its purification was achieved by this step, reverse-phase chromatography and sizing chromatography. Structurally, the growth factor contains 8 kDA of N-linked sugar; its precursor size after *in vitro* translation is also 22 kDA. Protease mapping of this species shows that it is distinct from the 18 kDa TGFα precursor previously described. It is not clear whether this species is derived from the TGFα gene or a closely-related gene [93].

MDGF1 AND ITS RECEPTOR

TGFα may not be the only stimulatory growth factor produced by the mammary epithelium. A new growth factor termed mammary derived growth factor 1 (MDGF1) has been recently purified to apparent homogeneity from human milk. The factor has an apparent

molecular mass of 62 kDa and a pI of 4.8 [28]. The factor is a pepsin-sensitive and reducing agent-insensitive protein. N-terminal sequence of 18 amino acids shows no homology to any known growth promoting peptides. An apparently identical factor has been isolated from primary breast cancer and human mammary tumor cells suggesting that MDGF1 might be an autocrine or paracrine growth factor for breast cancer [28, 29]. Studies have begun to evaluate the biological effects of and binding sites for MDGF1 on human normal and breast cancer cell lines. At a concentration of 10–25 ng/ml the factor stimulated the growth of estrogen receptor positive MCF-7 human breast cancer cells by 50%. In this cell line, MDGF1 also stimulated the synthesis of collagen IV, a basement membrane protein by 40%. It did not have any effect on estrogen receptor positive ZR75-1 and T47-D breast cancer cell lines or on receptor negative MDA-MB 231 breast cancer cells. The factor showed a biphasic effect on the estrogen receptor negative MDA-MB 231 breast cancer cells. The factor showed a biphasic effect on the estrogen receptor negative MDA-MB 468 cells at concentrations above 5 ng/ml. The growth of normal human mammary epithelial cells (184 strain) was enhanced by 35% by the addition of the factor, whereas benz[*a*]pyrene immortalized non-tumorigenic 184A1N4 human mammary epithelial cells was stimulated by about 60–70%. However, transformation of these cells by SV40-T, v-Ha-*ras* or v-*mos*, desensitized them to MDGF1. Iodinated MDGF1 binds to moderate affinity sites on the responsive MCF-7, MDA-MB 468 and 184A1N4 cell lines ($K_d = 6 \times 10^{-9}$ M). Cross-linking of [^{125}I]-MDGF1 to binding sites with discuccinimidyl suberate (DSS) followed by SDS gel electrophoresis revealed the presence of a major band of molecular weight of approx. 180–200 kDa in MCF-7 and MDA-MB 468 breast cancer cell lines. Labeling of this band was inhibited by excess unlabeled MDGF1 but not other growth factors. These data suggest that human mammary epithelial cell lines possess receptors but not other growth factors for MDGF1 of 120–140 kDa in size [27]. Recent studies have demonstrated that upon ligand stimulation, a protein of approx. 185 kDa in size becomes rapidly phosphorylated on tyrosine residue(s). The relationship between the binding protein and phosphoprotein remains to be determined, but it seems possible that the MDGF1 receptor has tyrosine kinase activity which is ligand-activated.

INHIBITORY GROWTH FACTORS: TGFβ FAMILY, MAMMASTATATIN AND MDGI

While growth stimulatory factors appear to be important in proliferation of normal and malignant breast epithelium, locally acting growth inhibitory factors almost certainly play an equally essential role. One of the best known of these is the TGFβ family. TGFβ is a potent local inhibitor of mammary end bud development when implanted in the developing gland [95]. Inhibition is associated with epithelium-dependent synthesis of type I collagen, glycosaminoglycan and chondroitin sulfate matrix components [96]. Both TGFβ and TGFβ$_2$ are effective inhibitors *in vitro* of breast cancer cell lines [17, 19, 97] and normal mammary epithelial cells in culture [99]. Inhibition of normal mammary epithelial cells by TGFβ is associated with profound morphological alterations [99], differentiation, as evidenced by induction of milk fat globule antigen [100] and *c-sis* protooncogene [101]. Normal mammary epithelial cells produce TGFβ, while breast cancer cell lines make all three family members [17, 98]. In hormone-dependent breast cancer cell lines, estrogen suppresses and antiestrogen induces growth inhibitory TGFβ [17].

A second growth inhibitory molecule called mammastatin has also been isolated from conditioned medium or normal breast epithelial cells in culture. A monoclonal antibody has been prepared which blocks the effect of this inhibitor. The release of the inhibitor is increased by treatment of cells with high dose, cytostatic levels of estrogen, and it is inhibitory for breast cancer cells in culture [102].

Finally, another growth inhibitor, MDGI (mammary derived growth inhibitor) has been isolated from lactating bovine mammary glands and milk fat globule membranes [103]. This 13 kDa inhibitor has been sequenced and cloned, and antibodies have been prepared. It shares sequence homology with a family of proteins which bind hydrophobic ligands such as retinoic acid or fatty acids. The growth inhibitor is synthesized in developing lobuloalveolar structures, and is particularly abundant in proximal parts of the terminally differentiated gland [104]. This molecule also reversibly inhibits proliferation of normal and malignant mammary epithelial cells *in vitro* [103].

CONCLUSION

To summarize, we have tried to suggest that not only are steroid hormones important in

proliferation and malignant progression of breast epithelium, but also the balance of locally acting stimulatory and inhibitory growth factors may come into play. Almost certainly, the steroids and growth factors must interact with a changing genetic background of the cells, taking on malignancy-associated functions as suppressor genes are lost and oncogenes are activated. We have reviewed the literature on four of these proto-oncogenes, c-*myc*, c-Ha-*ras*, EGF receptor and c-*erb*B-2 concluding that profound interactions occur with both endocrine hormones and local growth factors.

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