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Estrogen-Induced Lysosomal Proteases Secreted by Breast Cancer Cells: A Role in Carcinogenesis?

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In an attempt to understand the mechanism by which estrogens stimulate cell proliferation and mammary carcinogenesis, metastatic human breast cancer cell lines (MCF7, ZR75-1) were found to secrete a 52,000 dalton (52K) protein under estrogen stimulation. Following its purification to homogeneity, the 52K protein was identified as a secreted procathepsin-D-like aspartyl protease bearing mannose-6-phosphate signals. This precursor displays an in vitro autocrine mitogenic activity on estrogen-deprived MCF7 cells and is able to degrade basement membrane and proteoglycans following its autoactivation. The total protease (52K + 48K and 34K) was detected and assayed by monoclonal antibodies and was found to be highly concentrated in proliferative and cystic mastopathies. In breast cancer, its cytosolic concentration appears to be correlated more to tumor invasiveness than to hormone responsiveness. The mRNA of the 52K protease accumulates rapidly following estradiol treatment, as was shown by Northern blot analysis with cloned cDNA. The 52K cathepsin-D-like protease is the first example of a lysosomal protease induced by estrogens in cancer cells. Results obtained using different approaches suggest that two cysteinyl cathepsins are also related to cell transformation and invasiveness. It has been proposed that cathepsin-B is involved in breast cancer and metastatic melanoma, and its regulation by estrogen has been shown in the rat uterus. Cathepsin-L corresponds to the major excreted protein (MEP) whose synthesis and secretion are markedly increased by transformation of NIH 3T3 cells with Ki ras and are regulated by several growth factors. In addition to secreted autocrine growth factors and to other proteases (plasminogen activator, collagenase), lysosomal cathepsins may therefore play an important role in the process of tumor growth and invasion as long as their precursor is secreted abundantly.

Key words: cell proliferation, tumor invasion, MCF7 cells, cathepsin-B, -D, -L, breast cancer, lyosomal proteases

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The role of estrogens in stimulating the growth of estrogen receptor-positive breast cancer is well established [1]. However, the way in which these hormones act on tumor cells is poorly understood. It has been proposed that their action might be directly triggered by interactions of receptors with nuclear machinery, such as the nuclear matrix, to stimulate DNA replication or might be indirectly mediated by estromedins coming from other organs [2]. Recent studies showing that estrogens are able to stimulate directly the growth of breast cancer cell lines in vitro [3–5] after inducing the synthesis of several proteins suggest a third type of mechanism mediated by estrogen-induced factors or proteins directly secreted by breast cancer cells. The study of hormone-regulated proteins in cancer cell lines helps us to understand the mechanism of hormone action on gene expression [6–8], and we illustrate here that it can also guide us in understanding the control of cancer growth and invasion.

ESTROGEN-INDUCED AUTOCRINE MITOGENS

Steroid hormones regulate specific gene expression within minutes following their interaction with specific nuclear receptors [8]. In contrast, the mitogenic effect of sex steroids is observed only after a lag of approximately 1 day; during this time, the transcription of several genes has been stimulated, and different proteins have been induced. Among them, the proteins secreted by breast cancer cells are good candidates for mediating the effect of estrogens by interacting in turn on the plasma membrane of the same cells via an estrogen-regulated autocrine mechanism [9]. An autocrine mechanism has been proposed for cancer cells that acquire the ability to make and to respond to their own growth factors [10]. This concept has been applied to breast cancer cells, which remain under the control of sex-steroid hormones during the first steps of tumor progression [9].

One way to define growth factors and other mitogens involved in this autocrine mechanism is to characterize the proteins and peptides that are secreted by hormone-dependent human breast cancer cells and induced by estrogens, since estrogens are the only steroids with mitogenic activity in these cells [3–5]. This hypothesis was supported by the demonstration that glycoprotein fractions prepared from serum-free media conditioned by estrogen-stimulated MC57 cells increase the growth of resting MCF7 cells, whereas similar fractions from the conditioned media of estrogen-stripped MCF7 cells are inactive [11]. A mitogenic activity of estrogen-induced conditioned media has been confirmed by different groups [12,13]. Following the labeling of newly synthesized proteins by ^{35}S-methionine or ^{35}S-cysteine and analysis of the labeled proteins by SDS-PAGE, several estrogen-regulated proteins have been detected [for review, see 6,7,14]. In addition to the 52K protein [15], a 160K protein has been described, and a 65K protein has recently been identified as being α_1-antichymotrypsin [16], as has a 6–7K protein coded by cloned pS2 mRNA [8]. More classical growth factors such as EGF, IGF, and PDGF-like peptides are also secreted by these cells [17–18] (Fig. 1). One of the major challenges in the field of hormone-dependent cancer research is to define the protein(s) or peptide(s) responsible for the control of proliferation and invasion by these cancers.

Two strategies have been followed. The first was to characterize classical growth and transforming factor activities and to show that some of these growth factors were induced by estrogens in breast cancer cell lines [17,18]. The second was to purify and to identify the major proteins that were actually found to be regulated

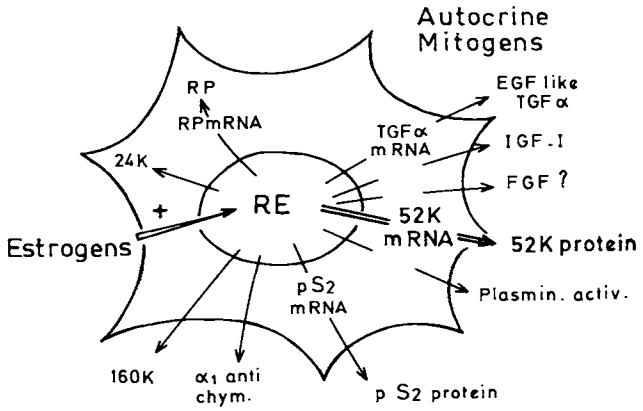

Fig. 1. Estrogens-regulated proteins and peptides in a human breast cancer cell. Estrogens stimulate the synthesis of several proteins via their nuclear receptor (RE). Some of the proteins whose functions are unknown were first identified by labeling and SDS-PAGE analysis and were designated by their molecular weight under denaturating conditions. The corresponding mRNAs have also in some cases been found to accumulate. Several of the secreted proteins and peptides recovered in culture media conditioned by estradiol-treated MCF7 cells are suspected of being the agent that stimulates cell proliferation and tumor invasion. RP progesterone receptor, α_1 antichymotrypsin. (Reproduced from Morisset et al [27] with permission of the publisher.)

by estrogens in these cells. This second approach lead us to identify a lysosomal protease with mitogenic and invasive potential.

REGULATION AND PURIFICATION OF THE 52K PROTEIN

The 52K protein, which we first described in 1979 [19], is secreted in small amounts into the culture medium by estrogen-treated MCF7 cells (5 ng/10^6 cells/hr) and by other ER-positive breast cancer cells under estrogen control (T47D, ZR75-1). It is constitutively produced without requiring the influence of estrogen in ER-negative cell lines (MDA-MB231, BT20) [20]. In ER-positive cells, the protein is specifically regulated by hormones (estrogens and high doses of androgens) that can bind to and activate the estrogen receptor, but not by glucocorticoids, progestins, or androgens at low concentrations [15]. The effects of the antiestrogens tamoxifen and hydroxytamoxifen suggested that this protein was in some way related to the mitogenic activity of estrogens. In wild-type MCF7 cells, the antiestrogens totally inhibited the synthesis of the protein, whereas they partially stimulated the synthesis of the progesterone receptor. In contrast, in the antiestrogen-resistant variants of MCF7 cells (R27 and RTx6) cloned for their ability to grow in 1 μM tamoxifen, the antiestrogens became able, like estrogens, to increase the production of the 52K protein but remained unable, as in wild-type MCF7 cells, to stimulate the production of the estrogen-regulated 160K secreted protein and of pS2 mRNA [21] (Table I). In these cell lines, the 52K protein was therefore a better candidate for being a mitogen than the pS2 and 160K proteins. A possible mechanism for the antiestrogen resistance was that cells had acquired a growth advantage resulting from the tamoxifen-induced increased production of autocrine growth factors such as the 52K protein.

We applied a three-step strategy to purify the 52K glycoprotein. Using concanavalin-A-Sepharose chromatography, we partially purified it from 22 liters of conditioned medium from MCF7 cell cultures. From this partially purified fraction, we

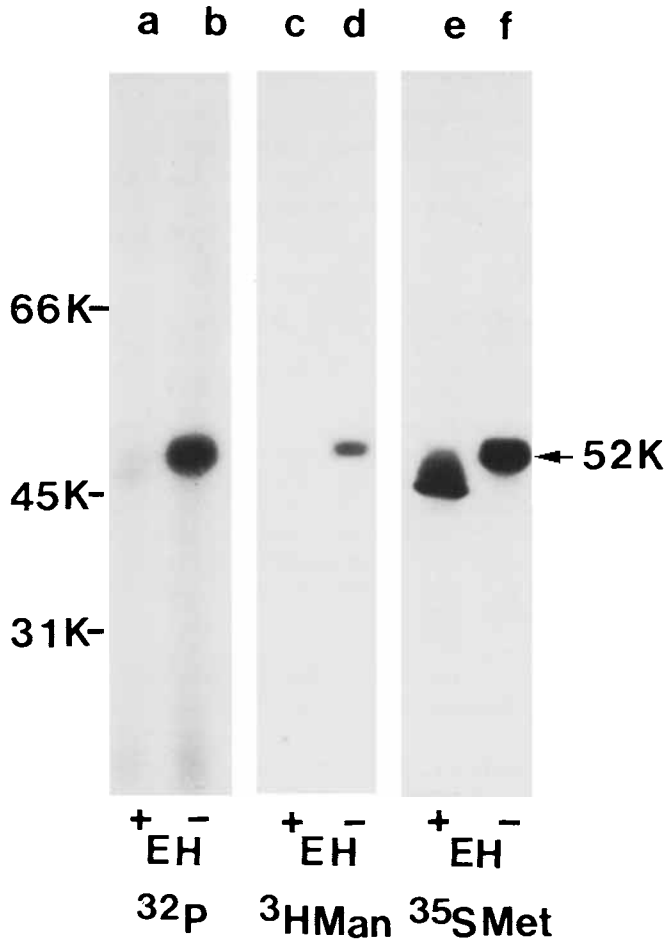

Fig. 2. Estrogen-treated MCF7 cells were labeled with ^{35}S-methionine, ^{32}P-H_3PO_4, or ^{3}H-mannose. Media were immunoprecipitated with the M1G8 antibody to the 52K protein and were analyzed by SDS-PAGE. The immunoprecipitated secreted 52K protein was (+) or was not (−) digested with endoglycosidase H (EH) and the TCA-precipated proteins were electrophoresed. (Reproduced from Morisset et al [29] with permission of the publisher.)

TABLE I. Effect of Tamoxifen on Three Estrogen Receptor-Positive Cell Lines*

Cell line	R_P	pS2 mRNA	160K protein	52K protein	Cell proliferation
MCF7	+	−	−	−	Inhibition
R27 RTx6	+	−	−	+	Resistance

*Summary of the effect of tamoxifen on five estrogen-regulated responses in the antiestrogen-sensitive (MCF7) and -resistant (R27 and RTx6) cell lines showing that only the 52K secreted protein behaves differently in the antiestrogen-resistant variants, in which it acquires the ability to be induced by antiestrogens. Results are detailed in Westley et al [21].

obtained several mouse monoclonal antibodies [22]. Finally, using an immunoaffinity column, we purified the 52K protein to apparent homogeneity (1,000-fold purification) in both its secreted and its cellular form [23]. The purification of 52K protein made it possible to study its activity on cell growth, to determine its structure and identity, and finally to clone its corresponding cDNA sequences.

By immunopurification without detergent, a homogeneous secreted 52K protein was obtained, which was shown to stimulate the growth of estrogen-deprived recipient MCF7 cells [24]. This stimulation was dose-dependent and occured at concentrations (1–10 nM) similar to those found in the culture medium. However, time-course experiments indicated that both estradiol and the 52K protein required the same (18 hr) lag before stimulating ^{3}H-thymidine incorporation. Like estradiol, the 52K protein was also able to stimulate the number and length of microvilli on the cell surface. This mitogenic activity of the purified 52K protein could be intrinsic or could be due to a contaminant not visible by silver staining overloaded gels. However, ^{35}S-cysteine-labeling experiments have excluded the possibility that the activity could be produced by newly synthesized peptides incorporating cysteine residues such as TGFα, pS2 protein, or IGF1 [24]. The in vitro mitogenic activity of the purified 52K protein was in agreement with an estrogen-regulated autocrine mechanism.

STRUCTURE AND IDENTIFICATION AS A LYSOSOMAL PROTEASE

Study of the co- and posttranslational modifications of the 52K protein helped us to define its structure and enzymatic activity. After exposure of cultured MCF7 cells to ^{32}P, the 52K protein is intensely labeled. Most of this label can be removed by endoglycosidase-H treatment, which deletes two N-glycosylated chains of the protein (Fig. 2). Mannose-6-P signals have been identified on these chains [25]. Pulse-chase experiments and Western blot analysis show that the 52K protein is the precursor of a lysosomal enzyme that accumulates in lysosomes as a stable 34K protein. About 40% of the cellular 52K precursor is secreted, whereas about 60% is successively processed into a 48K and a 34K + 14K protein [23,26] (Fig. 3). Part of

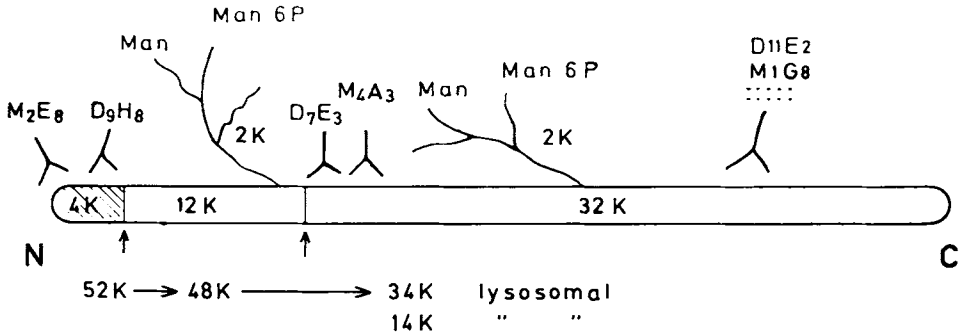

Fig. 3. Structure and processing of the 52K protein of MCF7 cells. The secreted 52K protein corresponds to a lysosomal procathepsin. In the cell, it is successively processed into a 48K active enzyme and a 34K + 14K enzyme, which normally function in the lysosomes. Two N-glycosylated oligosaccharide chains are represented bearing an accessible mannose-6-P signal (Man-6-P). The sugar composition of each chain and number of man-6-P sites per chain are not known. The overall structure is similar to that of procathepsin-D. Sequencing of cloned cDNAs is in progress [27].

the secreted 52K protein can be taken up and processed by MCF7 cells, but this binding is specifically inhibited by mannose-6-P [25] (Fig. 4).

The presence of mannose-6-P signals indicated that the protein is normally routed to lysosomes, where it exerts its usual function [28]. In testing several enzymatic activities corresponding to lysosomal hydrolases of similar molecular weight, we found that both the purified secreted 52K protein and the corresponding cellular proteins (52K, 48K, 34K + 14K) displayed a strong proteolytic activity at acidic pH, which was mostly inhibited by pepstatin [25,29]. There are similarities with the previously described cathepsin-D [30]. Antibodies to the 52K protein interact with liver cathepsin-D, and anticathepsin-D immunoprecipitates the 52K protein. The pH and inhibitor sensitivities of the two proteases are very similar, as are their

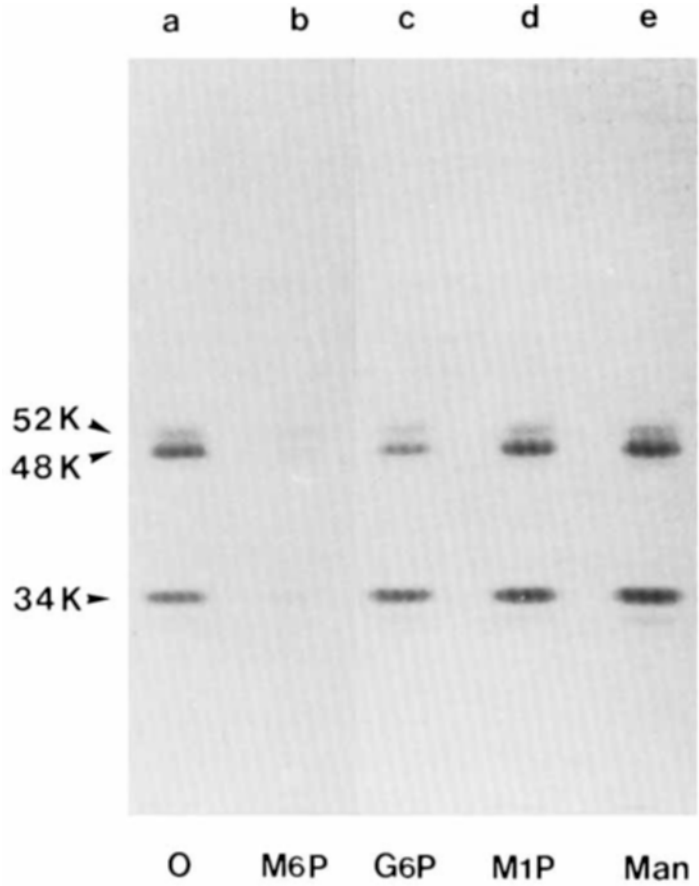

Fig. 4. Inhibition by mannose-6-phosphate of the uptake and processing of the 52K protein by MCF7 cells. Confluent MCF7 cells (1×10^6 cells) maintained in F12/DEM containing 1% FCS/DCC were rinsed twice in R12/DEM plus 0.1% BSA. They were then incubated with 400 μl of conditioned medium containing ^{35}S-methionine-labeled proteins secreted by MCF7 cells (300,000 TCA-precipitable counts) for 24 hr in F12/DEM with **(lane a)** 0.1% BSA alone (O), or **(lane b)** 10 mM mannose-6-phosphate or **(lane c)** 10 mM glucose-6-phosphate (G6P), or **(lane d)** 10 mM mannose-1-phosphate, or **(lane e)** 10 mM mannose (Man). The labeled cells were then washed three times in F12/DEM plus 0.1% BSA, and the cell lysate proteins were immunoprecipitated with the M1G8 anti-52K monoclonal antibody. The immunoprecipitates of each series were analyzed by SDS-PAGE and revealed by fluorography. (Reproduced from Capony et al [25] with permission of the publisher.)

molecular weights [25]. In addition, the first 15 amino acids as determined by microsequencing the N-terminal of the molecules are identical (P. Ferrara, unpublished data).

However, there are some differences compared to normal cathepsin-D [30]. 1) The tissue distribution appears to be different from that reported for cathepsin-D [31]; immunoperoxidase staining is mostly positive in breast cancer, melanoma, and liver but not in endometrium or other tissues [32]. This may be due to a markedly higher concentration of the protease in proliferative mammary cells compared to other cells. 2) Its secretion is tenfold greater in breast cancer cells than in normal mammary cells in culture (Capony, unpublished data). 3) The endo-H sensitivities of the oligosaccharide chain are different; the breast cancer 52K protein is fully endo-H-sensitive, unlike cathepsin-D from liver or placenta [25]. Available cDNA clones [27] will allow us to determine the complete coding sequences of the MCF7-52K cathepsin-D, and to detect any alteration in the structure of the protease, compared to the normal cathepsin-D sequence [33]. The study of gene promoter may also show differences related to its regulation by estrogens in cancer.

This secreted protein may be regulated by estrogens at a different level. Using a cloned 52K-cDNA probe and Northern blot analysis of poly-A+ RNA of the MCF7 breast cancer cell line, we have recently shown that estradiol rapidly induces the accumulation of a 2.2 kb 52K mRNA [27] (Fig. 5). The degree of stimulation was six to ten fold, and there was a basal level of the 52K-mRNA in the absence of estrogens. It is therefore likely that, in the case of other estrogen-regulated genes [8], the cathepsin-D-like enzyme is transcriptionnaly regulated by estrogens.

POTENTIAL ROLE OF THE 52K-CATHEPSIN-D PROTEASE IN MAMMARY CARCINOGENESIS

Some of the characteristics of the 52K protease, ie, its high concentration in proliferative and tumoral cells, its induction by estrogen, and the large proportion of its secreted form, suggest that it has a major function(s) in mammary carcinogenesis related to stimulating tumor growth and/or invasion via its proteolytic activity (Fig. 6). The mechanism of the mitogenic action of the 52K cathepsin-D-like enzyme is not yet understood, but it can be approached using specific inhibitors and several monoclonal antibodies [24,34]. Proteases are known to be potential mitogens. Several mechanisms are possible; they may act indirectly by releasing growth factors from precursors or the extracellular matrix via their enzymatic activity and/or by activating growth factor receptors. In this respect, proteolytic cleavages are needed to detach the TGFα precursor from the plasma membrane [35] and to activate the secreted TGFβ precursor [36]. The proteases responsible for these cleavages are unknown. Proteases may also act more directly via specific receptors, as in the case of thrombin [37], and they do in fact contain sequences analogous to growth factors.

Clinical studies indicate an association between high cellular concentrations of the 52K protease and cell proliferation of ductal mammary tissue [32,38]. These findings are consistent with a mitogenic activity, but the high 52K protein level may be a consequence rather than a cause of cell proliferation.

The major normal functions of cathepsins occur in lysosomes at very low pH, where they degrade endogenous proteins [39]. Since a procathepsin-D enzyme is secreted in large amounts at the periphery of cancer cells, the enzyme may acquire

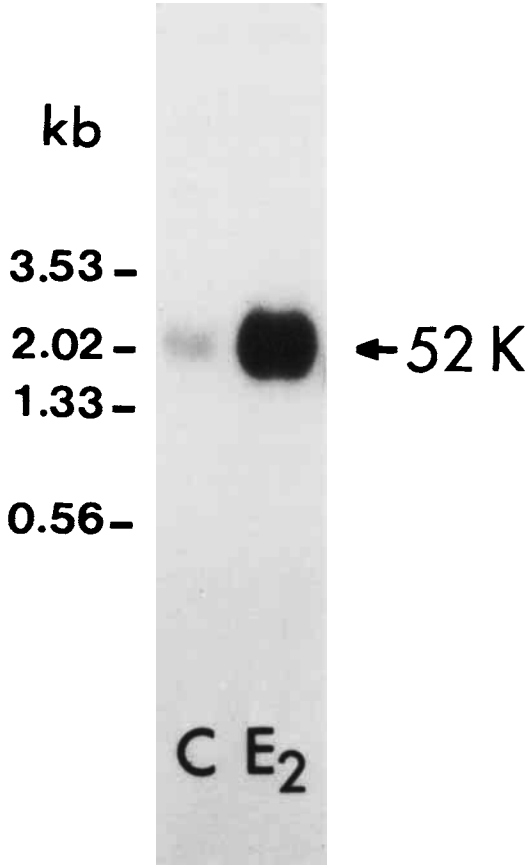

Fig. 5. Effect of estradiol on the 52K protease mRNA in MCF7 cells. Six hours following addition of estradiol (E_2) or solvent (C) to MCF7 cells, 10 μg of total RNA as analyzed in each track by 1% agarose gel electrophoresis. The 52K mRNA was detected by hybridization with a cloned cDNA isolated from a λgt11 cDNA library of estrogen-treated MCF7 cells (a gift of Dr. P. Chambon). The molecular weight of markers is shown in kilobases [27] (also see Cavaillès et al, in preparation).

abnormal functions, such as facilitating cancer cell migration and invasion by digesting basement membrane, extracellular matrix, and connective tissue. Cathepsin-D is secreted as an inactive proenzyme, but, at acidic pH, it can be autoactivated by the removal of a small part of the N-terminal profragment [40]. The same is true of breast cancer 52K protein [25]. Culture media conditioned under serum-free conditions by estradiol-treated MCF7 cells contain potential proteolytic activities that can digest methemoglobin [30] and extracellular matrix prepared from bovine corneal endothelial cells [25,41]. This activity appears to be due entirely to the 52K procathepsin-D since it is inhibited by pepstatin [25]. Between pH 5 and 6, and following its activation, the 52K protease can then digest the extracellular matrix of bovine corneal cells and human proteoglycans [25,41]. It is conceivable that breast cancer cells develop a sufficiently acidic microenvironment close to the extracellular matrix, where the secreted cathepsin-D-like 52K then behaves as a protease [41].

Clinical studies have recently supported the hypothesis of a role of the 52K protease in mammary carcinogenesis. Using different monoclonal antibodies to the

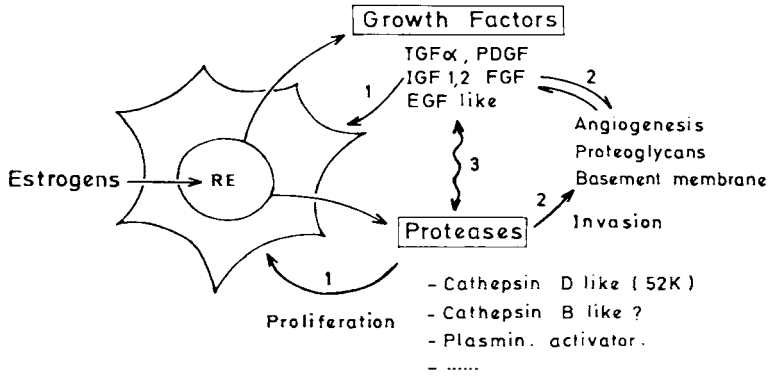

Fig. 6. Putative functions of estrogen-regulated secreted proteins and peptides secreted by breast cancer cells. Estrogens, via their nuclear receptors (RE), induce several proteins and factors that are secreted by breast cancer cells. One category (growth factors) may act as autocrine factors that stimulate the growth of the same cells [17]. Other proteins, such as proteases, can potentially act as mitogens and may also facilitate cancer cell migration, invasion, and possibly metastasis. The 52K protein is the precursor of a cathepsin-D-like protease, and its secretion, as well as that of cathepsin-B, appears to be increased in mammary cells following their transformation.

total 52K protein and its precursor, we were able to detect the protein in the cytoplasm of cancer tissue by immunoperoxidase staining of frozen sections and to assay total 52K protein (52K + 48K + 34K) or only its precursor in the cytosol of breast cancers [for review, see 42].

In a prospective study of 183 patients, high concentrations of the 52K protein ($\geqslant$700 U/mg protein) were found to be correlated with axillary lymph node involvement but not with estrogen receptor content [43]. A retrospective study involving a 5 year follow-up of 150 postmenopausal patients [44] indicated that the patients had a significantly shorter disease-free interval when the 52K protein concentrations in primary tumors were superior to 400 U/mg cytosol protein than when the concentrations were lower. The bad prognostic significance of high concentrations of the protease may be related to a subsequent increase in its secretion.

OTHER LYSOSOMAL PROTEASES IN CARCINOGENESIS

Three types of cathepsins have most often been studied in relation to cancer. Cathepsin-D-like enzymes have also been shown to be secreted by pancreatic [45] and ovarian [46] cancers. No steroid hormone regulation has previously been demonstrated in cancer tissue, but cathepsin-D has been reported to be induced by progesterone in the rat uterus [47].

It has been proposed that cathepsin-B-like enzymes are involved in breast carcinogenesis [48]. They are thiol proteinases with an optimal pH of 5–6. Cathepsin-B activity and antigenicity are greater in mammary cancer cells than in normal cells [48,49]. The proenzyme (40K) is secreted in cancer cells, but it is inactive and requires a pepsine-like enzyme to be activated by removal of the profragment. A regulation by prolactin has been suggested [49] but not confirmed in the T47D cell line [50]. Cathepsin-B was also proposed to be correlated with the potential metastatic activities of human melanoma cells [51] and with the mitogenic activity of human

mammary tumors. The relationship between estrogen and lysosomal enzymes has long been suggested by the pioneering work of Clara Szego and her colleagues [52]. Cathepsin-B was shown to be stimulated by estrogens in the rat uterus and was proposed to be related to the mitogenic activity of estradiol [53].

Using a totally different approach, another lysosomal protease, the major excreted protein (MEP), has been extensively studied and characterized by Gottesman and colleagues [54]. MEP is specifically secreted by mouse NIH 3T3 transformed fibroblasts but not by normal untransformed cells [54,55]. It is a cysteinyl proteinase, mostly active at pH 4–5, recently identified as a cathepsin-L by its substrate specificity [56] and by cDNA sequencing [57]; its synthesis and secretion are stimulated by several mitogens (phorbol ester, PDGF. . .) [58].

In these three examples, cathepsins (-D, -B, or -L) appear to be secreted in higher proportions in cancer cells than in normal cells. Their synthesis and secretion appear to be stimulated by mitogens, and they are secreted by cancer cells as higher-M_r proenzymes, which can be auto activated by partial proteolysis at acidic pH [25,55]. The reason for this greater secretion of lysosomal protease by cancer cells is not known but may be important in explaining invasiveness of cancer cells.

CONCLUSIONS AND PERSPECTIVES

It has often been proposed that proteases are involved in the process of invasion and metastasis by cancer cells [for review, see 59,60]. Collagenases [60] and plasminogen activator [61] have been the most extensively studied and have been considered to be involved in the process of metastasis. However, clinical data have not confirmed a correlation between plasminogen activator activity and the prognosis of breast cancer [62]. Tissue plasminogen activator appears to be correlated with estrogen and progesterone receptor sites more than with prognosis [63,64]. In contrast, evidence is converging favoring the idea that cathepsins are closely related to the metastatic and invasive processes in cancer. Cell transformation and several mitogens have been shown to stimulate the secretion of the precursors of MEP-cathepsin-L, cathepsin-B, and 52K procathepsin-D-like protease. Moreover, our first clinical results indicate that high concentrations of the cathepsin-D-like antigen in breast cancer cytosol are associated with rapidly developing breast cancers. These results strongly support the idea that secreted lysosomal proteases are involved in the process of tumor growth and invasion. As has been suggested in the case of secreted proteases in several cancer cells [59], we propose that the increased synthesis and secretion of 52K cathepsin-D by estrogens may facilitate the invasion of basement membrane, connective tissue, and blood vessels by breast cancer cells and consequently increase the frequency of metastasis [25,65]. In support of this hypothesis, estradiol appears to stimulate the migration of MCF7 cells through a reconstituted basement membrane [66]. Other cancers may also be controlled by the enzyme, but immunoperoxidase staining has revealed high concentrations of the protein mostly in breast cancer cells and melanoma [34], suggesting that it acts more specifically in these two types of cancer, which frequently result in metastases. The regulation of lysosomal enzymes by sex-steroid hormones may have similar consequences in other hormone-regulated cancers (prostate, endometrium). The involvement of proteases in the control of cancer growth has important therapeutical implications [59]. Since the 52K protease, like other autocrine growth factors, is produced by both hormone-dependent and

-independent breast cancer cells, the neutralization of its activity (by antibodies, analogs, enzyme inhibitors, etc) could be of considerable importance in treating breast cancer and melanoma and in preventing metastasis, in the same way that small-cell lung cancer is treated by neutralizing bombesin-like growth factors [67]. These antimitogenic treatments would be of more general use than antihormone therapy, whose efficacy is restricted to hormone-dependent cancers. Further studies are needed to prove the biological function of these proteases in carcinogenesis and to discover how to inhibit their putative destructive effects.

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