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Growth Regulation of Human Breast Carcinoma Occurs Through Regulated Growth Factor Secretion

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We describe studies on human breast cancer in which it is shown that specific growth factors (IGF-I, TGFα, PDGF) are secreted by human breast cancer cells and likely to be involved in tumor growth and progression. These activities are regulated by estradiol in hormone-dependent breast cancer and secreted constitutively by hormone-independent cells. These growth factor activities can induce the growth of hormone-dependent cells in vivo in athymic nude mice. Hormone-dependent breast cancer cells also secrete TGFβ, a growth-inhibitory substance, when treated with antiestrogens. TGFβ functions as a negative autocrine growth regulator and is responsible for some of the growth-inhibitory effects of antiestrogens.

Key words: **breast cancer, growth factors, estrogen, IGF-I, TGF, PDGF**

Estrogens play a central role in growth regulation of both normal and neoplastic breast tissue. At puberty and throughout menstrual life including pregnancy-lactation, estrogen exerts mitogenic, anabolic, and secretory effects on mammary epithelium. Estrogen treatment of males will induce breast development at any age. Breast cancer occurs in women who have never had functional ovaries with only 1% of the frequency of that in women with intact ovaries. Thus estrogens play a critical role, at least initially, in nearly all breast cancers. Metastatic breast cancer growth is strongly regulated in about one-third of clinical cases by therapies which alter concentrations or activities of estrogens [1]. This hormonal component of growth control appears to be a remnant of the normal control of epithelial proliferation. While estrogen is a proximate mitogen for either normal or malignant breast epithelium, the hypothalamus-pituitary axis is indirectly in control of ovarian estrogen secretion by virtue of GnRH and gonadotropin stimulation [2]. In addition, the pituitary gland (or other

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organs) may secrete as-yet-undefined direct- or indirect-acting mitogens. Such hypothetical, estrogen-induced, endocrine-acting mitogens have been termed estromedins [3,4]. The hormonal control of cancer cell proliferation has recently received an additional potential regulatory component with the proposal of autocrine or self-stimulating polypeptide growth factors [5].

Our laboratory has devoted itself to studies on the biochemical and molecular events, induced by estrogen, which are associated with direct stimulation of proliferation of human breast cancer cell lines in vitro and of breast cancer in vivo. Using clonal lines of cells, usually derived from pleural or ascites fluid of patients, we have succeeded in demonstrating receptors for and direct proliferative responses to physiologic doses of 17β estradiol (E_2) (a result which has subsequently been confirmed in numerous other laboratories) [6–9]. Several estrogen-responsive human breast cancer cell lines exist, including MCF-7, T47D, MDA-MB-134, ZR-75-1, and CAMA-1 [10]. MCF-7 is probably the best characterized of these. We will review the hormonal responses of these cell lines and the mechanisms by which such cells respond to estrogens and examine some recent experiments whereby the tumorigenic properties of MCF-7 cells are enhanced by v-ras^H oncogene transfection, bypassing estrogen controls. We will also examine another aspect of growth regulation—the fact that growth inhibitors exert their negative effects on cell proliferation, at least in part, by secretion of growth-inhibitory substances which fulfill negative autocrine loops.

RESPONSES OF HUMAN BREAST CANCER TO ESTROGENS AND ANTIESTROGENS

Because mitogenic effects are a central part of regulation by estrogens, many enzymes involved in macromolecular synthesis have been examined. A systematic search in MCF-7 and other breast cancer cells has led to observations that E_2 induces a large number of enzymes involved in nucleic acid synthesis, including DNA polymerase, thymidine and uridine kinases, thymidylate synthetase, carbamyl phosphate synthetase, asparate transcarbamylase, dihydroorotase, and dihydrofolate reductase [11,12]. Physiologic concentrations of E_2 stimulate DNA synthesis through both scavenger and de novo biosynthetic pathways. In two instances recently reported, estrogen regulates thymidine kinase and dihydrofolate reductase at the mRNA level [13,14]. Regulation of thymidine kinase mRNA occurs, at least in part, at the transcriptional level [14]. Estrogens appear to modulate many enzyme activities involved in growth. Whether growth is induced by generalized induction of numerous genes or by pleiotropic or cascade mechanisms is not known. The existence of "second message" regulatory systems in this process is possible and any of the data to be reviewed here are supportive of such a pathway. We have also recently observed that E_2 stimulates the turnover of phosphatidyl inositol in MCF-7 cells [15]. In a variety of other model systems, this metabolic effect is quite rapid and tightly coupled to growth control by proteases and hormones, particularly the polypeptide growth factors [16,17]. In breast cancer cells, induction of phosphatidyl inositol turnover is slower. Estrogen induction of growth factors (to be described below) could explain the delayed time course of the phospholipid effects. Thus, phosphatidyl inositol turnover, with its associated stimulation of protein kinase C and Ca^{++} fluxes, could be a fundamental metabolic mediator of mitogenic effects of E_2.

Others have identified the progesterone receptor [18] as an additional protein induced by estrogen. However, progesterone is apparently not directly growth modulatory of human breast cancer at least in vitro and physiologic concentrations of progestins do not exert major effects on breast cancer growth in patients. The presence of the progesterone receptor does, however, appear tightly coupled to functional growth regulation by estrogen. Thus progesterone receptor content of human breast tumors is used (alone with the estrogen receptor) as a marker for estrogen and antiestrogen responsiveness of tumors in clinical therapy [1].

In addition to regulation of these essential growth-controlling enzymes and the progesterone receptor, estrogens (and antiestrogenic compounds) alter the cellular or secreted activity of several other proteins whose function in growth control remains less well characterized. These include tissue plasminogen activator and other collagenolytic enzymes [19], several relatively abundant secreted proteins, including a 24-kDa protein described by McGuire and colleagues [20], 52- and 160-kDa glycoproteins described by Rochefort and colleagues [21], a 39-kDa glycoprotein complex [22], a 7-kDa protein initially identified by Chambon and colleagues by detection of an estrogen-induced mRNA species (termed pS2) [23], and the cytoplasmic enzyme LDH [24]. Tissue plasminogen activator (along with other proteases) is thought to contribute to tumor progression and growth by allowing the tumor to digest and traverse encapsulating basement membrane [25]. While this is likely, it is conceivable that proteases may serve additional role such as facilitating release of mitogenic growth factors like IGF-I (somatomedin C) from carrier proteins, or processing inactive precursor growth factor and proteases to active species [26]. Interestingly, one of the major secreted proteins, the 52-kDa glycoprotein, is also reported to have biologic activity in purified form—it is mitogenic for MCF-7 cells when tested in vitro [27]. These investigators have recently discovered that purified 52 kDa has cathepsin D–like proteolytic activity and thus its mitogenic effects may be linked to cell-surface proteolysis. The activities of the 160-, 39-, 24-, and 7-kDa proteins are unknown at present. It is of note that at least the induction of the 160-, 52-, and 7-kDa secreted proteins may apparently be dissociated from estrogen and antiestrogen modulation of MCF-7 cells growth by using two MCF-7 clonal variants aberrant in their growth response to these hormones [28–30]. These three protein species are decreased by antiestrogen to the same extent in MCF-7 and LY2, a stable, antiestrogen-resistant variant of MCF-7. This suggests that a significant reduction in secretion of these proteins has no impact on in vitro growth in the case of LY2. In I-13, an MCF-7 clonal variant which is growth arrested by physiologic concentrations of E_2, the same three proteins are induced to the same extent as in MCF-7.

In summary, while estrogens may exert a considerable number of influences in vivo which may indirectly alter breast cancer progression, direct effects of estrogens on isolated breast cancer cells in vitro are also well established. These effects includes growth regulation itself as well as modulation of enzymes and other activities thought to mediate mitogenic and metastatic events. Later we will consider estrogenic influences on a class of secreted proteins which although relatively minor in abundance are very active biologically—the polypeptide growth factors. These factors, along with some of the above-mentioned major secreted proteins, are likely candidates as "second messengers" in the actions of estrogen on breast cancer. Milk is an abundantly rich source of growth factor activities [31,32]. These factors in milk may be important in neonatal development and nutrition or may have additional actions on

the mammary gland. Since breast cancer cells produce and respond to these growth factors, it seems possible that growth factor secretion either by itself or in the presence of some as-yet-undefined transforming event may play a critical factor in neoplastic progression.

The triphenylethylene antiestrogen prototype known as tamoxifen has become a mainstay in adjuvant breast cancer therapy of postmenopausal women as well as in advanced disease for all estrogen-receptor-positive women and is effective either by itself or when used in combination with cytotoxic chemotherapy. In contrast to cytotoxic agents, antiestrogens appear to be cytostatic rather than cytocidal and have a remarkably low incidence of significant side effects. Many investigators have noted the close correlation between the clinical response to antiestrogens and the presence of the estrogen receptor (and its induced product—the progesterone receptor). Since antiestrogens and their active metabolites have a high affinity for the estrogen receptor, the most likely explanation of antiestrogen action appears to be simple antagonism of the growth-promoting effects of estrogen [33,34]. However, alternate views involving other microsomal binding sites for antiestrogen have been presented [35]. The failure to observe antiestrogen responses in estrogen-receptor-negative cells [7,11,12] is the strongest argument in favor of the centrol role of estrogen receptor in antiestrogen action.

Antiestrogen treatment of estrogen-dependent breast cancer leads to cell-cycle blockade (early G_1) of most of the cells in vitro and to arrest of tumor growth in vivo [33,36–38]. It had been initially observed that MCF-7 cells responded in vitro (though oppositely) to estrogens and antiestrogens under cell culture conditions in which estrogens were not thought to be present [39]. While these experiments could be interpreted to suggest that antiestrogens could act (to arrest growth) independently of an occupied estrogen-receptor complex, recent work by Katzenellenbogen and coworkers has clearly shown that high concentrations of phenol red present in the culture medium of the cells in these studies provided a significant estrogenic stimulus [39]. Removal of phenol red, whose structure resembles that of certain nonsteroidal estrogens, abrogated antiestrogen action on MCF-7 cells and dramatically enhanced the responsiveness of the cells to estrogen induction of cell growth and progesterone receptor. There remains little compelling evidence at present that antiestrogens act in any fashion other than by direct antagonism of the initiation of signals generated by an agonist-occupied receptor. These studies provide strong evidence for the direct estrogen responsivity of human breast cancer cells.

The principle limitation with respect to the clinical utility of antiestrogens is the gradual resistance which develops in tumors treated with these agents. While in some cases antiestrogen-resistant tumors lack the estrogen receptor, it is not likely that loss of the estrogen receptors explains the majority of instances of in vivo loss of antiestrogen sensitivity during treatment. If the cell requires estrogen action through the receptor for growth, then loss of receptor will be accompanied by slowing of growth. Clearly estrogen independence must come first and loss of receptor is either random or results from hormone independence. It is of interest that in a model system for acquired resistance—a stable clone of MCF-7 cells stepwise selected in vitro for antiestrogen resistance—high levels of the estrogen receptor and estrogen responsivity for some functions not apparently directly involved in growth are still maintained [29]. These data suggest that positive and negative growth control elements lie distal to the estrogen receptor; data supporting this contention follow.

GROWTH REGULATION OF EPITHELIAL CELLS

In order to study growth regulation of human breast cancer by polypeptide growth factors we used previous work to identify likely starting points. A well-established system in the study of polypeptide growth factor action has been the growth of rodent fibroblasts in vitro. Studies were initially carried out in cell monolayers on plastic surfaces. Smith, Scher, and Todaro, among others, identified "restriction points" in the cell cycle of "normal" (but immortalized) fibroblasts. Various growth factors abrogated these restriction points, allowing the cell cycle to progress [40]. Platelet-derived growth factor (PDGF), a "competence" growth factor, allowed cells to pass a restriction point in early G_1, epidermal growth factor (EGF or the related transforming growth factor α, TGFα) acted later, while insulinlike growth factor-I (IGF-I or somatomedin C) acted still later in G_1 [41]. EGF and IGF-I are "progression" growth factors. Malignant transformation was proposed to result from production of autostimulatory growth factors, abolishing both competence and progression restriction points in a cell's own cycle. One consequence of autocrine growth factors appears to be the serum-independent growth of some cancer cells [42–44].

Factors inducing "anchorage-independent" growth were also studied by using agar or agarose suspensions of cells. It had been observed that the ability of cells to grow in colonies under anchorage-independent conditions was correlated with their tumorigenicity or state of malignant "transformation" [45]. Research from a number of laboratories over the past few years had identified at least four growth factor activities which together can reversibly induce the transformed phenotype in murine fibroblasts. These studies have identified PDGF, EGF (or TGFα), IGF-I (or IGF-II), and an additional growth factor, transforming growth factor β (TGFβ) [40,46,47]. These growth factors are considered likely to be involved in cancer growth control for this reason. However, it should be emphasized that the murine fibroblast model system may not apply to cancers of other tissues or species of origin.

The principle restriction points for epithelial cell growth are unknown. A major departure from the fibroblast model, however, is the fact that TGFβ is a growth inhibitor for many types of primary and malignant epithelial cells [48,49]. Therefore, it is likely that while some of the same growth factors may facilitate traverse of the cell cycle in fibroblasts and epithelial cells, control of anchorage-independent growth may involve another less well-defined growth factor(s). A candidate for such a growth factor is provided by the work of Halper [50]. Basic pituitary FGF can fulfill such a function in cloning of an adrenal carcinoma cell line (SW13), and epithelial cancers produce a related activity which remains relatively uncharacterized at present [50]. A variety of other growth factors have been described [5,41,45].

GROWTH FACTOR PRODUCTION AND GROWTH REGULATION BY HUMAN BREAST CANCER CELLS

Notwithstanding the unknown features of cell-cycle control in breast cancer, we have begun the analysis of its secreted growth factors with study of representative members of all of the above-mentioned activity classes: PDGF, TGFα, IGF-I, TGFβ, and an epithelial transformation factor. We have placed special emphasis on the regulatory effects of estradiol in the activities of these growth factors.

We and others have shown growth regulation of MCF-7 cells in monolayer culture by a variety of lipid-soluble trophic hormones other than estradiol. These

include glucocorticoids, iodothyronines, androgens, and retinoids. MCF-7 cells have receptors but very little growth response to progesterone and vitamin D. Additional studies have demonstrated receptors for and responses to the polypeptides, insulin, EGF, and IGF-I. Receptors, but little mitogenic response, have been demonstrated for other hormones, such as prolactin and calcitonin [51]. The multiplicity of growth-stimulating hormones for breast cancer cell culture systems in vitro has several interpretations. Obviously, multiple hormones may influence breast cancer growth. Alternatively, serum borne factors induced by E_2 (estromedins) may play important contributory roles in vivo [52]. Finally, growth factors with a similar spectrum of activities could be elaborated by the breast cancer cells themselves. E_2 is an absolute tumor growth requirement for two human cell lines, MCF-7 and T47D, and a growth stimulator for a third cell line, ZR-75-1, in vivo in the nude (athymic) mouse model system [19,37,53]. McGrath and his colleagues have further defined this system by showing that E_2 need not enter the systemic circulation in nude mice to promote sufficient MCF-7 tumorigenesis; elevation of local E_2 concentration near the tumor sufficed [54]. This suggests that if estrogen acts by inducing changes in the host which permit tumor growth, the production and action of such factors is probably restricted to the local area of the tumor. The mammary stroma is, however, likely to provide an as-yet-unidentified contributory factor(s) in vivo for full mitogenicity of estrogen [55]. Other human breast cancer cell lines, such as Hs578T and MDA-MB-231, lack estrogen receptors and form rapidly growing, estrogen-independent tumors in the nude mouse [10,53,56]. Hs578T is derived from a carcinosarcoma and not a true epithelial tumor. These five human cell lines (MCF-7, ZR-75-1, T47D, Hs578T, MDA-MB-231) have been studied in detail by our laboratory in an attempt to better understand growth regulation of breast cancer in vivo and in vitro.

Our attention was directed to the possible involvement of secreted growth factors in growth control of breast cancer by an observation made with MCF-7 cells plated at various densities. We found that initial growth rate was proportional to number of cells plated [57]. While multiple interpretations of these data are possible, they are consistent with the production of autostimulatory growth factors by the MCF-7 cells. In preliminary experiments we found that conditioned medium harvested from MCF-7 cells treated with E_2 (CME_2) was capable of stimulating thymidine incorporation and proliferation of other MCF-7 cells. The residual E_2 was removed from CME_2 prior to its use as a mitogen. This kind of result had also been obtained by Vignon and Rochefort and their colleagues [58], who had noticed that MCF-7 cells grew faster with less frequent medium exchanges as compared to cells in which medium was changed every other day. They had also noted that CME_2 was directly capable of stimulating other MCF-7 cells.

GROWTH FACTOR PRODUCTION—TRANSFORMING GROWTH FACTOR ALPHA

We therefore began the fractionation and purification of CM from MCF-7 and other breast cancer cell lines to identify the growth factors present. These cell lines secrete stimulatory activity for MCF-7 and 3T3 fibroblast monolayer cultures as well as "transforming growth activity" (TGF) for anchorage-independent growth of NRK and AKR-2B fibroblasts in soft agar culture [53,59,60]. In initial studies using acid Biogel P60 and P150 chromatography we identified a 30-kDa apparent molecular

weight peak of transforming activity for NRK fibroblasts. This peak also coincided with a peak of MCF-7-stimulating activity and the principle species of EGF receptor-competing activity [53,59]. This peak of activity was also identified by an antibody specific for TGFα species but not cross reacting with EGF. Thus, this activity may be related to TGFα, but it appears to be larger than the cloned and sequenced 6-kDa species from transformed fibroblasts [61]. The 30-kDa TGFα-like species is induced by E_2 treatment of MCF-7, T47D, and ZR-75-1 cells two to eightfold depending on cell type and culture conditions. This observation is consistently seen with bioassays of transforming activity, EGF radioreceptor assays, radioimmunoassays, and assays of TGFα mRNA [53]. Current experiments are focused on regulation, purification, and characterization of this activity. The expected 4.8-kb TGFα mRNA species has been detected by Derynck and co-workers in MCF-7 and some other human breast cancer cell lines [61,62]. It is of interest that all estrogen-independent epithelial breast cancer cells secrete high levels of the TGFα -like activity [53]. Hs578T, which is hormone independent but not epithelial, does not secrete TGF α. Preliminary experiments in which cell growth is inhibited by using EGF-receptor-blocking antibodies (kindly supplied by J. Kudlow) and anti-TGFα antibodies (generously supplied by J. Tam) are strongly consistent with the hypothesis that TGFα secretion is part of an autocrine loop.

Both EGF and TGFα can act via the EGF receptor on both diploid and immortalized cell lines. Many groups of investigators have detected the EGF receptor in human and rodent mammary tumor biopsies and malignant cell lines [63,64]. The apparent molecular size in breast cancer cells is 170 kDa and the kinase domain is unaltered as determined by S1 ribonuclease analysis [63]. At the present time little work has addressed the state of phosphorylation or the tyrosine kinase activity of the receptor.

GROWTH FACTOR PRODUCTION—INSULINLIKE GROWTH FACTOR I

Using radioimmunoassay, we and others have noted that a second potential autostimulatory mitogen, IGF-I, is also secreted by all human breast cancer cells examined to date [65,66]. This species, partially purified from MCF-7 cells, comigrates with authentic serum-derived IGF-I after acid ethanol extraction. IGF-I mRNA species were also detected with Northern blot analysis [68] by using a DNA probe to authentic IGF-I [67]. One of these, a 600-bp mRNA, corresponded to the smallest of three RNA transcripts observed in poly A selected RNA from human liver in the same study. We initially observed no E_2 induction of secreted IGF-I in standard culture conditions employing phenol-red-containing medium although antiestrogens inhibited IGF-1 secretion. Subsequent studies, utilizing the more substantially estrogen-depleted phenol-red-free medium, have observed a fivefold IGF-I induction with E_2 treatment [67]. IGF-I secretion is inhibited by growth-inhibitory antiestrogens (in phenol-red-containing medium) and glucocorticoids. Current work is focussed on the mechanism of IGF-I induction and its possible biological role(s). Interestingly, two highly malignant estrogen-receptor-negative breast cancer cell lines (MDA-MB-231 and Hs-578T) secrete high levels of IGF-I and have low responsiveness to exogenous IGF-I [66].

IGF-I mitogenesis is mediated by its receptor, a close homologue of the insulin receptor. The receptor in a variety of cell types consists of a 450-kDa complex (two

α chains of 130 kDa and two β chains of 85 kDa) [5]. The receptor has tyrosine kinase activity [5]. Its mechanism of action is largely unknown but is thought to stimulate growth by some as-yet-undefined posttranscriptional mechanism [69]. IGF-I receptors of the expected size have been reported on several human breast cancer cell lines [70]. The quantities of IGF-I secreted into the medium are more than sufficient to saturate the IGF-I receptors found on all of the breast cancer cell lines we have thus far studied. We conclude that IGF-I is a hormonally regulated autocrine growth stimulator. This is further substantiated by nude mouse data to be described later. We are currently performing an extensive series of experiments attempting to prove that IGF-I secretion is part of an autocrine loop. These experiments involve use of antibodies against IGF-I and antibodies against the IGF-I receptor. In addition, we have performed a series of transfections involving the introduction of antisense cDNA for IGF-I in a regulatable expression vector. These experiments preliminarily suggest that IGF-I secretion is required for continued cell growth.

GROWTH FACTOR PRODUCTION—PLATELET-DERIVED GROWTH FACTOR

In addition to IGF-I and the TGFα species previously mentioned, all breast cancer cell lines which we have examined to date secrete a PDGF-related activity detected by anchorage-dependent growth stimulation of 3T3 fibroblasts in the presence of platelet-poor plasma [71,72]. Immunoprecipitation of metabolic labeled MDA-MB-231 breast cancer cell extracts and medium detected the expected 28-kDa and 14-kDa species [71]. The secretion of the PDGF-like species is estrogen regulated by biologic, immunologic, and nucleic acid assays. PDGF acts through its 185-kDa tryosine kinase receptor on a variety of mesenchymal cell types [5]. The receptor has recently been purified, cloned, and sequenced [73]. Human breast cancer cells are not known to be growth regulated by PDGF and we do not have detectable PDGF receptors [71]. Therefore, PDGF may have a paracrine role in nature. Interestingly, the highly tumorigenic MDA-MB-231 cell line produces the most PDGF of the cell lines examined so far [71].

A NOVEL ANCHORAGE-INDEPENDENT EPITHELIAL GROWTH FACTOR

The hormonal controls on the cell cycle for epithelial cells are only poorly understood. While it is known that EGF and IGF-I are commonly mitogenic and TGFβ commonly growth inhibitory for epithelial cells, the corresponding restriction points in the cell cycle where these growth factors might act is largely unknown. In addition, the controls for anchorage-independent growth are also mysterious. Halper [50] has established a model system with human SW-13 adrenal carcinoma cells in soft agar culture. These cells clone poorly unless basic fibroblast growth factor (FGF) or conditioned medium from certain epithelial cancers such as SW-13 is applied. No other growth factors are known to be active. This activity has been only partially characterized from kidney but appears to be 40–42 kDa in size.

We have begun to purify a related activity by using a previously described assay [50] from human breast cancer cells [74]. The most tumorigenic lines MDA-MB-231 and Hs578T produce high levels of the activity, while estrogen-receptor-containing lines produce much lower levels. The activity from MDA-MB-231 cells is very acidic in its isoelectric point, and approximately 60 kDa in size by gel filtration and gel

electrophoresis. It has been extensively purified by an acid-ethanol extraction, isoelectric focussing, and HPLC sizing. Current work is directed toward complete purification and characterization of this activity and examination of its regulation [74]. This activity has some similarities to a growth factor described by Kidwell [13] but preliminary data suggest that they are distinct.

In summary, we have observed that estrogen regulation of MCF-7 cells is associated with inductions of TFGα and IGF-I and repression of TGFβ, to be discussed later. It is possible that estrogen-antiestrogen regulation of MCF-7 cells is at least partly mediated by coordinant effects on growth-stimulatory and growth-inhibitory growth factor "second messengers." Future studies with blocking antibodies against growth factors and these receptors should help evaluate this hypothesis. Two other growth factors are also secreted by MCF-7 cells—PDGF and a partially characterized epithelial transforming factor purified to near homogeneity. These two activities are produced in very large amounts by estrogen-receptor-negative, highly tumorigenic lines. Estrogen-independent cancers are associated with increased output of a large number of growth factor activities. The critical growth factors in this type of cancer will undoubtably require extensive future study. In the next section we will further evaluate growth factor secretion as it relates to malignant status in a nude model system for tumor progression.

TUMORIGENESIS IN ATHYMIC NUDE MICE

Previous work has shown that MCF-7 cells were absolutely dependent upon estrogen supplementation for tumor formation in nude mice [19,37,53]. We wanted to determine if CM proteins induced by estrogen were capable of acting humorally in vivo in the nude mouse to stimulate MCF-7 tumorigenesis, thus replacing the requirement for estradiol [75]. For this purpose we developed a serum-free culture system which has supported cell growth for all five above-mentioned cell lines for up to 1 wk. The medium consists of Richter's IMEM + 2 mg/liters transferring + 2 mg/liters fibronectin. MCF-7 cells $\pm$ E_2 pretreatment (10^{-9}M, 4 days) were used to condition serum-free medium, collected over a subsequent 2-day period (CM and CME_2). Media were dialyzed extensively against 1 M acetic aid, lyophilized, reconstituted in phosphate-buffered saline, and the precipitated protein was removed. This extraction also removed 99.98% of the residual E_2. Reconstituted CM and CME_2 were infused into athymic female oophorectomized mice via Alzet minipumps. The equivalent of 10 ml of CM or CME_2 per day for 4 wk was infused from a mid-dorsal, subcutaneous location. MCF-7 cells were injected ($2\text{–}5 \times 10^6$ cells/injection) at four different mammary fat pad locations in each mouse. Small tumors (up to 0.5 cm diameter) appeared at MCF-7 sites within 2 wk. Tumors in CME_2-infused animals appeared with two to threefold greater frequency than in CM-infused animals; animals innoculated with only MCF-7 cells and sham pump implantations did not have tumors. CM- and CME_2-supported tumors reached maximum size in 2–3 wk of treatment, usually declining in size thereafter, whereas E_2-pellet-implanted animals have continuously growing tumors for at least 4 wk and they do not regress. CM- and CME_2-induced tumors were verified as adenocarcinoma by histologic analysis. While the CME_2 supported tumor growth, uterine weight was unaffected. In addition, CME_2 activity was decreased by treatment with trypsin, a reducing reagent, or heating to 56°C for 1 hr. Therefore the tumor growth-promoting substance(s) in CME_2 was

unlike E_2 and likely to be similar to a polypeptide growth factors(s). These data suggest that cultured human breast cancer cells under estrogenic stimulation release a tumor-promoting factor(s) which can act in vivo after release into the general circulation of the athymic mouse. We do not know why the tumors induced by CM regress. There appear to be four potential explanations. First, during the process of growth factor purification and concentration one or more essential activities are lost. Second, it should be recalled that these growth factor activities infused via minipump are acting via an endocrine route. Thus any of a large number of pharmacologic explanations may explain our failure to induce sustained tumor growth. Third, it remains reasonable that estrogens do exert systemic effects which their induced growth factors do not. Thus estrogen effects on the immune system, etc, are possible. Finally, estrogens may induce many effects of tumor cells themselves which cannot be induced secondarily by secreted growth factors.

As an independent line of investigation to evaluate the possibility of autoregulatory growth factors, we have also utilized the MCF-7 cells grown as xenografts in the nude mouse to study the activity of individual growth factors. As previously above, conditioned medium extracts from E_2-treated MCF-7 cells stimulate limited growth of MCF-7 tumor in the absence of E_2 itself. As a test of the hypothesis that E_2-induced growth factors may mediate this effect, we have directly infused human EGF (1 μg/day), human IGF-I (0.6 μg/day) into female oophorectomized nude mice injected at four mammary fat pad locations (2–5 $\times$ 10^6 cells/injection site) with MCF-7 cells. These concentrations correspond to those observed in the conditioned medium extracts utilized in the previous studies. As before, growth factors were infused with Alzet minipumps, and the experiment was carried out for 2 wk. Both growth factors induced tumors, but EGF induced more than twice the tumor incidence as IGF-I. EGF supported development of tumors to 0.5 cm in diameter. As expected, E_2-pellet-implanted control animals had a high incidence of continously growing tumors to 0.8 cm over the time of the experiment [75]. Thus, based on these experiments with authentic growth factors, it is likely that breast-cancer-produced and closely related IGF-I and TGF-like species have some autostimulatory actions on tumor growth in vivo. In addition, the TGFα species induced by E_2 may be relevant in E_2-stimulated tumor growth. Greater availability of TGFα, TGFβ, PDGF, and epithelial-transforming activity in the future should facilitate the testing of these activities in this in vivo reconstitution system.

In other studies [76] investigating mouse mammary carcinogenesis, Oka and co-workers have recently demonstrated a likely role of EGF in both mammary tumor onset and subsequent growth support. Using a mouse strain highly susceptible to spontaneous mammary tumors, removal of the submandibular glands (sialoadenectomy) dramatically reduced the incidence of tumor formation and/or the rate of growth of the breast tumors allowed to form. The submandibular gland is a major source of EGF in mice and reinfusion of EGF into such sialoadenectomized mice returned tumor incidence and growth rate of tumors to their normally high level. TGFα- and EGF-like activities thus may have endocrine functions in tumor support. As the data with MCF-7 cells show, one mechanism of tumor progression might involve local production (estrogen regulated) of TGFα by the tumor. Clearly, TGFα- or EGF-like growth factors are likely to be important regulators of mammary tumor progression by a variety of possible mechanisms. A large body of literature already exists demonstrating that EGF has both tumor promotional and immunosuppressive activities [77].

Interestingly, at least some of the growth factor products of breast cancer appear related to growth factors in milk [31,32,78]. One example is TGFα. The function of such factors may be related to offspring growth rather than parental mammary growth, since TGFα (and EGF) can promote eyelid opening in mice (ref). Though growth factors such as IGF-I and TGFα may be capable of autocrine stimulation of tumor cells, they and other growth factors may also subserve paracrine functions on surrounding non-neoplastic tissue. PDGF promotes fibroblast growth and chemotaxis and its secretion may contribute to the marked stromal proliferation characteristically surrounding breast carcinoma [79]. In addition, TGFα and TGFβ stimulate bone resorption and hypercalcemia, also characteristic of breast cancer [80]. Other effects of paracrine growth factors might be immunomodulatory in nature. Finally but potentially the most important paracrine function secreted by cancer is angiogenesis factor(s). Though many activities may contribute, both growth factors and proteolytic degradation products of basement membranes are likely candidates [81–83]. The principle components secreted by breast cancer leading to vascular infiltration of the tumor have not yet been identified. However, Vallee and co-workers have recently isolated, sequenced, and cloned an angiogenic protein secreted by human colon carcinoma cells [84]. Substantial additional work is required to sort out which growth factor activities are growth related and which, if any, directly contribute to the malignant phenotype.

EFFECTS OF RAS GENE TRANSFECTION IN HUMAN BREAST CANCER CELLS

Recent studies carried out in rodent systems have implicated specific genetic alterations leading to malignant transformation and tumor progression. In the carcinogen-treated rat model system, activation of the oncogene known as the Harvey ras (c-ras^{H}) occurs by point mutation [85]. At the present time, no such unifying statements can be made about human breast cancer. Rather, diverse observations of oncogene activation suggest a plethora of mechanisms at work in malignant progression. In one human breast cancer cell line, Hs578T, an activated c-ras^{H} oncogene has been observed, as predicted based on the rat model system [86]. However, this potential mechanism appears far from universal. Second, a whole series of cellular proto-oncogenes is observed to be expressed in diverse studies employing cell lines and tumor specimens [64,87]. These oncogenes (all members of the ras family, as well as myc, myb, fms, fos, fes) include those localized in plasma membrane, nucleus, and cytoplasm. Two other oncogenes, c-erb b (the EGF receptor) and neu (or c-erb b_2), are closely related to the EGF receptor and have also been detected in breast cancer cell lines and tumor biopsies [64,88]. Interestingly, c-erb b (the EGF receptor) is expressed to the greatest extent in estrogen-receptor-negative cell lines and tumor biopsies [63,89]. It may represent a new marker for dedifferentiation or increased malignant potential in breast cancer. It is not yet known whether overexpression of c-erb b in cancer directly contributes to the transformed phenotype or indirectly mediates the effects of EGF (or TGF) produced in an autocrine-type loop. Finally, as previously mentioned, PDGF, partially the product of the c-sis protooncogene, is expressed by a variety of breast cancer cell lines [71,72]. Though PDGF itself is not generally growth stimulatory of epithelial cells it may contribute in other ways to the transformed phenotype (such as through paracrine actions). It is possible

that additional oncogene activities will be observed in breast cancer by using different techniques in the future. One such possibility is that an epithelial cell test system will detect transforming genes which go unrecognized by the well-established NIH 3T3 fibroblast test system.

The diversity in observations of activated oncogenes and expressed cellular proto-oncogenes may suggest that many mechanisms or steps exist in the malignant progression of breast cancer. Alternatively, observations of expression of some of these cellular protoonocogenes could reflect malignant status rather than induce it. Clearly, to test hypotheses concerning oncogene activity in breast cancer it is necessary to directly insert the oncogene of interest into a relevant cell test system. This objective may recently have been approached by using normal diploid human mammary epithelium first immortalized with brief benzo[a] pyrene treatment and then transfected with oncogenes [90]. Stampfer has observed that treatment of normal mammary epithelial cells in culture with benzo[a]pyrene achieved immortalized but nontumorigenic lines. These lines appear nearly normal by several criteria. Subsequently, using retroviral vectors, Clarke has inserted various oncogenes into one of these lines to determine the phenotype effects [91]. Insertion of v-ras^H, v-mos, and SV40 T antigen rendered the cells capable of growth in high levels of serum but did not confer tumorigenicity. Transfectants containing SV40 T plus either v-ras or v-mos were strongly tumorigenic in nude mice. Interestingly, in unpublished studies, we find that all of these cell lines including the diploid human mammary epithelium secretes biologically active TGFα.

While estrogens are critical in the pathogenesis of nearly all breast cancer it is unfortunate that by the time metastases are clinically apparent at least 50% of all breast cancer is hormone independent and after selective pressure with endocrine therapy all breast cancer becomes hormone independent. In order to be able to study this process in detail we attempted to convert hormone-dependent breast cancer to a hormone-independent phenotype.

For this purpose we chose to permanently transfer DNA from the tumor-causing retrovirus Harvey sarcoma virus to MCF-7 cells. The tumor-inducing portion of this viral DNA (the oncogene) is called v-ras^H, the most commonly detected activated oncogene in highly malignant human cancers. MCF-7 cells did not initially contain this oncogene, but one estrogen-independent cell line, Hs578T, does [86]. We transferred the v-ras^H oncogene to MCF-7 cells by the calcium phosphate method [92].

MCF-7 cells containing stably integrated v-ras^H genes in their DNA (MCF-7_{ras}) had five to eight times the level of ras mRNA as in control cells, and had detectable phosphorylated p21 (the protein which is the ras gene product). The cellular p21 is not a substrate for phosphorylation. MCF-7_{ras} cells displayed unaltered growth rate under control conditions in vitro but had resistance to growth inhibition by antiestrogens. The transfected cells were tumorigenic in the absence of estrogen in 85% of inoculated female oophorectomized nude mice [92]. Interestingly, the MCF-7_{ras} cells also exhibited increased rates of turnover of phosphatidyl inositol, analogous to E_2 treatment of MCF-7 cells [15]. In addition, these cells also expressed increased levels of the laminin receptor on their surfaces and increased invasiveness [93].

We next assayed for secreted growth factors by MCF-7_{ras} cells. CM prepared from MCF-7_{ras} cultures as compared with control cultures contained three to fourfold-elevated levels of radioreceptor assayable TGFα and bioactive TGFα as assayed by anchorage-independent growth of NRK fibroblasts. A single peak of TGFα -like

activity was eluted at an apparent MW of 30 kDa from acid gel chromatography of MCF-7_{ras} CM. Also, secretion of immunoreactive IGF-I and TGFα was augmented three to fourfold in MCF_{ras} cells, but PDGF secretion was further not elevated. MCF-7_{ras} tumors in the nude mouse were able to induce the development of small tumors derived from MCF-7 cells separately implanted at a distant site in the nude mouse [94]. That is, when MCF-7_{ras} cells were inoculated on one side of a nude mouse and wild-type cells on the other, tumors appeared nearly 100% of the time on the MCF-7_{ras} side and about 40% of the time on the wild-type side. These do not represent metastases. They do not contain v-ras sequences. When removed from the animal and growth in culture they are still hormone dependent. We can repeat this experimental result with an entirely in vitro model system in which MCF-7_{ras} cells are used as a feeder layer. Thus, the presence of MCF-7_{ras} tumor is able to temper growth of previously hormone-dependent cells without permanently altering their phenotype. Ras gene activation could bring about phenotypic and tumorigenic changes in human breast cancer cells, some of which may also be induced by estrogens. However, the cells retained the capacity to bind estrogen and respond to estrogens as shown by E_2 induction of the progesterone receptor. Thus ras gene transfection bypasses estrogen activation of the transformed phenotype but induces that phenotype via a pathway which appears to be similar but not identical to the E_2 induction pathway. Future studies will more clearly define the similarities and differences between E_2- and v-ras^H-induced malignant progression of MCF-7 cells.

GROWTH INHIBITION BY TRANSFORMING GROWTH FACTOR BETA

We will now address one last hypothesis—that growth inhibition (for example by antiestrogens) not only occurs by a down regulation of growth-stimulatory activities, but in addition by enhanced production of growth-inhibitory substances. Our attention was drawn to this possibility for two reasons. First, in work with glucocorticoid-sensitive lymphoblasts evidence has been presented that effects occur through positive induction of gene products capable of inducing cell lysis. Second in preliminary experiments we found that conditioned media derived from antiestrogen-treated MCF-7 cells was capable of inducing growth inhibition of estrogen-receptor-negative, antiestrogen-resistant MDA-MB-231 cells. Based on apparent molecular weight of some of this activity, we considered TGFβ as one potential negative regulator.

Breast cancer cells secrete a TGFβ-related activity [95]. A major peak of radioreceptor-competing and AKR-2B-fibroblast-transforming activity comigrates with authentic platelet-derived TGFβ on acid Biogel chromatography. In contrast to its transforming effects on some fibroblasts, authentic TGFβ is growth inhibiting for many breast cancer (and other epithelial-derived lines) [48,49]. All breast cancer cells examined expressed the expected 2.5-kb mRNA species. Though estrogen deprivation can induce TGFβ secretion more than 30-fold there are no changes in mRNA concentration for TGFβ. Interestingly, TGFβ secretion is inhibited by treatment of MCF-7 cells with growth-stimulatory E_2 and insulin. Growth-inhibitory antiestrogens and glucocorticoids strongly stimulate its secretion. Intracellular TGFβ did not appear to be modulated. TGFβ from antiestrogen-induced MCF-7 cells strongly inhibits the growth of another estrogen-receptor-negative cell line, MDA-MB-231. This growth inhibitor was reversed in the presence of a polyclonal antibody directed against native TGFβ. Interestingly, in the antiestrogen, but not TGFβ-resistant, resistant MCF-7

variant LY2, antiestrogens do not significantly induce TGFβ secretion. These cells contain identical amounts of TGFβ mRNA to wild-type MCF-7 cells. Current work is further addressing the mechanism of TGF regulation [96].

TGFβ acts through a high molecular weight (615 kDa) receptor complex. The receptor subunits have been reported as identical 330-kDa species. This receptor has not yet been purified, cloned, or sequenced, but it is not reported to have tyrosine kinase activity [5]. High-affinity binding sites for TGF have been reported on responsive (growth inhibited) human breast cancer cell lines. Taken together we believe that these data suggest that growth regulation of some breast cancers (and as a speculation in normal mammary cell(s)) may be modulated by secretion of potent growth inhibitors.

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