Prognostic Significance of the Estrogen-Regulated Protein, Cathepsin D, in Breast Cancer

An Immunohistochemical Study

J. A. HENRY, BSc, MB ChB,*† ANNE L. McCARTHY,* B. ANGUS, BMed Biol, MB ChB,* B. R. WESTLEY, BSc, PhD,* FELICITY E. B. MAY, BSc, DPhil,*‡ S. NICHOLSON, MB BS, FRCS,§ J. CAIRNS, BSc, FIMLS,* A. L. HARRIS, BSc, MB ChB, DPhil, MRCP,‖ AND C. H. W. HORNE, DSc, MD, FRCPath*

Expression of the estrogen-regulated lysosomal protease, cathepsin D, was studied in a series of 94 breast cancers using an immunohistochemical technique. Granular staining of tumor cell cytoplasm was detected in 62 cases. Positive staining was associated with a significant increase in overall time to relapse and when survival was analyzed in terms of intensity of cathepsin D staining there was a significant trend for both increased time to relapse and increased length of survival. The presence of estrogen receptor was associated with positive cathepsin D immunostaining, and in the subgroup of estrogen receptor-positive tumors cathepsin D staining was associated with significantly prolonged survival; this was not the case for estrogen receptor-negative tumors. Positive cathepsin D immunostaining was associated with significant prognostic advantage in patients with confirmed lymph node metastasis but not in node-negative patients. It is suggested that cathepsin D expression reflects the functional integrity of the estrogen response pathway. Cathepsin D may prove a clinically useful adjunct to assessment of estrogen receptor status.

***Cancer* 65:265–271, 1990.**

THE ENZYME, cathepsin D, is a lysosomal aspartyl endopeptidase with an acid pH optimum and a molecular weight of approximately 42 kilodaltons.[1] Cathepsin D has a wide tissue distribution but, interestingly, it appears to be regulated by estrogens in breast cancer. The 46-KD glycoprotein, first described by Westley and Rochefort,[2] the synthesis of which is induced by estrogens in certain breast cancer cell lines, has been shown to be cathepsin D.[3] More recently, during a search for estrogen-regulated messenger RNA sequences in breast cancer cell lines, the mRNA pNR-100 was isolated, sequenced, and found to encode cathepsin D.[4]

Estrogen-regulated proteins are of considerable interest in breast cancer, a tumor which has long been known to be estrogen responsive. A variety of proteins,[1,5–7] growth factors,[8] and mRNA sequences[4,9,10] have been found to be regulated by estrogens in breast cancer cell lines. Although the presence of estrogen receptor alone is of value as a prognostic indicator in breast cancer[11] it has been suggested that estrogen-regulated proteins, either alone or in conjunction with estrogen receptor status, may be better prognostic indicators, because their presence may provide evidence of the functional integrity of the estrogen response.

Progesterone receptor is the most notable example of a prognostically advantageous estrogen regulated protein.[12] More recently, tissue type plasminogen activator, also regulated by estrogens in breast cancer cells,[6] has been shown to be associated with better prognosis in breast cancer.[13] The prognostic significance of other nonestrogen-regulated gene products has been studied, in particular amplification and expression of the c-erbB-2/neu oncogene[14,15] and expression of epidermal growth factor receptor,[16] both of which are associated with poor prognosis. In this report we have used a polyclonal antibody to cathepsin D[17] to study the immunohistochemical expression of cathepsin D in a series of 94 cases of breast cancer.

From the Departments of *Pathology, §Surgery, and ‖Clinical Oncology, University of Newcastle-Upon Tyne, Newcastle-Upon-Tyne, United Kingdom.

Supported by The North of England Cancer Research Campaign.

† Recipient of the Wellcome Trust for a Research Training Fellowship.

‡ Recipient of a 1983 University Research Fellowship from the Royal Society.

The authors thank Dr. W. A. Reid, Department of Pathology, University of Leeds, Leeds, UK, for his generous gift of antibody to cathepsin D. The authors also thank Mr. S. Brabazon and Mr. W. Robinson for assistance with illustrations.

Address for reprints: J. A. Henry, BSc, MB ChB, Department of Pathology, Royal Victoria Infirmary, Queen Victoria Road, Newcastle-Upon Tyne, NE 1 4 LP, United Kingdom.

Accepted for publication July 21, 1989.

Materials and Methods

Antibody Specificity

Cell culture and immunoprecipitation: MCF-7 cells were plated onto 8-mm microwells and grown to confluence in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10^{-8} mol/l estradiol, and 1 μg/ml insulin and then incubated with ^{35}S-labeled methionine in serum-free medium for 6 hours. The medium was collected and the cell extract prepared by lysis in non-ionic detergent. Labeled protein (10^5 cpm of medium or 5×10^5 cpm of cell extract) was incubated overnight at 4°C with 1 μl of rabbit polyclonal cathepsin D antiserum, a gift from Dr. W. A. Reid, Department of Pathology, University of Leeds, Leeds, UK. Antigen–antibody complexes were removed with protein-A sepharose and subsequently analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Tumors and Immunocytochemical Staining

Ninety-four cases of primary operable breast cancer were studied. Patient age ranged from 31 to 80 years old (mean, 57.8 years). Sixty-five patients were older than 50 years and were considered postmenopausal (mean, 64.6 years); 29 patients were aged 50 years old or less (mean, 42.6 years).

All patients were initially treated by surgical tumor resection and all lumpectomy and node-positive patients received postoperative radiotherapy. Adjuvant tamoxifen was not used but on relapse 37 received the antiestrogen tamoxifen. Patients were followed clinically for up to 60 months and deaths due to breast cancer recorded. Samples of fresh tumor tissue were taken at the time of primary surgery for estrogen receptor assay as described previously[16]: tumors with cytoplasmic estrogen receptor levels in excess of 5 fmol/mg protein were considered estrogen receptor positive. The remaining tumor tissue was fixed promptly in phosphate-buffered formalin. Blocks were selected, postfixed in formal sublimate, embedded in paraffin wax, and 5-μm sections cut. After digestion in 0.1% trypsin for 10 minutes at 22°C, sections were stained using a diaminobenzidine peroxidase–antiperoxidase technique[18]: sections were incubated with cathepsin D antiserum at 1/300 dilution for 30 minutes at room tem-

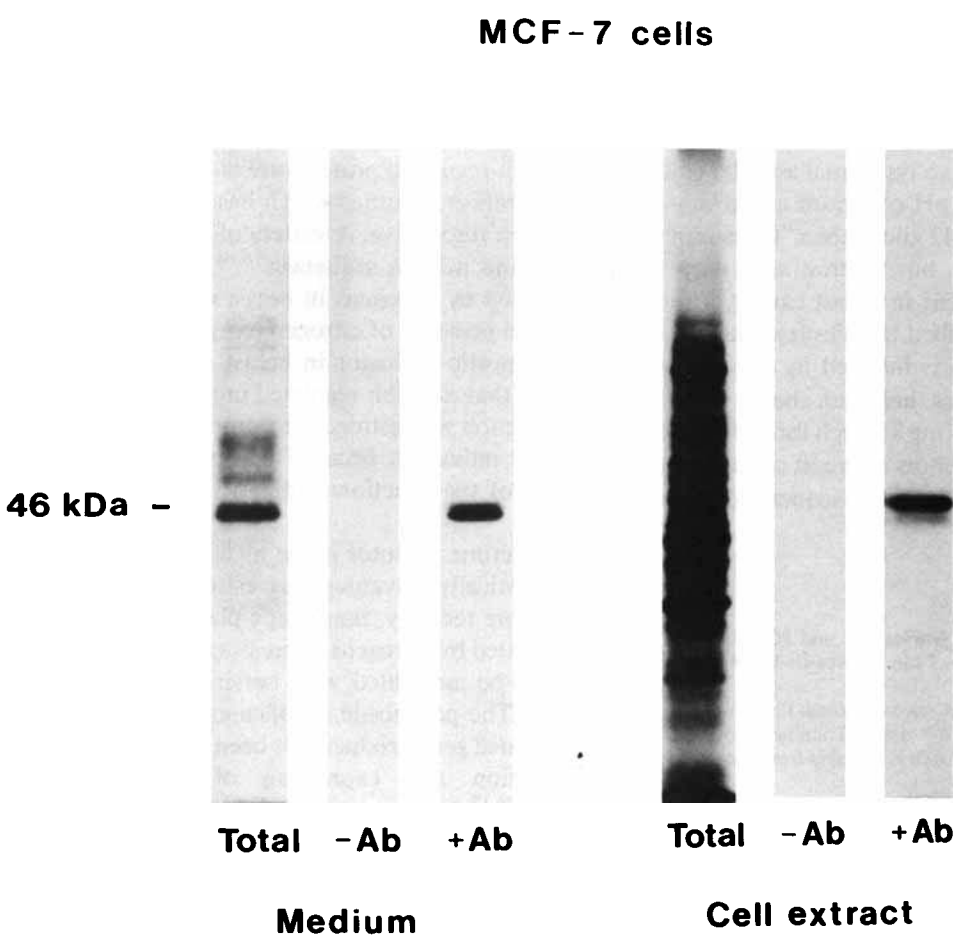
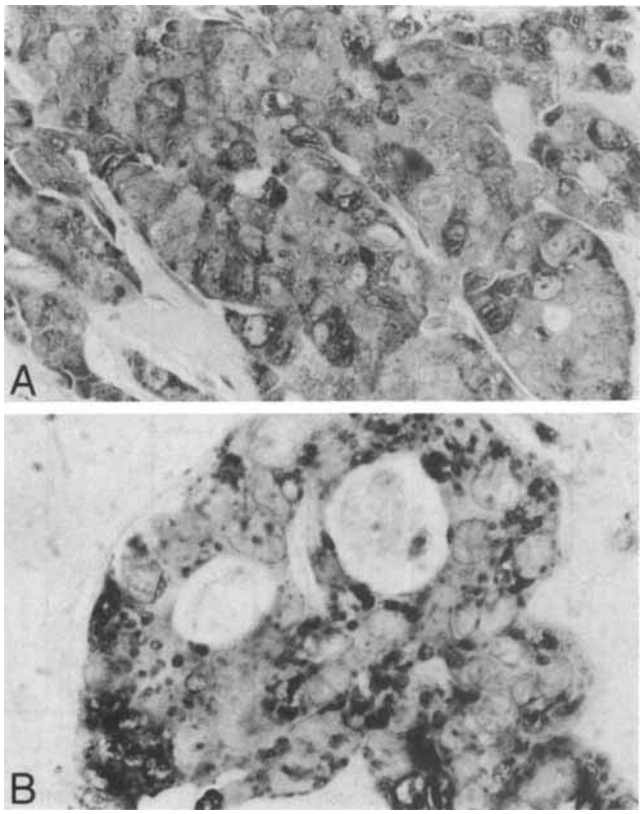


FIG. 1. Immunoprecipitation of MCF-7 cell secreted and cellular proteins by cathepsin D antiserum. Medium: 10^5 cpm of labelled protein, total; 10^5 cpm were precipitated in the absence, −Ab, or presence, +Ab, of cathepsin D antiserum. Cell extract: 2.5×10^4 cpm of labelled protein, total; 5×10^5 cpm were immunoprecipitated in the absence, −Ab, and presence, +Ab, of cathepsin D antiserum.



FIGS. 2A AND 2B. Invasive ductal carcinoma, immunoperoxidase stain for cathepsin D. (A) Granular cytoplasmic staining in tumor cells only. (B) At increased magnification, lysosomal localization of cathepsin D staining is apparent.

perature. The stained sections were scored by two independent observers for intensity of staining using a three-point scale: 0, no cells staining; +, weak or moderate staining; and ++, strong staining. Discrepant results were reviewed. Positive staining of macrophages was ignored.

Statistics

Analysis was carried out using a program for Acorn/BBC microcomputers (Acorn Computers Ltd., Cambridge, England).[19] Survival curves were prepared by the life table method and compared by the log-rank test and the chi-square test for trend.[20] The relationship between the various tumor parameters was examined using the chi-square test.

Results

The MCF-7 human breast cancer cell line contains cathepsin D mRNA and synthesises and secretes cathepsin D.[3] To determine the specificity and sensitivity of the cathepsin D antiserum, it was used to precipitate the ^{35}S-labeled secreted and cellular proteins. The pattern of the total secreted protein (Fig. 1) is similar to that reported previously,[2] with a prominent 46-KD band. This protein, which has been identified as cathepsin D, was precipitated quantitatively by the antiserum, with no precipitation of other proteins. The pattern of the proteins in the cell extract was more complex: in this case three bands were identified which correspond to the intracellular forms identified by Morisset and associates,[3] with no precipitation of other bands. Using sections of human liver as a positive control, this polyclonal antibody to cathepsin D was found to be effective on formalin-fixed, paraffin-embedded tissue sections, therefore a study using routinely processed archival tissue was possible.

Cathepsin D immunostaining was detected in breast cancers from 62 of the 94 patients studied. Interpretation of staining was reproducible: the two independent scorers disagreed over tumor positivity in only nine of 94 cases (90% concordance). Staining was predominantly cytoplasmic and granular (Figs. 2A and 2B) and closely resembled the pattern shown by lysozyme (muramidase) in the lysosomes of histiocytes. Stromal macrophages also stained positively (Fig. 3) but their characteristic morphologic features facilitated recognition and elimination. The intensity of tumor staining was variable and tumors were graded as strongly positive (17%, Figs. 2A and 2B), positive (49%), or negative (34%).

Cathepsin D expression was independent of patient age/menopausal status: there was no significant difference in the incidence of cathepsin D expression when premenopausal and postmenopausal patients were compared (chi-square = 1.3).

Positive cathepsin D immunostaining was associated with significantly increased overall time to relapse (chi-square [log-rank] = 4.119, $P < 0.05$: Fig. 4): an increase in time to death was also seen although this fell short of significance (chi-square [log-rank] = 3.342, $P < 0.1$). When survival was analyzed in terms of the intensity of

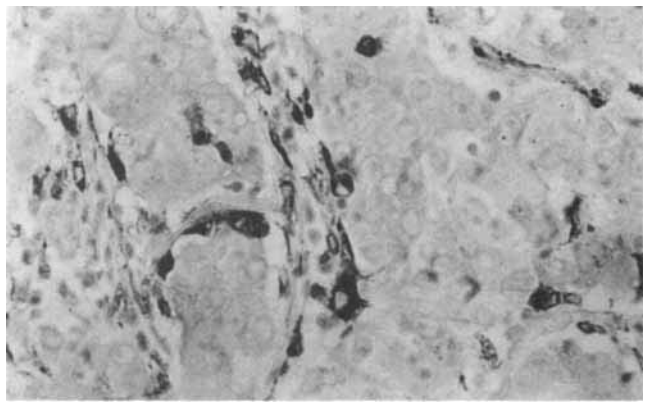


FIG. 3. Positive staining for cathepsin D in stromal macrophages of an otherwise negative tumor.

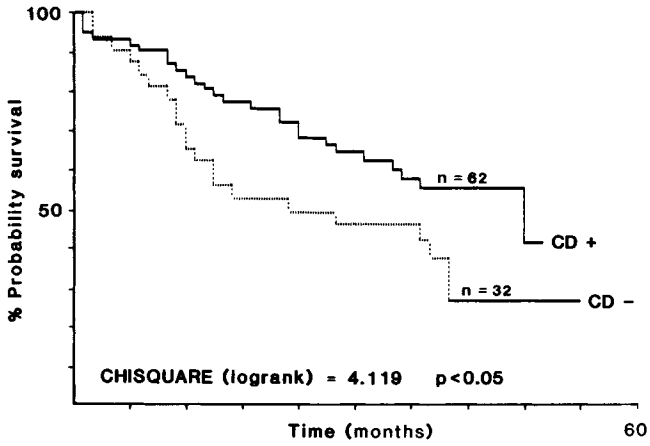


FIG. 4. Time to relapse in breast cancer related to immunocytochemical detection of cathepsin D in tumor cells. The longer time to relapse in the cathepsin D positive group is significant.

cathepsin D staining scored on a three-point scale there was a significant trend for both increased time to relapse (chi-square [trend] = 5.155, $P < 0.025$) and increased length of survival (chi-square [trend] = 5.941, $P < 0.025$: Fig. 5) in the more strongly staining tumors.

Other variables of prognostic significance studied in this patient group comprised estrogen receptor status and lymph node metastatic status: the significance of cathepsin D immunostaining was determined in the subgroups defined by these variables. The presence of estrogen receptor was associated with positive cathepsin D immunostaining (chi-square = 4.160, $P < 0.05$: Fig. 6). In the subgroup of patients with estrogen receptor-positive tumors which also stained positively for cathepsin D survival was significantly longer (chi-square [log-rank] = 6.180, $P < 0.025$: Fig. 7). In estrogen receptor-negative patients positive cathepsin D immunostaining was not associated with any

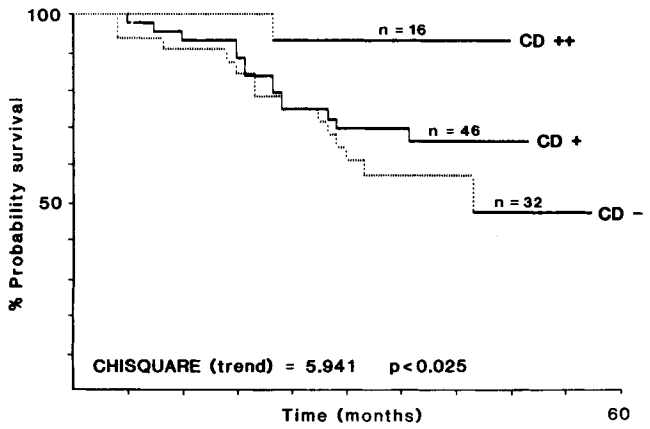


FIG. 5. Survival in breast cancer related to intensity of immunocytochemical staining for cathepsin D in tumor cells. The superior survival with increased intensity of cathepsin D staining is significant.

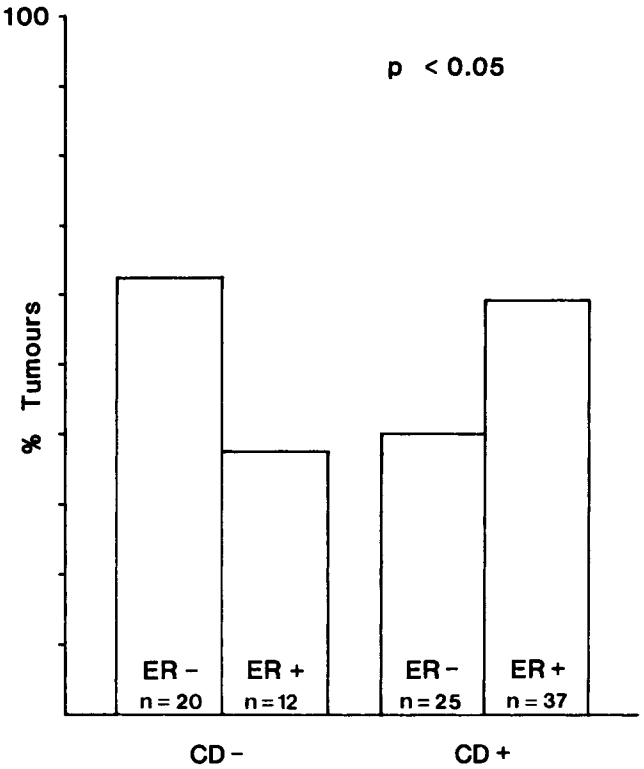


FIG. 6. Primary tumor estrogen receptor status related to immunocytochemical staining for cathepsin D (chi-square = 4.160, $P < 0.05$). Tumors staining for cathepsin D tend to be estrogen receptor positive.

significant survival advantage (chi-square [log-rank] = 0.016, $P > 0.1$). When the presence of lymph node metastasis was considered, in node-positive patients, positive immunostaining for cathepsin D conferred a significant advantage in terms of both time to relapse (chi-square [log-rank] = 5.937, $P < 0.025$: Fig. 8) and death (chi-square [log-rank] = 5.937, $P < 0.025$); in node-negative patients the presence or absence of cathepsin D immunostaining did not significantly influence these parameters.

The relationship of cathepsin D expression to antiestrogen response was considered. No patients had received adjuvant tamoxifen but 37 had received tamoxifen on relapse: response data was available for 36, of whom ten had shown some objective response (complete response/partial response/stable disease). There was no significant relationship between cathepsin D expression and response to antiestrogens on relapse (chi-square = 0.14).

Discussion

We have used an immunohistochemical technique to study the expression of the lysosomal aspartyl protease cathepsin D in a series of 94 breast cancers. We have found that the presence of immunohistochemically de-

tectable cathepsin D is associated with a significant prognostic advantage which, moreover, is related to the intensity of cathepsin D staining. In this series, immunohistochemical positivity for cathepsin D is significantly associated with positive estrogen receptor status, and in patients with estrogen receptor-positive tumors, expression is associated with significant prognostic advantage. Even in the prognostically poor subgroup of patients with confirmed lymph node metastases, cathepsin D staining confers prognostic advantage.

To our knowledge, this is the first immunohistochemical study of cathepsin D expression and its relationship to prognosis in breast cancer. The polyclonal antibody employed was effective on formalin-fixed, paraffin-embedded material (unlike the monoclonal antibodies employed by Garcia *et al.*,[21] effective only on frozen sections) hence an archival study was possible. The immunohistochemical approach has the advantage of precise tissue localization of antigen: until now the only studies of cathepsin D expression in breast tumors have used an immunoenzymatic technique to assay levels of the antigen in tumor extracts.[22,23] As cathepsin D is a widely distributed enzyme, notably also present within the inflammatory cells found in variable numbers in association with most breast tumors, the specificity of any assay based on tumor extracts will be low. Immunohistochemical studies are advantageous in that nontumor cells staining positively for cathepsin D can be identified readily and disregarded, greatly improving specificity.

Cathepsin D promises to be a useful biochemical prognostic marker in breast cancer. Ninety percent of patients whose tumors stained strongly positive for cathepsin D were alive 5 years later compared with only 45% of those whose tumors contained no detectable cathepsin D. As a prognostic marker, cathepsin D is comparable to estrogen

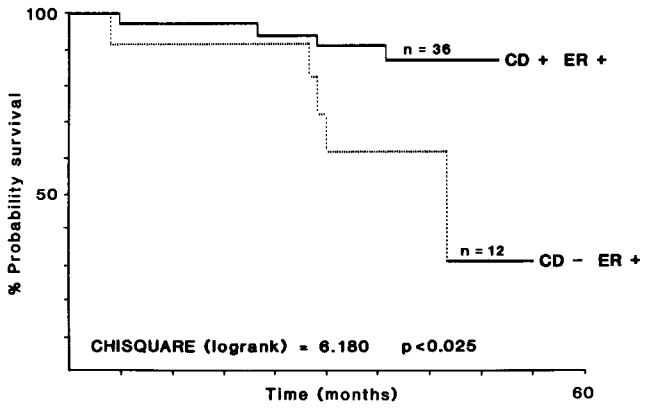


FIG. 7. Survival of patients with estrogen receptor-positive tumors related to immunocytochemical staining for cathepsin D. The superior survival seen in the subgroup with immunocytochemically detectable cathepsin D is significant.

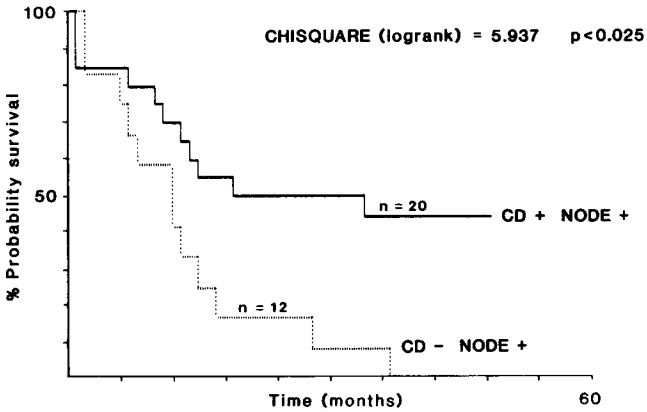


FIG. 8. Survival of patients with confirmed lymph node metastasis related to immunocytochemical staining for cathepsin D. Significant superior survival is found in the subgroup with immunocytochemically detectable cathepsin D.

receptor,[24] progesterone receptor,[25] and tissue type plasminogen activator[13] in that its expression confers prognostic advantage. These favorable prognostic markers contrast with epidermal growth factor receptor[16] and c-erbB-2/neu oncogene[14] whose expression is prognostically deleterious.

Our finding, that cathepsin D expression is associated with prognostic advantage, is in conflict with the findings of two other studies. Maudelonde *et al.*[22] report that high levels of cathepsin D expression in primary breast cancers are associated with an increased propensity for lymph node metastasis. Thorpe[23] found that high concentrations of cathepsin D predict poor prognosis in primary breast cancer. Both of these studies used an immunoenzymatic technique to measure levels in extracts of breast tumors, with consequent limitations to specificity, as mentioned previously.

The prognostic advantage associated with cathepsin D expression seems paradoxical in view of what is known of the nature of this enzyme. Cathepsin D is a proteolytic enzyme which may be secreted by breast cancer cells to facilitate tumor invasion[3,26] and may also be an autocrine mitogen,[27] hence it is attractive to hypothesize that cathepsin D expression might be associated with poor prognosis. However, to be effective as a protease enhancing metastatic potential, the precursor form of cathepsin D requires autoactivation at low pH[26]: such acid conditions may not necessarily arise, particularly in the spreading, viable edge of tumors. Cathepsin D is known to be under estrogen regulation in breast cancer cells[4] and it is this property which we believe accounts for the prognostic advantage conferred by the expression of cathepsin D.

In the current study a significant association was found between positive estrogen receptor status and immunohistochemically detectable cathepsin D. This finding is in

general agreement with work by Maudelonde *et al.*,[22] who report a similar association, but no overall correlation, between the concentrations of estrogen receptor and cathepsin D. This, and the known estrogen regulation of cathepsin D, strongly suggest that cathepsin D belongs to the family of estrogen-regulated proteins which also includes the progesterone receptor[5] and tissue type plasminogen activator.[6,28] The presence of both of these proteins is associated with improved prognosis in breast cancer[13,25] in a manner similar to cathepsin D. Detection of estrogen-regulated proteins such as progesterone receptor, tissue type plasminogen activator, and cathepsin D provides additional information on the functional integrity of the estrogen response pathway which is of prognostic and therapeutic importance.

Further support for this hypothesis is obtained by examining the effect of cathepsin D expression on prognosis in estrogen receptor-positive and estrogen receptor-negative tumor subgroups: in patients with estrogen receptor-positive tumors cathepsin D expression was of particular prognostic advantage, perhaps reflecting hormonal regulation of cathepsin D. The absence of cathepsin D expression in some receptor-positive tumors may be evidence of an incomplete estrogen response pathway, therefore the associated poor prognosis, despite receptor positivity. Cathepsin D was also expressed by a proportion of estrogen receptor-negative tumors but in this subgroup expression was not associated with prognostic advantage. Such expression in estrogen receptor-negative tumors is possibly constitutive and unregulated, as has already been described in some nonestrogen-responsive breast cancer cell lines.[4]

The improved prognosis of patients expressing cathepsin D in this study may be a consequence of functioning estrogen receptor reflecting a better differentiated or less aggressive tumor phenotype.

Cathepsin D expression was also a marker of good prognosis in patients with confirmed lymph node metastases: the prognosis for these patients is normally poor. This observation is difficult to explain but again, may be indicative of a less aggressive tumor phenotype.

The superior survival associated with cathepsin D expression does not appear to be related to antiestrogen response. The patients did not receive adjuvant tamoxifen and the improved survival was seen in terms of time to relapse, therefore there could be no antiestrogen effect. In the group of 36 patients who received tamoxifen on relapse there was no evidence of any correlation between cathepsin D expression in the primary tumor and subsequent antiestrogen response.

In conclusion, we have shown that expression of cathepsin D in breast cancer is associated with a significant prognostic advantage. In view of the known estrogen regulation of cathepsin D in breast cancer it is likely that cathepsin D is an additional marker of estrogen responsiveness, comparable to progesterone receptor and tissue type plasminogen activator. The immunocytochemical approach utilized allows accurate assessment of the presence of cathepsin D in tumor cells hence the contribution of inflammatory cells to overall cathepsin D levels can be disregarded. It is hoped that cathepsin D assessment will prove a clinically useful adjunct to estrogen receptor status in the management of breast cancer. We plan to extend our series and further examine cathepsin D expression in relation to endocrine response and to examine the relationship of cathepsin D expression to other markers of prognostic significance.

REFERENCES

1. Barrett AJ. Cathepsin D and other carboxyl proteinases. In: Barrett AJ, ed. Proteinases in Mammalian Cells and Tissues. Amsterdam: Elsevier/North Holland, 1977; 209–248.

2. Westley B, Rochefort H. Estradiol induced proteins in the MCF-7 human breast cancer cell line. *Biochem Biophys Res Commun* 1979; 90:410–416.

3. Morisset M, Capony F, Rochefort H. The 52-kDa estrogen induced protein secreted by MCF-7 cells is a lysosomal acidic protease. *Biochem Biophys Res Commun* 1986; 138:102–109.

4. Westley BR, May FEB. Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cell lines. *Nucleic Acids Res* 1987; 9:3773–3786.

5. Horwitz KR, McGuire WL. Estrogen control of progesterone receptor in human breast cancer. *J Biol Chem* 1978; 253:2223–2228.

6. Butler WB, Kirkland WL, Jorgensen TL. Induction of plasminogen activator in a human breast cancer cell line (MCF-7). *Biochem Biophys Res Commun* 1979; 90:1328–1334.

7. Edwards DP, Adams DJ, Savage N, McGuire WL. Estrogen induced synthesis of specific proteins in human breast cancer cells. *Biochem Biophys Res Commun* 1980; 93:804–812.

8. Dickson RR, Huff KK, Spencer EM. Induction of epidermal growth factor related polypeptides by 17-B-oestradiol in MCF-7 human breast cancer cells. *Endocrinology* 1986; 118:138–141.

9. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Res* 1982; 10:7895–7903.

10. May FEB, Westley BR. Cloning of estrogen-regulated messenger RNA sequences from human breast cancer cells. *Cancer Res* 1986; 46: 6034–6040.

11. McGuire WL, Carbonne PP, Vollmer EP. Estrogen Receptors in Human Breast Cancer. New York: Raven, 1975; 37–56.

12. McGuire WL, Clark GM, Dressler LG, Owens MA. Role of steroid hormone receptors as prognostic factors in primary breast cancer. *NCI Monogr* 1986; 1:19–23.

13. Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fennelly JJ, Lijnen HR. Tissue type plasminogen activator: A new prognostic marker in breast cancer. *Cancer Res* 1988; 48:1348–1349.

14. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the Her-2/neu oncogene. *Science* 1987; 235:177–182.

15. Berger MS, Locher GW, Saurer S *et al.* Correlation of c-erbB-2 gene amplification and protein expression in human breast cancer with nodal status and nuclear grading. *Cancer Res* 1988; 48:1238–1243.

16. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL. Epidermal-growth-factor receptor status as predictor of early recurrence and death from breast cancer. *Lancet* 1987; 1:1398–1402.

17. Reid WA, Valler MJ, Kay J. Immunolocalisation of cathepsin D

in normal and neoplastic human tissues. *J Clin Pathol* 1986; 39:1323–1330.

18. Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG. The unlabelled antibody method of immunohistochemistry: Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase:antiperoxidase) and its use in the identification of spirochaetes. *J Histochem Cytochem* 1970; 18:315–333.

19. Angus B. Unpublished data, 1989.

20. Peto R, Pike MC, Armitage P *et al.* Design and analysis of randomised clinical trials requiring prolonged observation of each patient: II. *Br J Cancer* 1977; 35:1–39.

21. Garcia M, Salazar-Retana G, Pages A *et al.* Distribution of the Mr 52,000 estrogen-regulated protein in benign breast disease and other tissues by immunohistochemistry. *Cancer Res* 1986; 46:3734–3738.

22. Maudelonde T, Khalaf S, Garcia M *et al.* Immunoenzymatic assay of Mr 52,000 cathepsin D in 182 breast cancer cytosols: Low correlation with other prognostic parameters. *Cancer Res* 1988; 48:462–466.

23. Thorpe S, Rochefort H, Garcia M *et al.* High concentrations of 52K cathepsin D predict poor prognosis in primary, postmenopausal breast cancer. *Cancer Res* 1989 (in press).

24. Osborne CK, Yochmowitz MG, Knight WA, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 1980; 46:2884–2888.

25. Alexieva-Figusch J, Van Putten WLJ, Blankestein MA, Blonk-Van Der Wijst J, Klijn JGM. The prognostic value and relationships of patient characteristics, estrogen and progestin receptors and site of relapse in primary breast cancer. *Cancer* 1988; 61:758–768.

26. Briozzo P, Morisset M, Capony F, Rougeot C, Rochefort H. *In vitro* degradation of extracellular matrix with Mr 52,000 cathepsin D secreted by breast cancer cells. *Cancer Res* 1988; 48:3688–3692.

27. Vignon F, Capony F, Chambon M, Freiss G, Garcia M, Rochefort H. Autocrine growth stimulation of the MCF-7 breast cancer cells by the estrogen-regulated 52 k protein. *Endocrinology* 1986; 118:1537–1545.

28. Duffy MJ, O'Grady P, Simon J, Rose M, Lijnen HR. Tissue type plasminogen activator in breast cancer: Relationship with estradiol and progesterone receptors. *J Natl Cancer Inst* 1986; 77:621–623.