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Immunohistochemical expression of cathepsin D in correlation with extracellular matrix component, steroid receptor status and proliferative indices in breast cancer

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Abstract In 87 breast cancer patients, the immunohistochemical expression of the basement membrane (BM)-degrading enzyme cathepsin D (CD) was correlated with the expression of extracellular matrix components, with growth fraction, steroid receptor content and with the other conventional prognostic variables in breast cancer. Only 6.25% of tumours had laminin-defined BM, while 86.8% showed staining for fibronectin. CD was also identified in carcinoma cells (cancer cell CD; CCCD) and in stromal cells (stromal cell CD; SCCD). Forty-five percent of tumours showed CCCD and 47.5%, SCCD expression. CCCD expression was significantly correlated with positive oestrogen receptor content, with low Ki-67 and high PCNA score and with SCCD expression. There was no correlation with collagen type IV, laminin or fibronectin. SCCD expression was positively correlated with collagen type IV, laminin expression and tumour grade. The data suggest that the CD of tumour cells and the CD of tumour-associated macrophages have different roles in breast cancer. CCCD correlates with cell proliferation and is regulated by oestrogens, while SCCD relates to cell differentiation, is oestrogen-independent, and has a proteolytic role in the breakdown of BM components.

Key words Collagen type IV · Laminin · Fibronectin · Cathepsin D · Breast cancer

Introduction

Invasive growth characterizes malignant neoplasia and frequently determines the course of the disease. It is a complex multistep process with dissolution of the extracellular matrix, including the basement membrane (BM), as the initial event. In the development of neoplastic epithelial lesions progressing towards invasion, a complex and dynamic interaction occurs between epithelial tumour cells and extracellular matrix, prior to the breakdown of the BM, as demonstrated by morphological methods [13, 22, 26]. The BM-degrading proteases, including type IV collagenase and other proteases, such as plasminogen activators, cathepsins, and heparanases, form a cascade system of enzymes to facilitate the breakdown of the extracellular matrix [3, 22, 31]. The BM, however, is not a static structure that can only be destroyed: it may also be deposited. The presence of BM in a neoplasm might be an expression of the type of interaction of tumour cells with the extracellular matrix. Laminin and collagen IV [23] are the major intrinsic components of BMs. Both molecules have cell adhesion functions and are involved in the process of tumour invasion [3, 22, 23]. Tissue fibronectin, an extrinsic component of basement membranes, is found in the connective tissue in close apposition to the BMs [23] and is considered to be a glycoprotein with important adhesive functions [22, 28].

Cathepsin D (CD) is an oestrogen-induced lysosomal enzyme that can act either directly, by digesting the extracellular matrix, or indirectly by initiating the proteolytic cascade that may be responsible for the breakdown of BMs [27]. There are discrepancies and conflicting opinions about the relative value of CD; these are based on the use of different criteria, different techniques, different reagents, and different interpretations [8]. In the current study we evaluated the expression of BM antigens (collagen type IV and laminin), fibronectin and the BM degrading enzyme (CD) in comparison with two cell proliferation indices (Ki-67 and PCNA), steroid receptor status and the other conventional prognostic features (menopausal status, tumour size, histological types, tumour grading and lymph node status).

Materials and methods

A total of 87 unselected human breast cancer specimens from preoperatively untreated patients [67 no special type (NST) infiltrating ductal, 14 lobular and 6 mixed type], and 10 benign control cases were collected during the frozen section procedure. Tumour

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and control specimens were collected shortly after surgical removal, snap-frozen in isopentane liquid nitrogen, using an embedding medium for frozen tissue specimens, O.C.T. COMPOUND (Tissue Tek-Miles). The frozen specimens were stored at –80°C until processing. One parallel sample was fixed in formalin for 24 h and was subsequently processed with routine techniques and embedded in paraffin. Tumour histotype, size, lymph node status, patient age, and tumour grade based on the method of Blood and Richardson [5] modified by Elston and Ellis [10] were recorded for each case. The infiltrating lobular carcinomas were of classical type and were classed as grade II. The control specimens consisted of normal breast tissue, benign proliferative and fibrocystic breast disease.

For collagen IV, laminin, fibronectin, cathepsin D and PCNA immunostaining routinely buffered formalin-fixed and paraffin-embedded tissue blocks were sectioned at 4-μm-intervals. Paraffin sections were deparaffinized, dehydrated and pretreated with pepsin (0.1% in 0.1 N HCL for 30 min at room temperature). After blocking of endogenous peroxidase (20 min in methanol with 0.35 H_2O_2) and washing in phosphate-buffer saline (PBS) (3 × 5 min), the paraffin sections were treated with normal rabbit serum (1:20) for 30 min for nonspecific background. After washing in PBS the sections were incubated with polyclonal human anti-type IV collagen antiserum (DAKO) diluted 1:100 in PBS with 1% bovine serum albumin) for 1 h, with monoclonal mouse anti-human laminin (Dako-Laminin, 4C7) at 1:300 dilution for 30 min, rabbit anti-human fibronectin (A 245 Dako) at 1:400 dilution for 30 min, polyclonal rabbit anti-cathepsin D (013A Dako) at 1:300 dilution for 30 min and monoclonal PC10 (DAKO) at 1:500 for 30 min at room temperature in a moist chamber by using the three-step indirect immuno-peroxidase method as previously described. After washing in PBS, the paraffin sections were incubated for 30 min with a rabbit anti-mouse and for another 30 min with mouse anti rabbit peroxidase conjugate. Between each conjugation, sections were washed for 10 min with TRIS buffer. After final washing with PBS, a diaminobenzidine (DAB) containing 0.05% H_2O_2 solution was used to visualize the immunoreactivity. All secondary reagents were purchased from Dako. Paraffin sections with the specific monoclonal antibody omitted served as negative controls.

Ki-67 immunostaining was performed on frozen cryostat sections 5 μm thick, which were air-dried and fixed in absolute acetone. They were stained with monoclonal antibodies against Ki-67 (Dako) using a sensitive two-step indirect immunoperoxidase technique as previously described [11]. The sections were incubated with a primary antibody at a 1:10 dilution for 30 min, at room temperature in a moist chamber. After washing in PBS, the frozen sections were incubated for 30 min with a rabbit anti-mouse (Dako) and for another 30 min with swine anti-rabbit (Dako) peroxidase conjugate. Between each conjugation, sections were washed for 10 min with TRIS buffer. After a final wash with PBS, a diaminobenzidine-H_2O_2 substrate was used to visualize the immunoreactivity. The sections were washed, counterstained in Mayer's haematoxylin, and mounted. Cryostat sections with the specific monoclonal antibody omitted served as negative controls.

The amount of immunoreactivity of collagen IV at the tumour–stroma border was scored semiquantitatively. More than 50% immunoreactivity at the tumour–stroma interface was scored as extensive (+++) deposition, between 25% and 50% as moderate (++) and less than 25% as limited (+) for collagen IV immunoreactivity. The blood vessel staining for type IV collagen was estimated in the same way.

Three patterns of staining were estimated for laminin immunoreactivity. Tumours were classified as "positive" with regarded to the immunoreactivity when there was unequivocal immunostaining of the matrix components and the blood vessels in at least in one representative area of the tumour, "negative" when there was no staining and as having a third pattern when only positive blood vessels were found. As regards fibronectin expression, we also separated the cases into three groups, in accordance with the staining pattern at the periphery: strong positive connective tissue staining at the periphery of tumours, positive and negative areas of connective tissue staining pattern at the invading edge of the tumours, and negative connective tissue staining in the same areas.

CD expression was scored as the percentage of carcinoma-positive cells (CCCD) in the region of the carcinoma that stained for CD. For statistical analysis the cases of CCCD were divided into four categories of CD-positive carcinoma cell (0%, <10%, 10 to 50%, and >50%). The percentage of stromal CD only staining (SCCD), which consisted predominantly of carcinoma-associated macrophages, was also scored. For statistical analysis the cases of SCCD were divided into two categories for CD-positive stromal cells (<10% and >10%).

The evaluation of anti-PCNA and Ki-67 immunostaining was calculated as the percentage of PCNA-positive tumour cells in relation to the total number (about 1000) of the tumour cells in representative fields. Every stained nucleus was considered positive, irrespective of intensity. In cases where staining was heterogeneous in the tumour, the examined fields included those with the highest and those with the lowest percentage of stained cells. All slides were reviewed and scored in a blind test by two pathologists. The percentage of positively stained cells was recorded as Ki-67 LI (labelling index) and PCNA LI. and for statistical analysis we separated the cases in two groups, <15% and >15% for Ki-67 and <50% and >50% for PCNA.

Paraffin sections were immunostained with monoclonal anti-receptor antibodies (oestrogen and progesterone receptor immunocytochemical assay, ER-ICA and PgR-ICA Kits; Abbot Laboratories). The immunostaining was assessed on the basis of the visually estimated percentage of neoplastic cells with positive nuclear staining and on staining intensity. Staining intensity was graded as low (+), moderate (++), and intense (+++). All cases with negative staining and those with less than 10% of stained cells, were regarded as negative. For the purposes of statistical analysis, all cases with more than 10% stained cells, independent of the intensity, were regarded as positive. The association of continuous variables confirmed using nonparametric (Spearman, Kendall Tau) correlation. *P*-values smaller than 0.05 were considered statistically significant.

Results

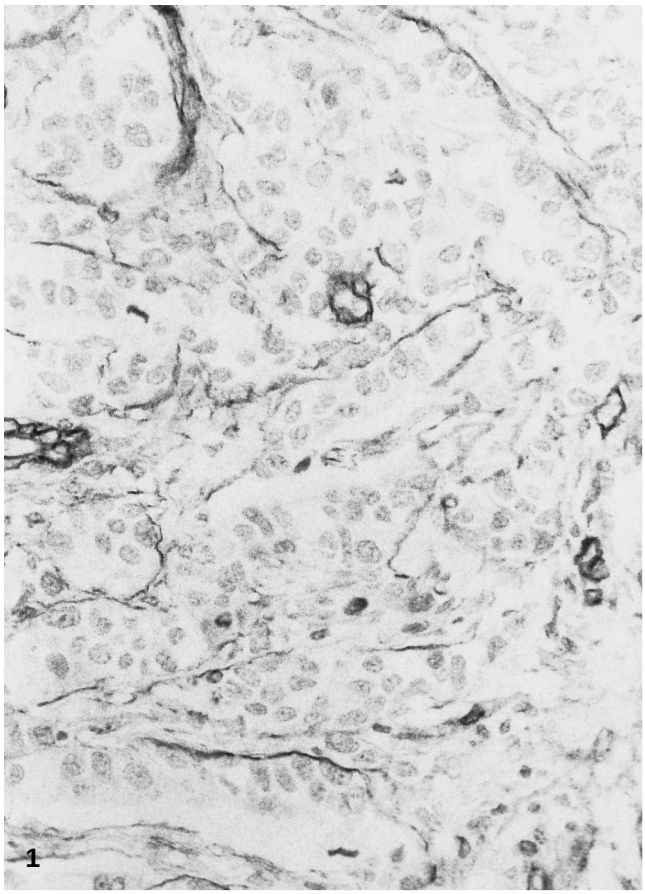
In all cases satisfactory immunohistochemical evaluation of collagen type IV was accomplished, which was assessed using blood vessels and fat and Schwann cells as an internal control. In 10 control specimens, BMs were strongly and continuously stained by anti-type IV collagen antibodies, as were the internal control, the normal ducts and ductules and the adjacent in situ component of the invasive carcinomas. Normal epithelial components and connective tissue elements showed no positive staining in their cytoplasm. In the invasive carcinomas, we observed different staining patterns of type IV collagen immunoreactivity, and the stained line appeared either conserved or fragmented in limited or wide parts of the tumour foci or absent or even around individual tumour cells (Fig. 1). In general, the pattern of staining was

Fig. 1 Type IV collagen immunoreactivity in a case of infiltrating ductal breast cancer. Immunoperoxidase, original magnification ×100 ▶

Fig. 2 Infiltrating ductal breast carcinoma with laminin-defined basement membranes around tumour cells. Immunoperoxidase, original magnification ×100

Fig. 3 Fibronectin staining, in a case of infiltrating ductal carcinoma, around tumour cell nests and between them. Immunoperoxidase, ×160

Fig. 4 Cathepsin D-positive expression in the cytoplasm of tumour cells and in benign stromal cells in case of infiltrating ductal carcinoma. Immunoperoxidase method ×160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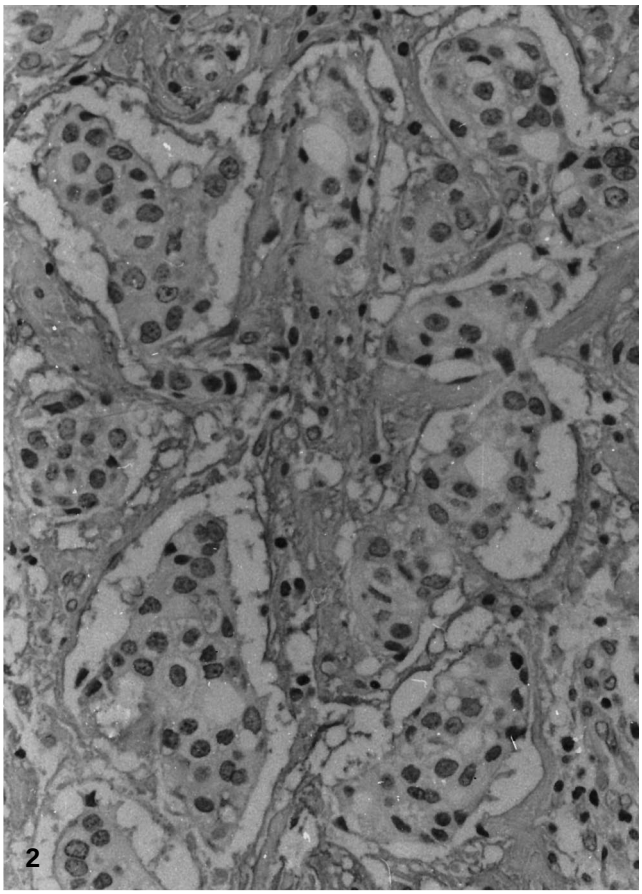
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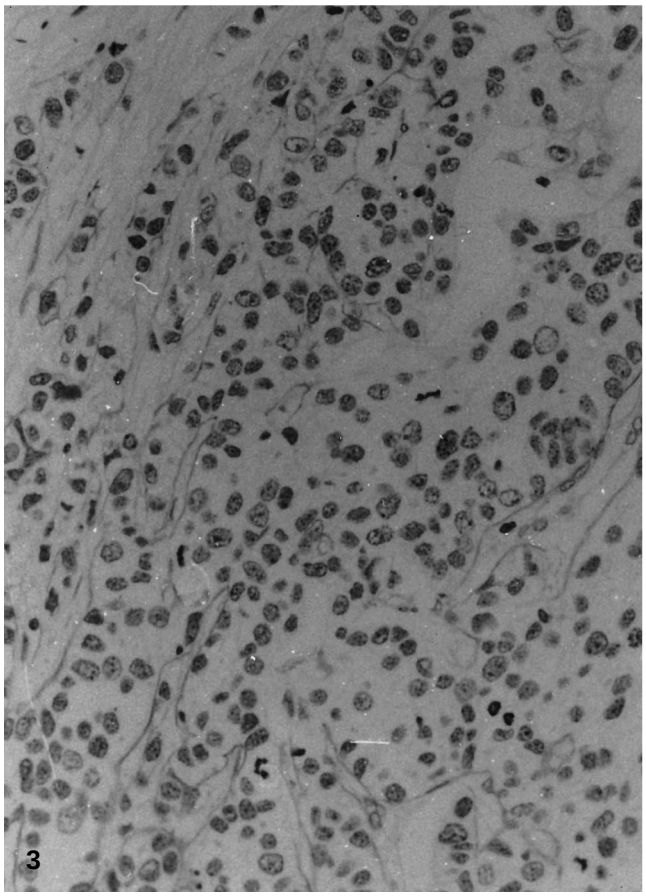
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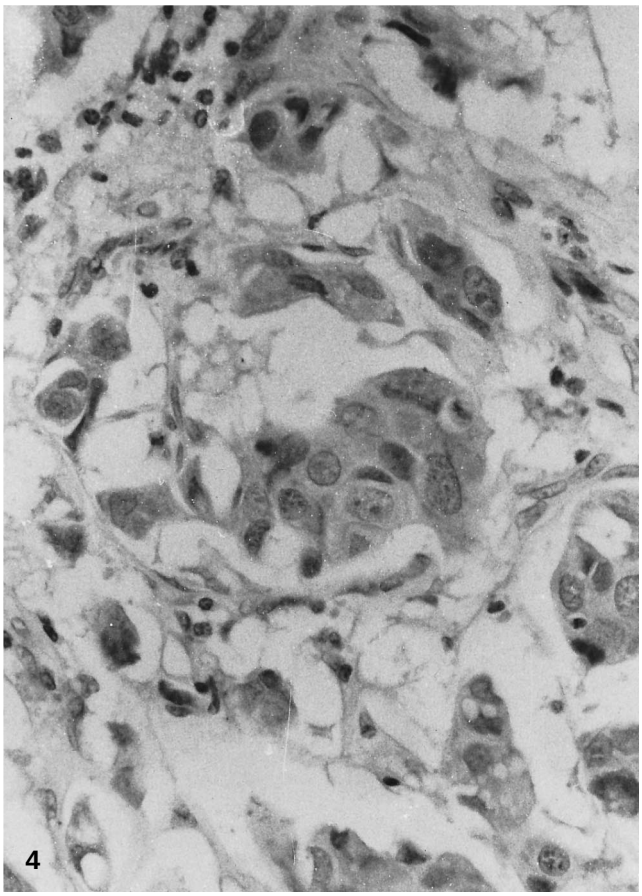
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Table 1 Immunohostochemical expression of extracellular type IV collagen with collagen IV blood vessel positive.

BVP	Type IV collagen expression			
	<10%	10–50%	>50%	*P*-Value
+	28	7		
++	3	21		<0.001
+++	5	10	7	

Table 2 CCCD (cancer cell cathepsin D) expression with clinicopathological variables in breast cancer (*NS* not significant)

	CCCD expression				
	(–)	<10%	10%–50%	>50%	*P*-value
Age (years)					
<50	17	7	8	4	NS
>50	29	13	10	5	
Tumour size (cm)					
<5	22	11	9	4	NS
>5	15	5	5	1	
Type					
Ductal	36	14	10	7	NS
Lobular	8	2	3	1	
Mixed	1	2	3		
Grade					
I	36	14	10	7	
II	8	2	3	1	NS
III	1	2	3		
Lymph node status					
–	16	6	9	1	NS
+	22	10	7	10	

Table 3 CCCD expression with the collagen type IV, laminin, fibronectin,SCCD expression, steroid receptors' status, Ki-67 and PCNA-LI.

	CCCD expression				
	(–)	<10%	10%–50%	>50%	p-value
Collagen IV					
+	16	9	7	1	
++	24	4	6	4	NS
+++	3	2	1	1	
Laminin					
(–)	42	14	12	7	NS
(+)	3	1	1		
Fibronectin					
(+)	11	7	3	3	
(–)	10	4	2		NS
(+),(–)	4	8			
ER					
(–)	12	2		1	<0.05
(+)	25	12	13	5	
PgR					
(–)	14	4	4	1	NS
(+)	19	7	8	7	
SCCD					
<10	32	6	5	3	<0.05
>10	17	10	10	4	
Ki-67					
<15	7	4	4	3	<0.05
>15	9	3	1		
PCNA					
<50	15	1	1	1	<0.05
>50	11	7	4	2	

nearly always heterogeneous. Infiltrating lobular carcinomas showed immunoreactivity around individual cancer cells in most cases. The expression of type IV collagen immunoreactivity was strongly positively correlated with blood vessel expression of collagen IV ($P < 0.001$; Table 1) and with laminin pattern expression ($P = 0.002$) but not with fibronectin or cathepsin D, Ki-67 or PCNA, steroid receptors content, patient age, tumour size, histotype, tumour grade, or lymph node status.

The antibody directed to laminin stained the BM of normal ductules or acini and vessels. Staining of the normal structures was used as an internal positive control. Only 6.25% of tumours (5/80) had laminin-defined BMs (Fig. 2). In all the positive tumours, there were areas in which no immunoreactivity for laminin was observed, either in the same slide or in different slides prepared from the same tumour. There were cases with no immunoreactivity in intra- and peritumour vessels. Laminin expression was correlated only with the collagen IV expression ($P = 0.002$).

In normal and benign control specimens, fibronectin was observed around ductules or acini and vessels with a BM-like pattern, and as irregular fibres in the intermediate stroma. Thirty-six (69.2%) tumours in our series showed staining of the connective tissue stroma among the epithelial tumour cells at the periphery of tumour. It was usually difficult to distinguish between stromal fibronectin and cell membrane- or BM bound fibronectin. Fibronectin was expressed around tumour cells and between them (Fig. 3). In rare cases, as in a few control specimens, cytoplasmic staining of the tumour cells was also observed. There were many different staining pattern of fibronectin immunoreactivity. There were cases with homogeneous positive staining patterns in the centre and in the connective tissue at the periphery of the tumours. Other cases showed positive centres and negative periphery, and the converse. There were also cases with positive and negative areas in the centres and in the periphery of the tumours and finally cases with positive or negative centre staining patterns and positive and negative areas at the periphery of tumours. No significant relationship was found between the staining patterns of fibronectin and the BM staining pattern either with CD expression or with proliferating indices and the other biological and clinicopathological variables.

Immunoreactivity for CD was seen as brown, fine to coarse granular cytoplasmic staining and was heterogeneous in intensity and localization in most invasive carcinomas (Fig. 4). Stromal staining was usually due to strongly positive histiocytes dispersed among cancerous nests, either in desmoplastic stroma or in association

Table 4 SCCD expression with the collagen type IV, laminin, fibronectin,steroid receptors' status Ki-67 and PCNA

	SCCD expression		
	<10	>10	*P*-value
Collagen IV			
+	21	12	
++	20	18	<0.05
+++	1	6	
Laminin			
(–)	39	33	<0.05
(+)		5	
Fibronectin			
(+)	13	11	
(–)	7	7	NS
(+), (–)	6	7	
ER			
(–)	9	7	NS
(+)	24	30	
PgR			
(–)	11	13	NS
(+)	16	19	
Ki-67			
<15	12	6	NS
>15	6	7	
PCNA			
<50	16	4	NS
>50	11	11	

with necrosis. There were 36 tumours (43.7%) that showed CD expression in the cancer cells, and 41 tumours (47.13%) that were "positive" for CD (>10%) in the stromal cells (SCCD). Results of CCCD and SCCD expression with BM proteins, fibronectin, steroid receptor status, proliferative indices and the other clinicopathological variables are shown in Tables 2–4. CCCD expression was positively correlated with oestrogen receptor content (P = 0.019), PCNA labelling index (P = 0.024) and SCCD expression (P = 0.04). A high Ki-67 labelling index was correlated with low CCCD expression (P = 0.045). There was no correlation with collagen type IV, laminin or fibronectin expression or progesterone receptor content or any of the other conventional clinicopathological variables studied. SCCD expression was also correlated with collagen type IV (P = 0.03), with laminin (P = 0.002) and with the tumour grade (P = 0.007).

Discussion

The ability of neoplastic cells to spread to distant sites is derived from multiple complex interrelated processes that include the dissolution of BM and extracellular matrix by degradative enzymes [13, 22, 26, 31]. Tumour production of CD, a lysosomal aspartyl protease, has been implicated in tumour invasiveness and metastatic dissemination through the breakdown of extracellular matrix, permitting vascular invasion [7]. CD activity has been shown to be useful in predicting relapse-free survival in breast cancer [16, 17, 30]. BM proteins may be involved in cellular adhesion to BMs and extracellular matrix. Fibronectin mediates the attachment of a variety of cell types [28], whereas type IV collagen and laminin may be primarily involved in the attachment of epithelial cells [18]. BMs are thought to form a protective barrier against the initial infiltration of tissues by malignant cells. However, the exceptional ability of metastatic cells to attach to the BM of endothelial cells may play a part in the spread of tumours via the circulation [19, 24]. Nonetheless, the use of microvessel density as a practical tool in determining the prognosis of breast cancer has not been validated [25]. In our study we found parallel tumour BM collagen type IV expression and blood vessel BM expression; in areas of tumour with collagen type IV expression there were blood vessels with collagen type IV immunoreactivity. We suggest that in this instance the process of metastasis is impossible. We propose that it is not the microvessel density that represents or provides an indicator of metastatic capacity, but the quality of the vessel and the protective barrier capacity of the basal lamina. We also found that infiltrating lobular carcinomas showed a cytoplasma-like membrane immunoreactivity in individual cancer cells in most cases; in a few tumours cytoplasmic staining of fibronectin was detected. This suggests the production or the derivation of this protein from the neoplastic cells themselves.

The structure and the changes of BM in breast lesions have been studied extensively [1, 9, 14, 29], and a variable loss of BM is a constant feature in invasion. Furthermore, to our knowledge, no other studies have been reported on the relationship of BM component, fibronectin, CD expression and other prognostic variables, including steroid receptors and proliferation indices. We did not find any correlations of CCCD expression with BM components or with fibronectin, at the in situ level, although CD has been implicated in the breakdown of extracellular matrix. However, we found correlations between SCCD expression and collagen type IV and between SCCD and laminin expression. It is probable that the CD of carcinoma-associated macrophages plays a proteolytic role in the breakdown of the BM matrix. Furthermore, we observed that SCCD expression has been correlated with tumour grade, and it therefore seems that the CD plays a part in tumour evalution. We also noted the correlation of CCCD with the growth fraction of the tumour, as estimated by two proliferation indices (Ki-67 and PCNA). This finding is in agreement with those of others, who have shown that CD has the ability to promote tumour cell proliferation [21, 32]. Ki-67 is considered to be a prognostic indicator in breast cancer [2, 6, 12]. The data on PCNA [4, 15] are limited and contradictory, and some authors believe that PCNA is deregulated in breast cancer. Although we, like others [20], have found no correlation between the two indices in our data, they were correlated with the CCCD expression. We also found a correlation with CCCD expression and oestrogen receptor content. It is known that CD is an oestrogen-in-

duced lysosomal protease, which is increased by oestrogens and inhibited by anti-oestrogens [30].

We observed that the CCCD expression was significantly associated with positive oetsrogen receptor status, with high PCNA labelling index, and with SCCD expression, while there was an inverse relationship with the Ki-67 labelling index. The SCCD expression was positively correlated with collagen type IV expression and tumour grade. Therefore, it is possible that the CD of tumour cells and the CD of tumour-associated macrophages have different roles in breast cancer. The CCCD correlates with cell proliferation rates (promoting or suppressing them) and is regulated by oestrogens. The SCCD relates to the cell differentiation, is independent of oestrogen, and has a proteolytic role in the breakdown of collagen type IV.

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