How to target estrogen receptor-negative breast cancer?

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Abstract

Estrogen receptor (ER)-positive breast cancers generally have a better prognosis and are often responsive to anti-estrogen therapy, which is the first example of a successful therapy targeted on a specific protein, the ER. Unfortunately ER-negative breast cancers are more aggressive and unresponsive to anti-estrogens. Other targeted therapies are thus urgently needed, based on breast cancer oncogene inhibition or suppressor gene activation as far as molecular studies have demonstrated the alteration of expression, or structure of these genes in human breast cancer. Using the MDA-MB.231 human breast cancer cell line as a model of ER-negative breast cancers, we are investigating two of these approaches in our laboratory. Our first approach was to transfect the ER or various ER-deleted variants into an ER-negative cell line in an attempt to recover anti-estrogen responsiveness. The unliganded receptor, and surprisingly estradiol, were both found to inhibit tumor growth and invasiveness in vitro and in vivo. The mechanisms of these inhibitions in ER-negative cancer cells are being studied, in an attempt to target the ER sequence responsible for such inhibition in these cancer cells. Another strategy is trying to inhibit the activity or expression of an oncogene specifically overexpressed in most breast cancers. This approach was recently shown by others to be efficient in breast cancer therapy with HER2-Neu oncogene amplification using Herceptin. Without excluding other molecular putative targets, we have focused our research on cathepsin D as a potential target, since it is often overexpressed in aggressive human breast cancers, including ER-negative tumors, and rarely associated with HER2-Neu amplification. Our first results obtained in vitro on cell lines and in vivo in tumor xenografts in nude mice, illustrate that the mode of action of cathepsin D in breast cancer is useful to guide the development of these therapies. In the past 20 years we have learned that the action of cathepsin D in breast cancer is complex and involves both intracellular and extracellular activities due to its proteolytic activity and to interactions with membrane components without catalytic activity. Each of these mechanisms could be potentially inhibited in an attempt to prevent tumor growth. Breast cancer is a very heterogeneous and multigenic disease and different targeted therapies adapted to each category of breast cancer are therefore required. Validated assays in the primary tumor of molecular markers such as ER, HER2-Neu and cathepsin D should help to predict which targeted therapy should be applied to cure breast cancer patients.

Introduction

The mechanism of breast cancer progression and metastasis is not fully understood. However, breast cancer frequency tends to increase. Moreover the ability of these cancers to metastasize and their heterogeneity, clearly require new therapies, targeted on specific genes and proteins actively engaged in the physiopathology of breast cancer rather than the conventional cytotoxic chemotherapy, which is often hampered by toxicity or resistance problems. These targeted therapies should allow action with more efficacy and less toxicity, but only on the relevant breast cancer subset as determined by assaying the corresponding predictive marker in the tumor (Gibbs 2000).

There are at least three key requirements for developing a new molecular target in cancer treatments: (i) the availability of clinical studies relating gene overexpression in the tumor and prognostic value in clinical follow-up studies so
as to determine which patients would benefit from this therapy, and to tailor treatments adapted to each breast cancer subset in patients; (ii) a full understanding of the mode of action of the molecular target involved in breast cancer carcinogenesis or progression; (iii) a means to specifically target the drug in the tumor rather than in normal tissues, thus lowering toxicity and increasing selectivity as compared with traditional cytotoxic chemotherapy.

Anti-estrogen therapy is the first successful cancer therapy targeting estrogen receptor (ER) expression (Rochefort 1987, Jordan & Murphy 1990). However, its efficacy, as well as that of aromatase inhibitors, is limited to ER-positive breast cancers, which generally have a better prognosis (McGuire 1978, Osborne 1998). Unfortunately ER-negative breast cancers are more aggressive (Sheikh et al. 1994) through a still unknown mechanism. Therefore other targeted therapies are urgently needed in these cancers. ER-negative invasive breast cancers are often considered to be the result of tumor progression from ER-positive premalignant lesions or ER-positive breast cancers by genetic alteration (gene instability, loss of heterozygosity LOH, exon deletion etc.) epigenetic alteration such as promoter methylation (Ferguson et al. 1995) or ER protein degradation in proteasome after hypoxia in non-vascularized tumor (Stoner et al. 2002). However, some invasive, human breast cancers may be directly ER-negative via a hormone-independent pathway. This pathway is based on the results of gene knock out studies in mice (Korach 1994, Hewitt et al. 2002) and on immunohistochemical studies in humans, showing that some proliferative ductal lesions and many high-grade ductal carcinoma in situ (DCIS) are ERα-negative (Roger et al. 2000).

Targeted therapy of ER-negative hormone-independent breast cancers could be a priori based on two general approaches: (i) to transform ER-negative into ER-positive cancer cells by gene therapy or ER gene reexpression; (ii) to target genes or proteins actively involved in the genesis or progression of the corresponding breast cancer subset.

To transform ER-negative into ER-positive breast cancer cells

In our laboratory, we used the MDA-MB 231 breast cancer cell line as a model of ER-negative breast cancers. This human cell line is particularly suitable for pre-clinical studies since it is highly aggressive both in vitro and in vivo (Price et al. 1990), it does not overexpress HER2-Neu, but it does overexpress and secrete cathepsin D (cath-D), a lysosomal protease extensively studied both in our laboratory (Rochefort et al. 1987, 1989) and in several independent clinical studies for its prognostic value (for reviews see Rochefort 1992, 1996, Rochefort et al. 2002).

Our first approach was very naively to transfected the ERα recombinant cDNA into the MDA-MB-231 ER-negative breast cancer cell line in an attempt to recover normal estrogen and anti-estrogen responsiveness. The unliganded ERα was found to inhibit the invasiveness and growth of two stably transfected MDA-MB 231 cell lines as compared with cell lines transfected with the vector alone. This was consistent with the lower invasiveness of ER-positive breast cancer cells (Thompson et al. 1992). However, and surprisingly, estradiol inhibited cell growth and invasiveness to a greater extent, both in vitro and in vivo (Garcia et al. 1992), while tamoxifen had no effect and the pure anti-estrogen ICI-16438480 abolished the favourable effect of transfected ERα (Rochefort et al. 1998). In order to study the mechanism of these inhibitions and particularly to define the domain and sequence in ERα responsible for inhibition, Platet and Garcia in our laboratory developed a method for transient co-transfection of ER or variants, with the luciferase expression vector, which enables monitoring of only the transfected cells without having to select stable transfectants (Platet & Garcia 1999). The first zinc finger in the C domain of ER was found to be responsible for this inhibition. The effect was specific to ERα and ERβ, while transfected glucocorticoid receptors glucocorticoid receptors (GR), androgen receptors (AR), vitamin D receptors (VDR), retinoic acid receptors (RARα) were inefficient (Platet et al. 2003). These studies suggest that ERα is protective, which is in agreement with the better prognosis of ER-positive breast cancers. The protective effect of ERβ might be even higher, since markedly decreased in earlier steps of carcinogenesis (Roger et al. 2001) and involved in the terminal differentiation of mammary glands (Forster et al. 2002). Estrogens and anti-estrogens unfortunately had an opposite effect on growth in naturally ER-positive breast cancer cells like MCF7 and in ER-negative breast cancer cells transfected with ERα like MDA-MB231 cells. However, the transcriptional activity of reporter genes was stimulated by estrogen in both types of cancer and some endogenous estrogen-regulated genes were also stimulated by estradiol after ER transfection (Toutou et al. 1990, Jeng et al. 1994). Understanding the difference in the two cell types (ER-negative or ER-positive) responsible for the inverse effect of activated ER may provide a clue for developing new targeted therapy aimed at replacing ER in order to inhibit the factor involved in the stimulation of proliferation and invasion in ER-negative breast cancers. More studies are required to pinpoint this factor, show its clinical significance in breast cancer patients and find a specific way to target the inhibitory sequence of ER in breast cancer tissue in vivo.

An interesting alternative is to reexpress the ER gene by demethylating its promoter or by using histone desacetylase inhibitors (Yang et al. 2001).

In both cases, however, an additional strategy will be required to target the drug specifically in mammmary cancer cells.
To target a protease actively engaged in the pathogenicity of breast cancer

There are a large number of possibilities to inhibit ER-negative breast cancers since the nature of genes responsible for the growth and spread of these tumors is not fully defined and vary according to the tumor subset (Ingvarsson 1999). For instance, overexpression and gene amplification of HER2-Neu and epidermal growth factor receptor have led to the development of new therapies targeting these proteins (Baselga et al. 1996, Pegram & Slamon 1999). These targets were initially proposed on the basis of clinical studies indicating their bad prognostic significance in breast cancer.

For 20 years our laboratory has been involved in studying another prognostic marker, cath-D, (for reviews see Rochefort et al. 2000, 2002) and in developing an alternative approach aimed at inhibiting cath-D expression or function in order to block the growth of breast cancer overexpressing this protease.

We will first present evidence that this lysosomal protease is a good potential therapeutic target and then review the different approaches adopted in an attempt to inhibit breast cancer growth and progression based on the dual mode of action of cath-D in cancer.

Cath-D is a good potential target for ER-negative breast cancers

Even though cath-D was initially shown to be induced by estrogen in ER-positive breast cancer cell lines, it is also constitutively overexpressed in ER-negative tumors as shown in both cell lines (Rochefort et al. 1989) and patients. Several clinical studies have shown an absence of correlation between ERα and cath-D level in invasive breast cancers (Rochefort 1992, Foekens et al. 1999) and DCIS (Roger et al. 2000). Cath-D overexpression was not correlated with HER2-Neu amplification (Brouillet et al. 1990), another target for ER-negative breast cancer.

When using a validated cytosolic assay, the specific overexpression of cath-D in ER-negative breast cancer patients based on several independent clinical studies, is frequently associated with shorter relapse-free survival and overall survival (for review see Rochefort et al. 2002). A relatively large fraction (about 60%) of ER-negative breast cancers overexpressing cath-D might therefore benefit from therapy targeted to cath-D.

This protease is not only associated with breast cancer progression, but appears to be also a rate-limiting factor in stimulating in vitro and in vivo tumor growth. Initial studies showed that specific overexpression by stable transfection of human pro-cath-D stimulated growth of a rat tumoral cell line derived from embryo fibroblasts transfected with an adenovirus, both in vitro and in vivo (Garcia et al. 1990). Experimental liver metastasis was also increased. More recent studies showed that cath-D antisense RNA, stably transfected in vitro into MDA-MB231 cancer cells, inhibited their proliferation, both in vitro (in a 3D matrigel system) and in vivo, when these cells are injected s.c. or i.v. in nude mice. The size and number of experimental lung metastases were also increased. The in vitro invasion conversely was not significantly decreased (Glondu et al. 2002). Therefore cath-D expressed in the human MDA-MB-231 breast cancer cell line is one of the rate-limiting factors required for the growth of micrometastases at distant sites.

One can argue that similar results were obtained in similar experiments with several growth factors and proteases, indicating that a large number of proteins are needed to promote tumor growth (Powis & Kozikowski 1991). However, since a proteolytic cascade is necessary for a tumor to spread and grow at a remote site, the inhibition of only one actor of the cascade may be sufficient to block the activation of other proteases. In this respect, cath-D has the potential to initiate a proteolytic cascade in vitro (Rochefort et al. 2000). However, the key argument is that very few potential breast cancer molecular targets have been clinically studied as extensively as cath-D for its prognostic value when specifically overexpressed in breast cancer (Ferrandina et al. 1997, Foekens et al. 1999).

Different approaches to inhibit cath-D production or action in breast cancer based on its mode of action

Cath-D can potentially act as a protease to degrade or activate substrates intracellularly and extracellularly. As an extracellular protease, cath-D may liberate growth and angiogenic factors entrapped in the extracellular matrix and consequently stimulate angiogenesis as shown with fibroblast growth factor-2 (Briozzo et al. 1991). This activity, however, requires an acidic pH. It has also the potential, mostly after secretion of the proenzyme to bind to membrane receptors at neutral pH through autocrine or paracrine mechanisms and to act as a ligand to trigger mitogenic signals (for review see Rochefort & Liaudet-Coopman, 1999). A priori, one can therefore inhibit cath-D production or cath-D action as a protease or as a ligand.

Cath-D gene expression can be inhibited via its gene regulation

In ER-positive breast cancer the efficacy of anti-estrogens and aromatase inhibitors may be partly, but not exclusively due to this mechanism, since tamoxifen is a partial estrogen agonist for cath-D regulation and other mitogens are also involved. In ER-negative breast cancer, the origin of cath-D over expression is unknown and might involve different mechanisms, including other enhancers stimulated by other transcription factors (Giamarchi et al. 1999).
Cath-D mRNA can be neutralized with antisense RNA
Antisense RNA, added in vitro to cancer cells, inhibits their growth both in vitro and in vivo as described in Glondu et al. (2002), but when added in vivo, it should be associated with an additional means of targeting. In order to reduce toxicity, cath-D inhibitors should act more specifically in breast cancer cells than in normal tissue. Some normal tissues also express cath-D such as liver, spleen etc. Liver toxicity was not observed in the young gene knock out mice (cath-D−/−) which die, however, after weaning with intestinal necrosis and thymocytes apoptosis (Saftig et al. 1995). An additional targeting strategy is based on the fact that in solid tumors such as breast cancer, pro-cath-D is secreted in an acidic micro environment (pH down to 6.2) due to hypoxia (Gerweck & Seetharaman 1996) and that both macrophages and ER-negative breast cancer cell lines such as MDA-MB-231 are able to acidify extracellular medium more strongly than an ER-positive breast cancer cell line such as MCF7 cells (Montcourrier et al. 1997).

Aspartyl protease inhibitors
Other approaches are based on the dual mode of action of this protease in cancer cells. It initially seemed obvious that cath-D, like most of proteases in cancer, acts intracellularly or extracellularly through its proteolytic activity. This is strongly suggested by the fact that the addition by directed mutagenesis of a KDEL reticulum endothelial retention signal at the C-terminal end of the protein, totally inhibited both the maturation of 52 kDa pro-cath-D into mature chains (34 kDa + 14 kDa) and the stimulatory effect of cath-D overexpression on cell growth (Liaudet et al. 1994). Moreover, at high cell density, tumor cells secrete a growth inhibitor which is degraded or inactivated intracellularly in acidic compartments by cath-D overexpression and maturation (Liaudet et al. 1995). Attempts have thus been made to use aspartyl protease inhibitors such as pepstatin, but currently without great success. This failure might be due to solubility or pH problems or to the fact that cath-D may not be acting as a protease but rather as a ligand by interacting with a membrane receptor to trigger a mitogenic transduction pathway for instance.

Inhibition of cath-D acting as a ligand
In order to determine whether cath-D proteolytic activity is necessary to stimulate cancer cells growth, we mutated its catalytic site by substituting the aspartic acid residue 231 of the 34 kDa chain with an asparagine residue. This mutation totally abolished cath-D proteolytic activity, but affected neither its expression level, processing nor secretion, nor its capacity to stimulate proliferation of cells embedded in Matrigel, colony formation in soft agar nor tumor growth in athymic nude mice (Glondu et al. 2001).

One characteristic common to all lysosomal proteases expressed in cancer cells (cath-D, -B, -L) is that they are secreted as proteolytically inactive pro-forms. They are then able to be re-endocytosed by tumoral cells (epithelial cancer cells, stromal cells ... etc.) by interacting with membrane receptor(s). In fibroblasts the receptors to endocytose pro-cath-D and other lysosomal hydrolases are, as expected, the Man6P receptors via the high mannose glycosylated chain(s) of the enzymes. However, in breast cancer cells, the lysosomal routing and endocytosis of pro-cath-D are Man6P-independent (Capony et al. 1994, Laurent-Matha et al. 1998).

We have successively excluded the Man6P/insulin-like growth factor-II receptor and the low density lipoprotein (LDL) receptor related protein (Laurent-Matha et al. 2002) but have not yet identified this interacting molecule. There are different potential means of interfering with this process, e.g. inhibiting secretion by neutralizing antibodies as described for cath-L in melanomas (Guillaumette et al. 2002), or inhibiting interaction with a Man6P-independent membrane receptor.

Others have proposed that a receptor which binds part of the cath-D pro-fragment is responsible and that neutralizing antibodies could inhibit its binding as well as tumor growth (Fusek & Vetryva 1994). This is to be confirmed by other laboratories. We propose that according to its site of secretion, the cancer cell density, and the environmental extracellular pH of the tumor, cath-D could act in vivo both as a ligand and as a protease. In this case the association of different types of inhibitors would be more efficient.

Conclusion
To conclude, the targeting of breast cancer on the ER has proven its efficacy and low toxicity with anti-estrogens and aromatase inhibitors. Systemic treatment of ER-negative breast cancers requires other molecular targets, based on alteration or over expression of a breast cancer oncogene or reexpression of a breast cancer suppressor gene. We have reviewed our attempts to transfect ER cDNA into ER-negative cell lines and to use cath-D as a target to inhibit the growth and progression of ER-negative breast cancer subset overexpressing specifically this protease. In addition to be potentially active on ER-negative breast cancers, cath-D inhibitors have also the potential to treat the anti-estrogen resistant ER-positive breast cancers which overexpress cath-D. A better understanding of the mechanisms by which cath-D overexpression stimulates tumor growth and progression and the development of means to inhibit these activities should in the near future, help in tailoring a treatment adapted to each breast cancer subset.

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References


Garcia M, Deroço D, Freiss G & Rochefort H 1992 Activation of estrogen receptor transfected in a receptor negative breast cancer cell line decreases the metastatic and invasive potential of the cells. PNAS 89 11538–11542.


Pegram MD & Slamon DJ 1999 Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant breast cancer. Endocrine-Related Cancer (2003) 10 261–266.