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Oestrogens, Proteases and Breast Cancer. From Cell Lines to Clinical Applications

H. Rochefort

Human breast cancer is characterised by its high frequency of metastasis and its oestrogen responsiveness, allowing specific anti-oestrogen therapy. Oestrogens are promoting agents that stimulate early steps of mammary carcinogenesis. The availability of several oestrogen receptor (ER)-positive and ER-negative human breast cancer metastatic cell lines has allowed characterisation of several hormone-regulated genes, some of which are involved in growth and metastasis. Moreover, these models have allowed examination of the mechanisms by which hormone antagonists (anti-oestrogens and anti-progestins) act on their respective receptors to inhibit tumour growth. By contrast, no convenient *in vitro* models are available to investigate the mode of action of oestrogens and anti-oestrogens on non-malignant mammary cells. Among the oestrogen-regulated genes, some are also regulated by growth factors, such as the cathepsin D gene, whose overexpression in primary breast cancers has been associated with relapse and metastasis in several retrospective clinical studies. The mechanism and consequences of cathepsin D overexpression on metastasis are reviewed. From these studies on cell lines, new immunological and genetic probes have been raised that can be applied to breast cancer tissue to titrate in patients expression of different genes involved in the control of mammary tumour growth and invasion. These tissue markers should help to stratify primary breast cancers according to their ability to metastasise and respond to therapies and consequently to choose the best therapy. Over the next decade, these studies should lead to new therapeutical approaches of breast cancers which resist classical systemic therapies.

Key words: breast cancer; oestrogens, cathepsin D, metastasis, phagocytosis
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INTRODUCTION

OESTROGENS HAVE been proposed to stimulate the growth of breast cancer since studies by Beatson [1] and Lacassagne [2]. The mechanism underlying this effect has been investigated by studying oestrogen receptor-positive (ER+) breast cancer cell lines [3]. The development of these model cell lines has provided extensive information, some unexpected, concerning mechanisms and clinical applications. Decisive steps in this area have been to define oestrogen-induced genes and proteins, to develop specific antibodies and cDNA probes, and to use these probes on tumour biopsy specimens for defining prognosis.

Present and future studies should reveal the mode of action of these markers in controlling decisive steps in tumour promotion and in defining the mechanism for their altered expression in breast cancer.

In this article, I will consider:

(1) The control of cell proliferation by oestrogen and anti-oestrogen and the associated oestrogen-induced proteins;
(2) The prognostic significance of overexpression of oestrogen regulated genes in primary breast cancer; and
(3) The biological significance of cathepsin D (cath-D) over-expression in breast cancer, including its possible role in metastasis.

CONTROL OF CELL PROLIFERATION BY OESTROGENS AND ANTI-OESTROGENS

The study of ER+ breast cancer cell lines, most developed from metastatic pleural effusions, has led to the observation that oestrogens can directly stimulate the growth of these cells. The mechanism involved is still under debate. Oestrogen-induced growth factors, which interact with their membrane receptor in an autocrine fashion to stimulate growth, have been a fruitful paradigm, leading to the description of several oestrogen-induced proteins and growth factor activities [4–6]. In addition, oestrogens induce nuclear transcription factors, such as c-*myc* and c-*fos* [7], that can, *via* their nuclear receptor, cross communicate with other transcription factors, such as the *fos/jun* complex, to increase expression of AP–1 regulated genes in addition to genes that are regulated via oestrogen response elements (ERE) [8]. Whatever the mechanism, it is clear that oestrogens in ER+ breast cancer cells can directly stimulate their growth independently of interaction with other cells or tissues.

Anti-oestrogens have provided precious tools in order to understand the mechanism of ER activation, and to approach the understanding of control of cell proliferation by oestrogens and growth factors. Studies of anti-oestrogens in ER+ cell lines have contributed to the development of their broad therapeutical use in ER+ breast cancers (Figure 1).

Among the oestrogen-induced proteins identified by comparing cells stimulated and those not stimulated by oestradiol, some of them, such as transforming growth factor (TGF) α, insulin-dependent growth factors (IGF)s and c-*myc* are of potential importance in cell growth stimulation. Several secreted proteins may also exert their action on other cells and be secreted in blood. This was the rationale for our laboratory to develop monoclonal antibodies to a 52-k glycoprotein which was found to be oestrogen-induced and secreted [9].

DIFFERENT OESTROGEN-INDUCED PROTEINS HAVE DIFFERENT PROGNOSTIC SIGNIFICANCE AS CYTOSOLIC MARKERS IN BREAST CANCER

The first oestrogen-induced protein characterised and used in the clinic was the progesterone receptor [10]. Using different approaches, our laboratory found that the 52-k oestrogen-induced protein, initially suggested to be an autocrine growth factor, was in fact the precursor of cath-D, an ubiquitous lysosomal protease [11]. The laboratory of Pierre Chambon, using differential screening of a cDNA library from oestrogen-treated MCF7 cells, described a pS2 mRNA encoding the pS2 secreted protein of 84 amino acids. PS2 is not expressed in normal mammary cells, but is expressed in gastric cells, and was found to be analogous to pancreatic spasmolytic protein [12].

The 52-k protein is a striking example of serendipity, since initially expected to be a circulating marker of hormone responsiveness, it was not found to be increased in plasma of breast cancer patients, but was revealed as a tissue marker correlated with risk of developing metastasis (for review, [13]). Cath-D concentration did not correlate wth ER status or pS2 status even though both cath-D and pS2 are stimulated by oestrogen in ER+ positive breast cancer cells. In fact, pS2 is more strongly correlated with progesterone receptor status, and has been found to be a useful indicator for responsiveness to anti-oestrogen therapy. This apparent paradox may be explained by the constitutive expression of cath-D in some ER-negative breast cancers [14]. The prognostic significance of c-*myc* expression, which is also oestrogen-regulated, is uncertain probably because the c-*myc* protein is not easily measured. However, c-*myc* is amplified in a fraction of breast cancer tumours. Cath-D is the first example of a protease whose immunoassay in the cytosol is available and can be readily standardised with quality control [15]. This tissue marker is independent from other markers associated with cell differentiation and cell proliferation, and is proposed, in addition to those markers, to help predict breast cancer prognosis.

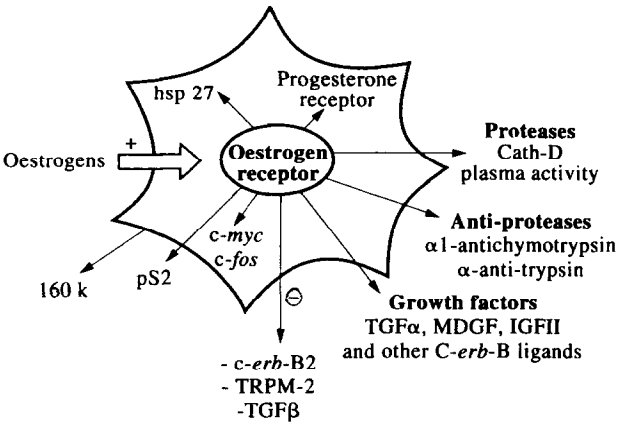

Figure 1. Oestrogen-controlled, coordinated expression of different genes whose expression are important for cell proliferation and tissue remodelling, such as growth factors, proteases, anti-proteases, transcription factors and membrane receptors. The first oestrogen-induced proteins are represented: among them, the progesterone receptor and pS2 are associated with good prognosis, while cath-D and c-*erb*-B2 are associated with poor prognosis. hsp27, a heat shock protein of 27 kDa molecular weight; MDGT, mammary-derived growth factor; 160 k, an unidentified secreted protein; Plasm. activ, plasminogen activators; $TRPM_{-2}$, testosterone-repressed prostate—message 2; TGF, transforming growth factor. IGF, insulin-like growth factor. c-*erb*-B2, $TRPM_{-2}$ and TGFβ have been shown to be regulated negatively by oestrogens and/or positively by anti-oestrogens.

HIGH CYTOSOLIC CATH-D ASSOCIATED WITH METASTASIS RISK: CAUSE OR CONSEQUENCE?

The fact that high cath-D concentration in cancer cells is predictive of recurrence and distal metastasis raises the question of its biological significance. It could be simply associated with metastasis, it could be causal or it may promote a rate-limiting step in metastasis. To approach this question, we have transfected a mammalian expression vector of human pro-cath-D or the control vector alone into a rat tumorigenic cell line (3YA12), which secretes no cath-D. Stable transfectant clones were selected, and those which produced high levels of human cath-D grew more rapidly at low serum concentrations and to a higher level of confluence than control vector clones. In addition, cells transfected with human cath-D had higher metastastic activity

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in the athymic mouse model compared to control transfected cells. Thus, in this experimental system, increased cath-D production facilitated metastasis [16].

The mechanism of facilitation of liver metastasis by cath-D overexpression [16] might involve either a simple physical interaction of the secreted pro-enzyme with the plasma membrane IGFII/M6P receptor or the proteolytic activity of cath-D may be required. To discriminate between these two possibilities, we inhibited secretion of the pro-enzyme by engineering a KDEL endoplasmic recticulum retention signal on to the human cath-D cDNA. Interestingly, the wild-type cath-D transfected clones and the control transfected clones, in which KDEL was replaced by the inactive signal KDAS, developed earlier and larger liver metastases than the KDEL transfected clones. In the KDEL transfected clones, pro-cath-D was retained in the endoplasmic reticulum, and did not mature, but its secretion and the addition of the M6P signal were not affected. This strongly suggested that cath-D maturation into a proteolytically active enzyme was required, and that a simple interaction with the M6P/IGFII receptor was not sufficient to stimulate metastasis [17].

PRO-CATH-D ACTIVATION AND METASTASIS

Since cath-D has a broad substrate specificity, there are virtually unlimited numbers of substrates whose proteolysis might explain facilitation of clinical metastases. A classical target for proteases to facilitate tumour invasion is the basement membrane [18]. In fact, both purified pro-cath-D and conditioned media from oestrogen-treated MCF7 cells digest extracellular matrix prepared from bovine corneal endothelial cells. Optimal activity occurs at acidic pH (4 to 5). Degradation of extracellular matrix by secreted proteases, present in conditioned media from different breast cancer cell lines, is mostly due to cath-D, since this degradation observed *in vitro* at an acidic pH is completely inhibited by pepstatin, but not by other inhibitors [19]. However, a role for cath-D in the digestion of basement membrane has not been directly demonstrated. Autoactivation of secreted pro-cath-D *in vivo* appears to require an acidic micro-environment, which occurs within cells (endosomes, lysosomes) rather than extracellularly.

Another interesting hypothesis is that pro-cath-D, following its autoactivation, could activate growth factor receptors, growth factor precursors or trigger a proteolytic cascade by activating proteases precursors. In contrast to other tissue proteinases, cath-D has no known endogeneous inhibitor and an acidic micro-environment appears to the major factor controlling its activation. Therefore, we have characterised the acidic compartments in which cath-D may be activated. Acridine orange fluorescence revealed large intracellular acidic vesicles (LAVs) of ≥ 5 μm in diameter to be the acidic compartments, in addition to lysosomes [20]. These LAVs were more frequently found *in vitro* in breast cancer cells than in normal mammary cells, and they contained high levels of cath-D but not pro-cath-D. LAVs were not restricted to cell lines and were also found in primary cultures of pleural effusions from breast cancers. Vesicles of the same diameter (>5 μm), that were highly concentrated with cath-D, were also observed *in vivo* by immunohistochemistry on paraffin sections of breast cancer biopsies [21].

PHAGOCYTOSIS AND INVASION: A HYPOTHESIS

To determine the significance of these acidic intracellular compartments (LAVs), we recently quantified LAV-positive cells before and after migration through Matrigel. Migration through Matrigel (Becton Dickinson) is a classical *in vitro* model to study invasion through an extracellular matrix. We found more LAV positive, MDA-MB231 breast cancer cells after migration through Matrigel compared to those cells before migration [22]. The LAVs were found to contain phagocytosed extracellular material, such as latex beads and partially digested extracellular matrix and can, therefore, be considered as large heterophagosomes. The ability of breast cancer cells to phagocytose extracellular material, including extracellular matrix, and to digest this material within heterophagosomes has not been previously observed, and was thought to occur mostly in specialised phagocytotic, such as macrophages and polynuclear neutrophiles. Phagocytotic activity of cancer cells was previously proposed and supported by *in vitro* invasion of chicken blastoderm by cancer cells engulfing hypoblast yolk [23]. The relatively higher proportion of LAVs we observed in breast cancer cells, following their migration through Matrigel, can be interpreted in two ways. Migration through Matrigel might induce LAVs formation, or conversely, these compartments might facilitate development of cancer cell colonies in distal parenchyma and consequently clinical metastasis. Since cath-D concentration in cells having migrated was also higher than in cells before migration, it is possible that the engulfed extracellular material in LAVs can be more efficiently digested when cathepsins concentration in these compartments is in excess. This might explain why cancer cells overexpressing cath-D, either naturally as in human breast cancers or following transfection in our experimental rat tumour model, have a higher ability to develop metastasis. Other mechanisms, however, are not excluded, such as a premature activation of pro-cath-D in endosomes, allowing activation of a transducing mechanism involved in cell proliferation or migration.

CONCLUSIONS AND PROSPECTIVE

Results obtained on cath-D in breast cancer illustrate an additional example of serendipity by which one working hypothesis leads to unexpected findings. In addition, studies on cath-D as well as on other proteins like pS2, show that metastatic breast cancer cell lines have been decisive in identification of markers which can be used in the clinic. By contrast, there are no convenient *in vitro* models to study, oestrogen regulation in normal or premalignant human mammary cells. However, it is known that oestrogens are important mitogens *in vivo* on mammary ducts, at the time of puberty raising the possibility that local overproduction of oestrogens at early steps of oncogenesis may facilitate development of invasive breast cancer. In further support of a role for oestrogen in premalignant tissue, it has been shown that adjuvant anti-oestrogen therapy prevents development of breast cancer in the controlateral breast [24], which is the basis of clinical trials of anti-hormonal prevention of breast cancer in high risk patients.

The aetiology of breast cancer is unknown, but its frequency has increased in western countries. In addition to hereditary factors, the environment and sex steroid hormones are important factors to consider. One hypothesis is that a continuous supply of active oestrogen, of constant activation of its receptor, could facilitate early steps of mammary carcinogenesis by increasing expression of genes that control cell proliferation and invasion. The fact that the *in vitro* cell culture systems used were of human origin was particularly useful, since it allowed development of probes for direct clinical applications (Figure 2). The long-term experience in standardisation and quality control of steroid receptor assays in breast cancer in the last 20 years has also

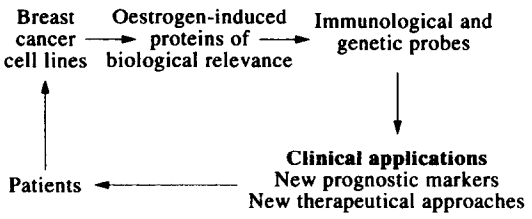

Figure 2. Interface strategies facilitating clinical applications *in vitro* cell culture studies. Medical applications are more rapid when human cell lines are available since the probes developed can be used directly on tumour tissues banks for retrospective studies and on tumour samples for prospective studies.

been decisive in facilitating the development of new prognostic markers. While more may be learned from the use of human breast cancer cell lines, it is important to consider that cancer development *in vivo* is more complex than a simple autocrine dysregulation of cancer cells. Paracrine interactions with surrounding cells, such as fibroblasts, endothelial cells and macrophages, as well as the complex interaction with the extracellular matrix and participation of immunological defense should also be considered.

Studies aimed to increase our understanding of mechanisms by which cell proliferation and metastatic dissemination are controlled should also lead to new therapeutical approaches. However, developing new drugs will probably take more time than time required to introduce new prognostic markers in the clinic.

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