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Special Paper

Cathepsin D and Breast Cancer

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INTRODUCTION

CATHEPSIN D is a proteolytic enzyme that is normally localised in the lysosomes and functions in protein catabolism. It belongs to the group of aspartyl proteases and is distinguished from other members of this group, such as pepsin, by a post-translational cleavage resulting in a molecule containing a heavy (34 kDa) and a light (14 kDa) chain and by the presence of N-linked oligosaccharides which are responsible for the targetting of the enzyme to the lysozomes via mannose-6-phosphate receptors. A number of disparate areas of research have identified cathepsin D as an important protease in a variety of disease processes including degenerative brain disease [1], connective tissue disease [2] as well as fundamental biological processes such as the presentation of antigens to class II major histocompatibility complexes [3]. Cathepsin D has also risen to prominence in breast cancer. The aim of the article is to review the importance of cathepsin D in breast cancer, in particular its biology, the regulation of its expression by oestrogen and the value of cathepsin D as a prognostic marker.

Interest in cathepsin D in breast cancer was first aroused by studies aimed at identifying proteins whose expression is regulated by oestrogens in breast cancer cell lines. A prominent oestrogen-regulated secreted protein was identified by analysis of ^{35}S-labelled proteins by one- and two-dimensional gel electrophoresis [4, 5] and this protein was subsequently shown to possess proteolytic activity at acidic pH [6, 7]. In 1987, cathepsin D mRNA was reported to be regulated by oestrogens in breast cancer cells [8], and in 1988 the oestrogen-regulated secreted protease was identified as cathepsin D, following protein sequencing and the sequencing of cDNA clones obtained from an expression library [9]. The original aim of identifying novel oestrogen-regulated proteins was to identify markers of oestrogen responsiveness that might have clinical value for predicting the likely response of breast cancer patients to hormone therapy. It is ironic, therefore, that despite the extensive literature on the regulation of cathepsin D expression by oestrogens in oestrogen-responsive breast cancer cell lines, most interest is now focused on the proteolytic activity of cathepsin D, the potential biological significance of the expression of this protease in the process of tumour growth and metastasis, and the possible value of cathepsin D expression as a marker of disease progression or poor prognosis.

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ROLE OF CATHEPSIN D IN TUMOUR GROWTH AND METASTASIS

Biological studies have focused principally on two aspects of the biology of cathepsin D: its effects on cell proliferation and its effects on metastasis.

Effect of cathepsin D on cell proliferation

Vignon and colleagues [10] immunopurified procathepsin D secreted from MCF-7 cells and showed that it increased the proliferation of MCF-7 cells which had been withdrawn from the effects of oestrogens by culturing in oestrogen-free medium. Procathepsin D increased cell numbers approximately 2-fold at concentrations of 2 ng/ml. Since this original observation, a number of studies have examined the mitogenic activity of mature and procathepsin D. Garcia and associates [11] reported that rat cells transfected with a cathepsin D expression vector showed increased cell proliferation in culture. The form of cathepsin D responsible for this effect is not known: a subsequent study [12] demonstrated that the cathepsin D was processed normally to the 34 and 14 kDa chains, possibly suggesting that mature rather than pro- cathepsin D is the mitogenic agent in this system. However, clones of cells overexpressing recombinant cathepsin D with a KDEL [12] sequence at the carboxy terminus were also analysed and these showed increased proliferation without maturation of the procathepsin D.

In surveys of the proliferative effects of a number of growth factors for breast cancer cell lines, Karey and Sirbasku [13] and Stewart and associates [14] reported that mature cathepsin D did not stimulate proliferation, and in a subsequent study, Stewart and colleagues [15] reported that procathepsin, purified from the culture supernatant of MCF-7 cells using pepstatinyl–agarose affinity chromatography, had no mitogenic activity. In contrast to these negative results, Vetvicka and associates [16] reported that procathepsin D, purified by immunoaffinity chromatography followed by ion-exchange chromatography, stimulated the proliferation of human breast cancer but not other types of cells.

Overall these studies are difficult to reconcile. At the one extreme, Vetvicka and colleagues [16] reported that the magnitude of the effect of procathepsin D was as great as the addition of fetal calf serum or IGF-II (insulin-like growth factor), whereas

Vignon and colleagues [10] reported that procathepsin D is considerably less mitogenic than IGF-II and, at the other extreme, Stewart and associates [15] reported no activity at all for procathepsin D.

There are similar controversies over the mechanisms involved in the reported growth stimulation. Vignon and associates [10] demonstrated that procathepsin D is taken up by breast cancer cells and processed into the mature form, and subsequent binding and crosslinking experiments [17] provided direct evidence that procathepsin D could bind to the type II IGF receptor which also functions as the mannose-6-phosphate receptor. A model was proposed in which procathepsin D acts as a partial IGF II agonist at the type II IGF receptor [17]. In contrast, Fusek and Vetvicka [18] reported that the proliferative effects are not inhibited by pepstatin or mannose-6-phosphate whereas they are inhibited by antibodies against the propeptide. This suggests an important role for the propeptide which would not be expected to bind to the type II IGF receptor. Chemically-synthesised propeptide was reported to stimulate the proliferation of three breast cancer cell lines and inhibit the binding of procathepsin D to MDA-MB-231 breast cancer cells [18].

In addition to mechanisms involving the interaction of the mannose-6-phosphate moieties [17, 19] and the propeptide of cathepsin D [16, 18], two other possible mechanisms have been suggested by which cathepsin D could stimulate cell growth. Conover and De Leon [20] have suggested that cathepsin D could interact with the IGF-I signalling pathway by degrading soluble IGF binding protein 3 (IGFBP-3), which is a potent inhibitor of IGF action through its ability to sequester IGF-I. The ability of mature cathepsin D to control the release of growth factor activity from the extracellular matrix has also been addressed. Cathepsin D also releases bFGF from the extracellular matrix in culture and the bFGF is subsequently internalised [21]. As bFGF is mitogenic for breast cancer cells [14], these experiments provide evidence for an indirect effect of cathepsin D on breast cancer cell proliferation. However, both these mechanisms require an acidic extracellular environment ($pH < 5.5$) to allow significant enzyme activity and there is doubt whether this pH is attained *in vivo*.

In summary, the ability of cathepsin D to act as a mitogen and the mechanisms that might be involved remain controversial. With the exception of the data of Vetvicka and colleagues [16], in which cell proliferation was measured using the MTT assay rather than cell counting, the effects of procathepsin D are insignificant or small in relation to the mitogenic effects of growth factors such as IGF-II and the overall importance of the proliferative effects of cathepsin D therefore also remain open to question.

Effects of cathepsin D on metastasis

The involvement of proteases in metastasis has been an increasingly active research area as this process involves the destruction of normal tissue architecture, the movement of tumour cells out of their site of origin into the lymphatic system or blood stream and the subsequent colonisation of other organs [22]. A number of observations have implicated cathepsin D in the metastatic process. Early studies using purified procathepsin D in which degradation of extracellular matrix was monitored *in vitro* showed that procathepsin D, either in conditioned medium from oestrogen-stimulated breast cancer cells or purified from conditioned media, could degrade extracellular matrix as long as procathepsin D was exposed to an acidic pH to allow autoactivation of the enzyme [23]. It has also been suggested that breast cancer cells might digest extracellular matrix by a mechanism involving ingestion and destruction within large acidic vesicles though this process has yet to be demonstrated *in vivo* [24]. Elegant studies by Garcia and associates [11] in which human cathepsin D was overexpressed in rat embryo cells (3Y1-Ad12) suggested that overexpression of cathepsin D in these cells is correlated with an increased propensity to form liver metastases when injected into athymic nude mice. Subsequent experiments were performed in which cathepsin D was modified in an attempt to change the cellular compartment in which it is expressed [12]. Cathepsin D containing a KDEL peptide at the C-terminus to localise it within the endoplasmic reticulum or a control peptide (KDAS) was expressed in 3Y1-Ad12 cells. This confirmed the high metastatic potential of cells expressing cathepsin D containing the control KDAS peptide, but the metastatic potential of cells expressing the KDEL cathespin D construct was significantly reduced concomitant with a dramatic reduction of the processing of procathepsin D into mature enzyme. This study therefore suggested that the involvement of cathepsin D in metastasis requires the mature enzyme and this may not be surprising as procathepsin D lacks proteolytic activity. In contrast to the results of Garcia and associates [11], Johnson and associates [25] have contested the role of cathepsin D in invasiveness as a result of a series of experiments in which secretion of procathepsin D was shown not to be correlated with invasion (measured by the ability of cells to invade an artificial basement membrane). This apparent discrepancy may simply emphasise the importance of the processing and maturation of cathepsin D, and the observation that secreted procathepsin D levels are not related to metastasis is in agreement with the data of Liaudet and colleagues [12] showing the ineffectiveness of intracellular unprocessed procathepsin D in promoting metastasis.

CATHEPSIN D AS AN OESTROGEN-REGULATED GENE—MECHANISMS INVOLVED IN CONTROL OF EXPRESSION

Oestrogens regulate the expression of a number of genes, and the mechanisms involved have been considerably clarified in recent years with the cloning of the promoter regions of oestrogen-regulated genes and the oestrogen receptor. Cathepsin D is of interest because its expression is regulated by oestrogens in certain cells, but it also has a variable level of constitutive expression. In addition, its regulation by anti-oestrogens is of interest. Although it was originally reported that cathepsin D is not induced by the partial oestrogen agonist tamoxifen [5], subsequent studies with cultured cells [26] and with breast cancer patients treated with tamoxifen [27] have shown that cathepsin D expression can be increased by tamoxifen in breast cancer cells. Of considerable interest from a mechanistic viewpoint was the observation that combinations of oestradiol and certain concentrations of tamoxifen are more oestrogenic for the induction of cathepsin D than either compound alone [26]. These features are in contrast to some other oestrogen-regulated genes such as the vitellogenin genes, which are expressed in the livers of oviparous vertebrates and whose expression is completely dependent on oestrogen and not induced at all by anti-oestrogens.

Cathepsin D cDNA clones were isolated and sequenced in the mid 1980s [8, 9, 28–32] and this allowed genomic clones to be isolated. Three groups have published the sequence of the promoter region of the cathepsin D gene [33–35] and the organisation of the promoter as reported by them is depicted in

Figure 1. Redecker and associates [35] identified no TATAA sequence (a consensus sequence for transcription initiation of regulated genes) in the promoter, but commented on the high G+C content typical of 'housekeeping' genes. May and colleagues [33] and Cavaillès and colleagues [34] both identified a TATAA sequence and May and colleagues [33] identified a near consensus CAAT upstream from the TATAA sequence. Consensus sites for binding a number of transcription factors, particularly SP1, were also identified. Although consensus oestrogen response elements (5'GGTCAnnnTGACC3') consisting of an inverted repeat were not identified, May and colleagues [33] identified five and Augereau [36] identified four perfect half palindromes, and such sequences have been implicated in the regulation of a number of other genes by oestrogen [37, 38]. Identification of the site from which transcription is initiated was addressed in all three studies. Redecker and associates [35] used RNA, extracted from U937 monocytic cells, in RNAse protection and primer extension assays to suggest that the initiation of transcription was at −68 (+1 being the A of the first AUG). Cavaillès and associates [34] identified a total of five sites of initiation of transcription between −20 and −72 bp with the major sites being at −20 and −72. May and colleagues [33] identified two start sites and these were mapped accurately at −14 and −63 using a series of deletion mutants. The three groups have therefore identified different start sites. It is possible that the −14 site of May and colleagues [33] corresponds to the TSS 1 of Cavaillès and associates [34] at −20 as both groups reported that transcription from this start site is regulated by oestradiol. The −63 start site of May and associates [33] may correspond to TSS V of Cavaillès and associates [34] at −72 and the start site identified by Redeecker and associates [35] at −68, as Cavaillès and associates [34] and May and colleagues [34] have both reported that transcription from this start site is constitutive.

Cavaillès and associates [39] created a series of chimeric recombinants containing a variety of fragments from the cathepsin D promoter region linked to a heterologous (thymidine kinase) promoter and a reporter (chloramphenicol acetyl transferase) gene. Transfection of these constructs together with the HEO oestrogen receptor expression plasmid allowed fragments which conferred increased expression of the reporter gene to be identified. A 7 kb insert containing the putative promoter together with the first intron and exon conferred oestrogen responsiveness and the region was then localised to −123/−364 on the basis of transfection experiments with constructs containing a number of restriction fragments from the cathepsin D promoter. The localisation of sequences conferring oestrogen responsiveness was subsequently refined by the study of Augereau and associates [36]. Transfection of TK/CAT constructs, in which sequences from the cathepsin D promoter

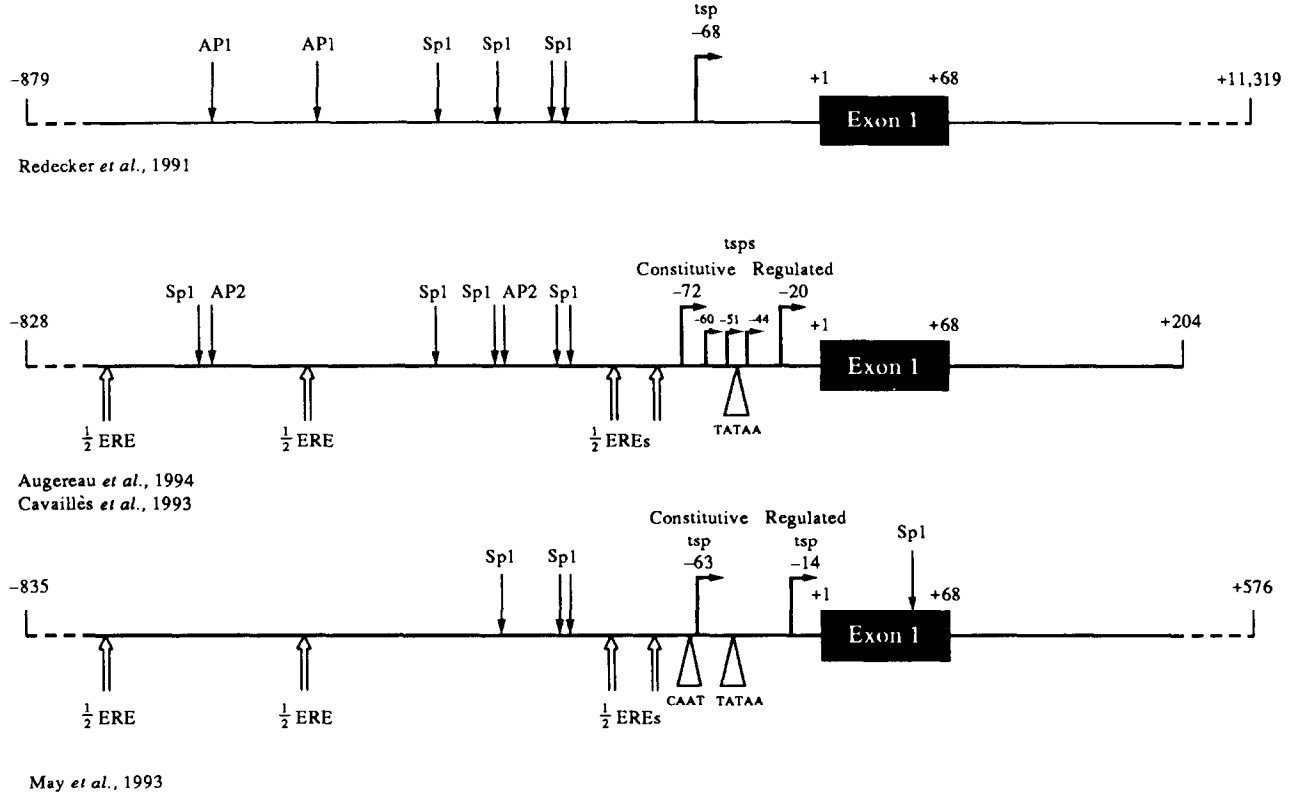

Figure 1. Promoter region of cathepsin D. Diagrammatic representation of the features of the cathepsin D promoter region identified by Redecker and associates [34], Augereau and associates [35], Cavaillès and associates [33] and May and associates [32]. The bold line shows the region around exon 1 from −400 to +200 (all sequences numbered with +1 being the first nucleotide of the AUG initiation codon). The numbers at either end of the dashed line show the length of the sequence contained in the publication. For clarity, the filled box which depicts exon 1 is shown to start at the first nucleotide of the AUG codon although exon 1 actually starts at the relevant transcription start point. The positions of transcription start points (tsp) are indicated with forward pointing arrows, consensus oestrogen receptor response element half sites (1/2 ERE) by upward pointing open arrows, consensus Sp1, AP1 and AP2 transcription factor binding sites by downward pointing arrows. The ERE half sites are numbered as in the original publications. The positions of consensus TATAA and non-consensus CAAT boxes, where identified in the original publications, are shown.

were artificially recombined, in some cases with oligonucleotides containing putative EREs, suggested that a sequence containing one of the perfect half palindromes (ERE E2) was necessary for oestrogen responsiveness. Transfection experiments with a construct containing a 29 bp oligonucleotide with the ERE E2 sequence embedded within it did not confer oestrogen regulation, suggesting that this sequence is not sufficient on its own. Further experiments showed that this region together with several others is protected from DNAse I digestion when incubated with nuclear extracts and recombinant mouse oestrogen receptor, and that oligonucleotides containing the E2 but not two other potential EREs (E1 and E3) bound mouse oestrogen receptor with a 10-fold lower affinity than the archetypal ERE of the *Xenopus laevis* A2 vitellogenin gene, which is a perfect palindrome. The experiments of Augereau and associates [36] focused on regions of the promoter containing perfect half sites as the potentially important regulatory sequences. Krishnan and associates [40] however, chose a region between −199 and −165 by inspection, which they claimed contained an oestrogen response element and an Sp1 site in close proximity, and demonstrated that this sequence bound oestrogen receptor and Sp1 and was able to confer oestrogen regulation on a heterologous promoter. In fact, the sequence identified does not contain a perfect half site and these experiments would suggest that sequences other than the perfect half sites are involved in the regulation of cathepsin D expression by oestradiol.

In conclusion, although many questions remain unanswered, these studies demonstrate that the cathepsin D promoter is particularly interesting because it has the hallmarks of both housekeeping (high G+C content and Sp1 sites) and regulated (TATAA box) genes. Further, the regulation of the expression of this gene by oestrogen cannot be ascribed to a perfectly palindromic consensus oestrogen response element, but probably involves a sequence which is recognised by the oestrogen receptor and other transcription factors. In addition, further analysis of the expression of this gene should reveal the way in which constitutive and regulated gene expression are coordinated in various cell types, and the mechanisms involved in the effects of triphenylethylene anti-oestrogens such as tamoxifen.

PROGNOSTIC VALUE OF CATHEPSIN D IN BREAST CANCER

Despite the interest in the oestrogen regulation of cathepsin D, and the biological function of cathepsin D in malignancy in general and breast cancer in particular, the largest number of publications on cathepsin D in recent years have been concerned with the expression of cathepsin D in tumours and tissue samples and the predictive and prognostic value of cathepsin D. These studies have been spawned by the availability of reagents with which to measure cathepsin D expression, including polyclonal antisera [41–43] and monoclonal antibodies [43, 44] for measuring the expression of cathepsin D in tissue sections by immunohistochemistry [41], and in tissue extracts by Western blotting [42], ELISA and IRMA assays [44–48].

Cathepsin D has aroused considerable interest because it has been proposed as a promising 'molecular' marker of prognosis in breast cancer that is independent of the more 'classical' markers such as tumour size, histological grade and lymph node status. Accurate markers of prognosis have been sought for several reasons. The first is that there has been a move to the use of some form of adjuvant therapy for the vast majority of breast cancer patients in the light of trial data and overviews [49, 50], demonstrating the effects of adjuvant therapy on survival. However, it is recognised that for some individuals, adjuvant therapy is of no benefit although the patient population as a whole does benefit. An example of this is the node-negative group which overall have a good prognosis and many women are effectively cured by primary treatment. There are, however, a proportion of women who despite the lack of nodal involvement at the time of diagnosis will relapse, and if these women could be identified then they could be treated more aggressively from the outset.

Another reason is that with the trend towards earlier diagnosis as a direct result of the breast cancer screening programme, a higher proportion of lesions are small and there is no nodal involvement. Thus by classical criteria, these lesions would be regarded as having a good prognosis (they tend to be small, well differentiated and have a low frequency of nodal involvement), but a proportion of women will relapse early and it would be helpful if this group could be identified and treated appropriately.

The first clinical studies which assessed cathepsin D expression in breast tumours produced counterintuitive results from those predicted by laboratory studies [51, 52]. The expectation had been that expression would be associated with steroid receptor status given the regulation of cathepsin D by oestrogens in cultured oestrogen responsive breast cancer cells. In fact, there was no significant relationship between cathepsin D expression and steroid receptor status, and the authors duly concluded that cathepsin D expression is not associated with hormone responsiveness.

A large number of subsequent studies have investigated the relationship between cathepsin D expression and a variety of clinical features of prognostic value including lymph node status, histological grade, tumour size and vascular invasion as well as the expression of other prognostically significant genes such as the steroid receptors and *C-ERBB-2*. Of the clinical studies which have also considered the relationship between cathepsin D expression and prognosis (Table 1), only one [53] found a relationship between cathepsin D expression and oestrogen receptor status, only one [56] found an association with grade and none found an association with tumour size. Four [57, 59, 61, 62] out of the 13 studies reported an association with nodal status, and in these four cases higher levels of cathepsin D was associated with nodal involvement. Although the general consensus that cathepsin D is an independent prognostic factors remains tenable, the relationship between cathepsin D expression and nodal status should be examined closely in future studies because of its well-established and strong prognostic value.

However, the most important question is not whether cathepsin D relates to other factors of known prognostic significance, but whether cathepsin D is a prognostic factor in its own right and is able to predict overall or disease-free survival. The rest of this review therefore focuses on studies which have attempted to answer this question.

To date, nearly all published studies have been retrospective and investigate the relationship between cathepsin D expression and other prognostic factors and survival in various cohorts of patients. However, the studies differ in the groups of patients analysed, the prevalence and types of treatment used, the method used to measure the expression of cathepsin D, the cut-off values used to define positive and negative tumours, and importantly the length of clinical follow-up and the way in which

Table 1. Prognostic value of cytosolic cathepsin D levels as measured by enzymeimmunoassay

[Ref.]	No. of patients	Median follow-up (months)	Total		Nodal status				ER status			
					+ve		−ve		+ve		−ve	
			DFS	OS	DFS	OS	DFS	OS	DFS	OS	DFS	OS
Thorpe *et al.* (1989)† [53]	396	48 (pre) 67 (post)										
Spyratos *et al.* (1989) [54]	122	55	Yes‡,§	Yes $P = 0.04$	No		Yes‡,§					
Romain *et al.* (1990) [55]	85	58 (overall) 30 (DFS)	Trend $P = 0.082$	Yes $P = 0.021$	Trend $P = 0.06$	Yes $P < 0.019$	No	No	Trend $P = 0.056$			Yes $P = 0.043$
Duffy *et al.* (1992) [56]	331	45 (CD+) 48 (CD−)	Yes $P < 0.05$	Yes $P < 0.01$	No	No	No	No	Trend $P = 0.06$	Yes $P < 0.025$	No	No
Namer *et al.* (1991) [57]	413	68		Yes $P = 0.03$	Yes $P < 0.02$	Yes $P < 0.008$	No	No				
Granata *et al.* (1991) (Node-negative only) [58]	199	87					No	No	Yes $P = 0.02$	Yes $P = 0.01$	No	No
Kute *et al.* (1992) (Node-negative only) [59]	162	29					Yes $P < 0.0001$	Yes $P < 0.0004$				
Spyratos *et al.* (1992) [60]	319	72	Yes $P = 0.007$									
Pujol *et al.* (1993) [61]	125	59 (mean)	Yes $P < 0.01$	Yes $P = 0.03$	Yes $P = 0.009$		No $P = 0.07$					
Foekens *et al.* (1993) [62]	710	48	Yes*,‖ $P = 0.001$	Yes*,‖ $P = 0.03$	Yes*,‖ $P = 0.01$		Yes*,‖ $P = 0.01$					
Gion *et al.* (1993) [63]	267	24–101 (range)	Yes* $P = 0.0003$	Yes* $P = 0.022$								
Seshadri *et al.* (1994) [64]	858	31	Yes $P = 0.018$		Yes $P = 0.005$		No		Yes $P = 0.046$		Yes $P = 0.037$	
Stonelake *et al.* (1994) [65]	83	16	No $P > 0.999$									

DFS, disease-free survival; OS, overall survival; ER, oestrogen receptor. An empty box indicates the analysis was not performed in the study. Yes indicates a statistically significant result. Trend indicates that the result was reported as approaching statistical significance. No indicates that the result was not statistically significant. Most *P* values were obtained from Log Rank tests of survival data. *indicates that the *P* value was obtained principally by multivariate analysis. †This study analysed survival by menopausal status only. ‡Positive values were defined in a variety of ways and the *P* value varied with the way in which positive tumours were defined. §Metastasis- rather than disease-free survival was measured. ‖Tumours containing >70 pg/mg protein were compared with tumours containing <30 pg/mg protein.

the data are analysed. These factors complicate objective analysis of the data.

A large proportion of the prognostic studies have used monoclonal antibodies to measure cathepsin D concentrations in tumour cytosols. With the increasing emphasis on early detection and the consequent decrease in the size of tumours at diagnosis, and the appreciation that the measurement of cathepsin D in the cytosol cannot give information on the cell types that express cathepsin D, there has been a move to develop immunocytochemical assays. Cathepsin D levels have also been measured using a semiquantitative blotting procedure and one study has measured the enzymic activity of cathepsin D.

As discussed by Ravdin [66], these different assays could be measuring aspects of cathepsin D expression that differ fundamentally in prognistic significance. The results of the prognostic studies are therefore grouped by assay type, although the majority have used either the commercially-available cytosolic assay or immunohistochemistry.

MEASUREMENTS OF CYTOSOLIC CATHEPSIN D BY ELISA OR IRMA

Early studies [52] used a two-site ELISA assay, but a two-site IRMA assay was subsequently developed which is somewhat more sensitive than the ELISA. This assay is commercially available and its performance has been validated in a pan-European study by EORTC [48]. The advantage of this assay is that cathepsin D levels can be measured in the same cytosols used for the measurement of steroid receptors.

The first published prognostic study by Thorpe and associates [53] examined the prognostic value of cathepsin D in cytosols prepared from tumours of women enrolled between 1977 and 1982 in the Danish adjuvant treatment protocol. The study included 396 women who were typical of the 1483 women enrolled in this programme at the time of the study. This study has been criticised [66] on the grounds of the selective way in which the data were analysed. Survival data were not presented for the whole group, but were analysed separately for pre- and postmenopausal women. In addition, the cut-off levels used to define low, intermediate and high cathepsin D levels were different in the two menopausal groups because they were based on the quartile values and these differed in the two groups. Despite, or perhaps because, the data were analysed in this complicated way, significant relationships were found between cathepsin D levels and survival. High cathepsin D levels were associated with poor relapse-free, but not overall, survival and these differences attained significance in postmenopausal women and approached significance in pre/perimenopauseal women. When survival was determined in lymph node positive and negative subgroups, lymph node negative but not positive pre/perimenopausal women had a shorter relapse-free survival whereas node-positive, but not node-negative, postmenopausal women had a significantly shorter relapse-free survival if they expressed elevated levels of cathepsin D. It is also noteworthy that, although cathepsin D expression was related to poor prognosis, it was significantly associated with expression of the oestrogen receptor, a marker of good prognosis, in pre/perimenopausal women.

At about the same time, a study involving a smaller number of women followed up for a median period of 4.6 years was published by Spyratos and associates [54]. In this study, two cut-off values (45 and 70 pmol/mg of protein) of cathepsin D were used. Strikingly, using a cut-off value of 70 pmol/mg protein, 20 out of 20 patients defined as cathepsin D positive developed metasases whereas only 20 out of 94 patients defined as cathepsin D negative developed distant metastasis, and the differences in metastasis and disease-free survival were highly statistically significant. When metastasis-free survival was analysed in node-positive or -negative subgroups, the worst survival was seen in node-negative women with high cathespin D levels. The observation that survival in this subgroup was worse than in either node-positive subgroup emphasised the potential of cathepsin D as a powerful prognostic indicator in the node-negative group which might allow the identification of women with a high risk of relapse.

These results led to further studies by a number of groups worldwide which were facilitated by the availability of a commercially available immunoradiometric assay which measures total cytosolic cathepsin D [53–65]. With the exception of the first study of Thorpe and associates [53], the majority of subsequent studies have reported the prognostic value of cathepsin D in all patients, although different cut-off values have been used to define positive and negative patients (Table 1). Two studies [58, 59] have only looked at node-negative cases. Twelve out of 13 studies have found a significantly worse prognosis in women with higher cathepsin D either in relapse-free survival, overall survival or both. The study which did not show a significant effect [65] had the shortest median follow-up of any study (only 16 months) and this could well explain the failure of this study to observe a significant effect. Overall, there does appear to be compelling evidence for a prognostic value of cytosolic cathepsin D in breast cancer patients.

The prognostic value of cathepsin D within subgroups is much more complex, partly because of the restricted number of patients within each subgroup. The results for two of the more commonly reported subgroups (nodal status and oestrogen receptor status) are also shown in Table 1. Nodal status is particularly important for the reasons already outlined and this has been addressed in the majority of studies.

Of the two studies which were restricted to node-negative tumours [58, 59], the study with the shorter median follow-up (29 months) [59] found a significantly worse prognosis in women with cathepsin D positive tumours. The study with the longer median follow-up (87 months) [58] did not demonstrate a significantly worse prognosis when all cases were considered, but within the oestrogen receptor positive group women with positive tumours did have a worse prognosis.

Overall, the majority of studies have not reproduced the findings of Spyratos and associates [54] which suggested that cathepsin D is of most value in node-negative women. Only Kute and colleagues [59] and Foekens and associates [62] in their large study of 710 women also showed a worse prognosis in the node-negative subgroup (they showed a prognostic effect in node-positive and -negative groups), while five studies have demonstrated a worse prognosis in the node-positive group. These conflicting results may have arisen for several reasons. First, Spyratos and associates [54] found the most significant effect for metastasis-free survival and no other study has used this endpoint. Second, longer follow-up is required to accumulate prognostic information on node-negative women as they constitute an inherently good prognostic group. Third, the type of and/or frequency of the use of adjuvant therapy may confuse the results. Nevertheless, current studies suggest that cathepsin D is of value in node-positive as well as node-negative patients, and may be of greater value in node-positive tumours. The clinical value of markers of poor prognosis in node-positive cases is questionable, since nodal status is in itself a powerful indicator

of a poor prognosis and node-positive patients are treated aggressively.

The presence of the oestrogen receptor is a marker of good prognosis. Cathepsin D was found to be a marker of poor prognosis in the oestrogen receptor positive subgroup in all the studies listed in Table 1 in which the prognostic value of cathepsin D was assessed in subgroups defined by oestrogen receptor status [55, 56, 58, 64]. Although oestrogen receptor status is not commonly used to determine treatment, this observation offers the possibility of identifying poor prognosis patients in this good prognosis subgroup.

MEASUREMENT OF CYTOSOLIC CATHEPSIN D BY WESTERN BLOTTING AND ENZYME ACTIVITY

Two studies have been published in which cathepsin D levels were measured in tumour cytosols by a semiquantitative Western blotting procedure [42, 67]. In the first study on 397 tumours [42], the prognostic significance of the expression of cathepsin D was not reported for the group as a whole, but increased expression was associated with shorter relapse-free and overall survival in node-negative but not node-positive patients. The value of cathepsin D was considerable with the relative risk of death being 3.9-fold higher than in women with low levels of cathepsin D. These results contrast markedly with a subsequent report [67] from the same laboratory on 927 node-negative patients in which cathepsin D was only found to have prognostic value in the subgroup of oestrogen receptor positive tumours. The conflict between these two studies is difficult to rationalise. One possibly important difference, which is also discussed in the following section, is the antibody. A polyclonal antiserum was used in the earlier study whereas a monoclonal antibody was used in the later study. Although the values obtained in a subset of the patients with both antibodies were similar, Ravdin and associates [67] commented that the monoclonal antibody gave higher values, and that these were more tightly clustered than values obtained using the polyclonal antibody.

Kute and associates [59] used a simple enzymic assay of cathepsin D activity in breast tumour cytosols. This technique has considerable appeal given the complexity of the intracellular processing of cathepsin D and the uncertainty of the molecular forms recognised by commercially-available antibodies. In this study, on predominantly node-negative cases, increased cathepsin D levels were associated with a highly significant decrease in relapse-free and overall survival (Table 1).

MEASUREMENT OF CELLULAR CATHEPSIN D BY IMMUNOHISTOCHEMISTRY

Immunohistochemical studies have used a variety of monoclonal and polyclonal cathepsin D antibodies to measure cathepsin D expression in tumour and stromal cells in breast cancer.

The first study by Henry and associates [68] used a rabbit polyclonal antiserum which reacted with mature and procathepsin D. This study reported an improved prognosis for women with cathepsin D positive tumours (Table 2). Cathepsin D positive tumours tended to express oestrogen receptor. Women with cathepsin D positive tumours had a significantly longer disease-free survival and increased overall survival. Cathepsin D expression was associated with an improved prognosis in node-positive cases only. This was the first study to demonstrate that stromal macrophages can express high levels of cathepsin D and that, depending on the level of infiltration, these cells could make a significant contribution to the levels of cathepsin D in tumour cytosols. However, the prognostic value of the expression of cathepsin D in stromal macrophages was not analysed in this study.

The results of eight other immunohistochemical studies are summarised in Table 2. Four found no prognostic value of cathepsin D expression in tumour cells, while the remaining three reported that high levels of cathepsin D expression in tumour cells was associated with poor prognosis. Of these studies, Winstanley and associates [71] measured cathepsin D expression in node-positive and -negative cases and showed a prognostic effect in all tumours. The study of Isola and colleagues [72] measured cathepsin D in node-negative cases only, but found a highly significant effect on relapse-free and overall survival. Kandalaft and associates [70] analysed a group of node-positive and -negative cases and reported that cases with increased cathepsin D expression showed a trend to decreased overall survival in node-positive cases only.

Four studies have analysed the expression of cathepsin D in tumour cells and stromal macrophages [72–75]. Three of the four studies reported no association of expression of cathepsin D in tumour cells with survival, but reported that increased stromal expression is associated with decreased survival. The study of O'Donaghue and associates [75] did not analyse subgroups largely because of the small number of cases in the study. Joensuu and colleagues [74] reported that stromal cathepsin D expression was associated with poor prognosis in the entire group and in node-positive but not node-negative cases. The study of Tetu and associates [73] was restricted to node-positive cases only and a trend was seen to reduced relapse-free survival.

The results of the immunohistochemical studies are, therefore, much more variable than those using immunoradiometric assay to measure cytosolic cathepsin D and this raises two principle issues. The first is the different conclusions reached by the immunohistochemical studies and the second is the discrepancy between the studies using immunohistochemical and immunoradiometric methods.

One source of variation among the immunohistochemical studies is the antiserum. The predominant epitopes recognised by the various polyclonal antisera have not been defined. Six of the studies used polyclonal antiserum raised against cathepsin D purified from human spleen whereas three of the studies used the mouse monoclonal antibody 1C11 (Triton Diagnostics, Alameda, California, U.S.A.) and one used a rabbit polyclonal antiserum (CRD2 11/23) in addition to 1C11. Of the six using polyclonal antisera, the study of Henry and associates [68] used a polyclonal antiserum raised by Reid and colleagues [41] against mature cathepsin D purified from human spleen, while five used a different antiserum also raised against mature cathepsin D. Different sources of antisera may account for the results of Henry and associates [68] but cannot account for the differences between the results of, for instance, Domegala and associates [69], Tetu and associates [73] and O'Donaghue and associates [75] who found no prognostic value of cathepsin D expression in tumour cells, and Kandelaft and colleagues [70] and Winstanley and colleagues [71] who found cathepsin D to be associated with a poor prognosis: all studies being performed using the same antiserum. Another variable is the method of scoring and it is noteworthy that these studies used a variety of scoring methods, including the use of a histoscore to define positive and negative tumours on the basis of intensity and numbers of cells staining [70], simple assessment of the proportion of cells staining [73] and relatively subjective assessments of overall positivity [68, 71]. This resulted in the adoption of different cut-off levels to define positive and negative tumours. In conclusion, further

Table 2. Prognostic value of cathepsin D expression in breast tumours as measured by immunohistochemistry

	Antibody	Cells analysed	No. of patients	Median follow-up (months)	Total DFS	Total OS	Node +ve DFS	Node +ve OS	Node −ve DFS	Node −ve OS
Henry *et al.* (1990) [68]	Rabbit poly*	Tumour	94		Yes (good) $P < 0.025$	Yes (good) $P < 0.025$	Yes (good) $P < 0.025$	Yes (good) $P < 0.025$	No	No
Domagala *et al.* (1992) [69]	Rabbit poly†	Tumour	136	84		No		No		No
Kandalaft *et al.* (1993) [70]	Rabbit poly†	Tumour	245	54	No	No	No	No	No	Trend (bad) $P = 0.072$
Winstanley *et al.* (1993) [71]	Rabbit poly‡	Tumour	359	132 (mean)		Yes (bad) $P < 0.025$		No		No
Isola *et al.* (1993) (Node negative only) [72]	Mouse mono§	Tumour	262	98					Yes (bad) $P < 0.0001$	Yes (bad) $P < 0.0001$
		Stromal	262	98					No	No
Tetu *et al.* (1993) (Node positive only) [73]	Rabbit poly†	Tumour	638	58			No	No		
		Stromal	638	58			Yes (bad) $P = 0.0647$	No		
Eng Tan *et al.* (1994) [43]	Rabbit poly‖	Tumour	218		No	No		No		No
	Mouse mono§	Tumour	224		Yes (bad) $P = 0.03$	No		No		No
Joensuu *et al.* (1995) [74]	Mouse mono§	Tumour	213	372		No		No		No
		Stromal	213	372		Yes (bad) $P = 0.007$		Yes (bad) $P = 0.04$		No
O'Donaghue *et al.* (1995) [75]	Rabbit poly†	Tumour	103	> 60	No	No				
		Stromal	103	> 60	Yes (bad) $P = 0.0001$	Yes (bad) $P = 0.0086$				

DFS, relapse-free survival; OS, overall survival. An empty box indicates that the analysis was not performed in this study. Yes indicates a statistically significant result. Trend indicates that the result was reported as approaching statistical significance. No indicates that the result was not statistically significant. *Antiserum raised against cathepsin D purified from human spleen and provided by W.A. Reid, University of Leeds, U.K. †Antiserum raised against cathepsin D purified from human spleen and obtained from Novocastra Laboratories, Newcastle upon Tyne, U.K. ‡Antiserum raised against cathepsin D purified from human spleen and provided by B. Westley and F.E.B. May, University of Newcastle upon Tyne, U.K. §Mouse monoclonal antibody from Triton Diagnostics, Alameda, California, U.S.A. ‖Antiserum raised against recombinant human cathepsin D and provided by G.E. Conner, University of Miami, U.S.A.

studies are required to determine which antibodies and which scoring methods are able to provide prognostic information. Only one study has attempted to address this issue by using more than one antibody [43]. Eng Tan and associates [43] showed that three different antibodies recognised overlapping subsets of cathepsin D positive tumours and analysis of nearly 500 cases using the two antibodies showed that they differed in their association with metastasis-free survival.

The agreement between the studies of O'Donaghue and associates [75], Joensuu and associates [74] and Tetu and associates [73] that the number of stromal macrophages is prognostically more important than staining of carcinoma cells is of considerable interest. Taken at face value, this suggests that stromal macrophages make a major contribution to the cathepsin D measured in tumour cytosols. In contrast to these findings, other studies [76, 77] have shown that there are good correlations between staining of tumour cells and cytosol levels of cathepsin D as measureand by IRMA.

In conclusion, cathepsin D is of considerable interest for elucidating the mechanisms involved in the regulation of gene expression by oestrogens and as a prognostic marker in breast cancer. Large-scale prospective studies are now required to establish definitively its prognostic value and to answer questions concerning the most appropriate assay. In addition, the source of the cathepsin D which is measured in tumour cytosols and the prognostic value of the expression of cathepsin D in the various cell types within a tumour remain to be established. Because elevated expression of cathepsin D is associated with a poor prognosis and because biological studies suggest that the poor prognosis may result from its proteolytic activity, it can be anticipated that the next phase of research on the clinical importance of cathepsin D will include the development of potent, non-toxic inhibitors of its proteolytic activity [78], which may be of value in slowing the metastatic spread of breast and other cancers.

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