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Comparative biochemical and immunohistochemical studies on the cathepsin D content of human breast cancer

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Abstract. Cathepsin D (CD) has been introduced as a predictor of prognosis in patients with breast cancer due to its mitogenic effect and its role in tumour metastasis. Commonly, the CD content of tumours is examined by means of a biochemical method based upon the use of monoclonal antibodies, and immunohistochemical visualization of CD has not been used extensively. The present study compares the biochemical and immunohistochemical findings in 216 cases of human breast cancer. CD may occur in tumour cells and/or macrophages. Correlation of immunohistochemically determined CD content and biochemical CD content is better in tumour cells than in macrophages. The possible causes of this observation are briefly discussed. Although a statistical correlation between the biochemical and the immunohistochemical CD findings exists, the results in individual cases vary within a wide range. Hence, the results of biochemical and immunohistochemical CD assay in an individual tumour cannot be compared directly.

Key words: Breast cancer – Cathepsin D – Biochemical assay – Immunohistochemical assay

Introduction

Since cathepsin D (CD) was found in human breast cancer cells (Capony et al. 1982) it has been extensively studied. CD is an acid lysosomal protease (aspartyl endoproteinase) present in numerous tissues and cells which normally degrades proteins (particularly high-molecular-weight proteins) in lysosomes at an acid pH (Rochefort 1990). Its inactive precursor is pro-CD, a 52 kDa protein bearing two *N*-linked oligosaccharide chains with mannose-6-phosphate signals at their extremities (Rochefort 1990). Pro-CD is bound to mannose-6-phosphate receptors and carried from the Golgi network to lysosomes via endosomes. The pro-CD-receptor complex dissociates, and while the receptor recirculates to the Golgi field the 52 kDa pro-CD is rapidly processed into a mature form (34 kDa and 14 kDa) via an intermediate form (48 kDa) (Morisset et al. 1986). While only negligible amounts of pro-CD are accumulated in or secreted by normal mammary cells, processing is markedly delayed in breast cancer cells (Rochefort 1990). Pro-CD and 48 kDa CD accumulate in the cells, and pro-CD secretion is increased by up to 50% (Capony et al. 1989). The second important difference between cancer cells and normal mammary cells is a 2- to 30-fold overproduction of total CD (pro-CD and mature CD) by the tumour cells (Capony et al. 1989) accompanied by an overproduction of 52 kDa mRNA (Cavaillès et al. 1988; Rochefort et al. 1989). The mRNA level is low in oestrogen-receptor-positive breast cancer cell lines but markedly increased by oestrogen treatment. It is naturally high in oestrogen-receptor-negative cell lines. An increased CD content of human breast cancer cells following administration of oestrogen or short-term treatment with tamoxifen has been described by Morisset et al. (1986), Maudelonde et al. (1989), and Touitou et al. (1991).

CD appears to play an important role in tumour invasion and in the production of metastases. It has a mitogenic effect possibly due to the release or activation of growth factors, or by the triggering of a plasma membrane receptor (Rochefort 1990).

Moreover, CD facilitates the development of metastases due to its proteolytic activity, but again direct proof that CD promotes some step in this procedure is lacking (Rochefort 1990). The cancer cells may internalize extracellular matrix by phagocytosis and endocytosis and degrade it in acid vesicles present in high numbers in the cytoplasm of the cells (Montcourrier et al. 1990). The direct extracellular digestion of proteins by CD has not yet been proven, and the possible role of other enzymes, such as collagenases, has not been settled (Liotta et al. 1980; Yee and Shiu 1986).

Overexpression of CD has been described as an important prognostic marker in several independent studies (Spyratos et al. 1989; Thorpe et al. 1989; McGuire et al.

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1990; Tandon et al. 1990). Two methods are available for the determination of the CD content of breast cancer tissue both using monoclonal antibodies. Cell extracts may be examined by the IRMA kit ELSA-CATH-D commercially distributed by CIS Bio International. Two antibodies (D7E3 and M1G8) recognize two epitopes on the large chain of the mature CD (34 kDa) and the same epitopes on the intermediate chain (48 kDa) and the pro-CD (52 kDa; Rochefort 1990). The homogenization technique used for the oestrogen and progesterone receptor assay extracts approximately 90–98% of the total CD (pro-CD + mature CD; Rochefort 1990). The second method is direct visualization of CD in the cells by immunohistochemistry. Again, this assay is based upon a commercial kit from CIS (HIS-CATH-D-AB-1), but only one of the two antibodies (M1G8) is used here.

Most studies on CD in breast cancer are based upon the biochemical assay, and only two papers deal with immunohistochemistry using other antibodies than those distributed by CIS (Henry et al. 1990; Domagala et al. 1992). We are not aware of any previous studies comparing biochemical and immunohistochemical results in the same tumours. It must be emphasized that the biochemical method measures the CD content of a certain tissue, irrespective of the cell types containing the enzyme, while immunohistochemistry visualizes the CD within the different cell types and thus may define the CD content of the tumour cells alone. The present study is aimed at answering this question.

Materials and methods

The study is based on 216 cases of human breast cancer [invasive ductal cancer (NOS), 179; invasive lobular carcinoma, 12; ductal carcinoma in situ, 5; invasive ductal carcinoma with predominant intraductal component, 6; mucinous carcinoma, 2; tubular carcinoma, 6; medullary carcinoma with lymphoid stroma, 3; papillary carcinoma, 3]. The paraffin blocks were taken from our files or supplied by the following pathologists: Professor Höer (Nordwest-Krankenhaus, Frankfurt am Main), Dr. Müller (Stadtkrankenhaus, Offenbach/Main), Professor Schäfer (Elisabeth-Krankenhaus, Cologne) and Professor Schubert (Städtische Kliniken, Wuppertal).

For *immunohistochemistry* we pretreated the slides with poly-L-lysin. The slides of the paraffin sections (4–5 μm) were kept at 37° C overnight and then deparaffinized, followed by protease digestion (protease type XIV P-5147, Sigma Diagnostics, Deisenhofen). They were then rinsed in ice-cold phosphate-buffered saline (PBS) and PBS at room temperature, followed by incubation with the primary antibody of the HIS-CATH-D AB-1 kit for 60 min, rinsing with PBS, incubation with the bridging antibody for 20 min, rinsing with PBS, and incubation with enzyme-labelled streptavidin. They were then again rinsed with PBS, followed by the addition of the chromogen-substrate solution for 15 min, rinsing with distilled water, counterstaining with haematoxylin, rinsing in tap water and mounting in Karion (Merck, Darmstadt). Sections from normal liver tissue served as positive controls and the macrophages in the breast cancer sections were used as an intrinsic control. In negative controls primary antibody was replaced by non-specific immune mouse serum.

The slides were examined microscopically by a single author (W.R.). Red granular cytoplasmic staining was evaluated as positive. An immuno-reactive score (IRS) was determined considering both the percentage of positively staining tumour cells and staining intensity. Primarily, the IRS was classified from 0 to 3+ with six intermediate grades. In order to avoid pseudoaccuracy of the subjective grading and to enlarge the number of cases per IRS grade, the intermediate grades were included in the main grades, and the final classification comprised four grades of IRS: 0, 1+, 2+, and 3+. Six weeks after the first classification, all slides were re-examined by the same author (W.R.) without knowledge of the first results. In both steps, the IRS was determined without knowledge of the biochemical results. The IRS for the macrophage CD was determined twice by the same procedure as used for the tumour cells.

Immunohistochemistry was usually confined to one slide per paraffin block. In a few cases, serial sections were performed in order to detect possible variations of the CD content in different portions of the tumour.

The biochemical studies were performed in all cases by Dianon systems [Stratford, Conn., USA (German branch: Bad Homburg)] by means of the IRMA kit ELSA-CATH-D. The results were kindly supplied by the German branch of DIANON.

The biochemical CD values (total CD, pmol/mg protein) were plotted against the final CD IRS for (a) the tumour cells alone, (b) the macrophages alone (macrophages $\times 1$), (c) the tumour cells + macrophages $\times 1$ and (d) the tumour cells + twice the IRS for macrophages (macrophages $\times 2$) since the macrophages have a much larger cytoplasm than the cancer cells and hence might influence the IRS stronger than it would appear from the combined IRS for tumour cells and macrophages $\times 1$. The following statistical values were calculated for (a) the total number of biochemical values per IRS grade and (b) the mean values and standard deviations for all biochemical values in the four IRS grades by means of the method of least squares according to Gauss including error weighting: linear regression, correlation coefficient (r) and probability for no correlation (PNC). Moreover, the biochemical quantiles were determined for each IRS grade.

Results

The relative percentage of tumour cells and macrophages staining positively by immunocytochemistry differed markedly between the individual tumours. The same was true for staining intensity and the percentage of CD-positive cells in both the tumour cells and macrophages (Fig. 1A–D). A moderate variation of the IRS for tumour cells and macrophages was observed when

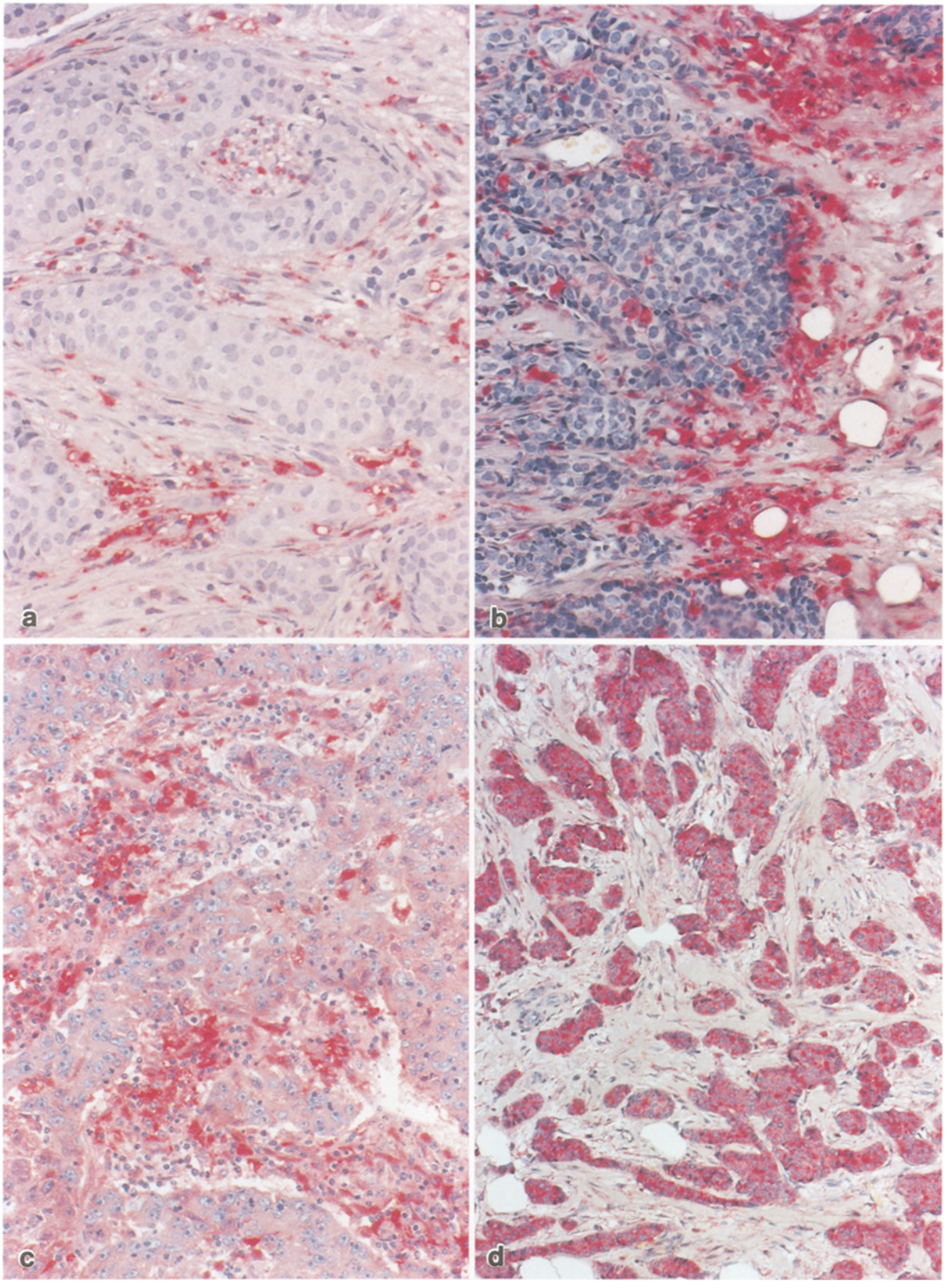

Fig. 1a–d. Immunohistochemical cathepsin D (CD) assay in four cases of invasive ductal breast cancer (NOS). Mouse monoclonal antibody HIS-CATH-D-AB-1 kit, paraffin section. **a** Cancer cells negative, macrophages 2+ (moderately positive), ×140. **b** Cancer cells negative, macrophages 3+ (strongly positive), ×140. **c** Cancer cells 2+ (moderately positive), macrophages 3+ (strongly positive), ×140. **d** Cancer cells 3+ (strongly positive), macrophages 1+ (occassionally positive), ×54

Table 1. Immunohistochemically determined cathepsin D (CD) content in step sections of breast cancer paraffin blocks: CD content varies within the same tumour

Case no.	0		1+		2+		3+		Total	
	No. of slides	(%)	No. of slides	(%)	No. of slides	(%)	No. of slides	(%)	No. of slides	(%)
1	0	0	1	7.1	9	64.3	4	28.6	14	100.0
2	0	0	7	21.9	12	37.5	13	40.6	32	100.0
3	0	0	4	13.8	23	79.3	2	6.9	29	100.0
4	9	81.8	2	18.2	0	0	0	0	11	100.0

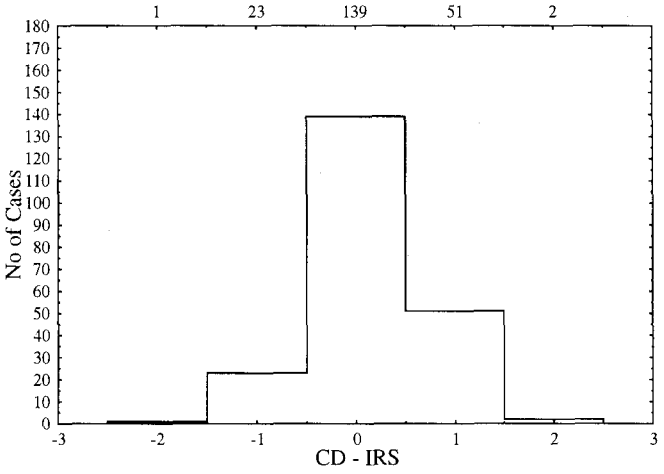

Fig. 2. Difference between the first and second evaluation of immunohistochemically determined cathepsin-D (CD IRS) (tumour cells)

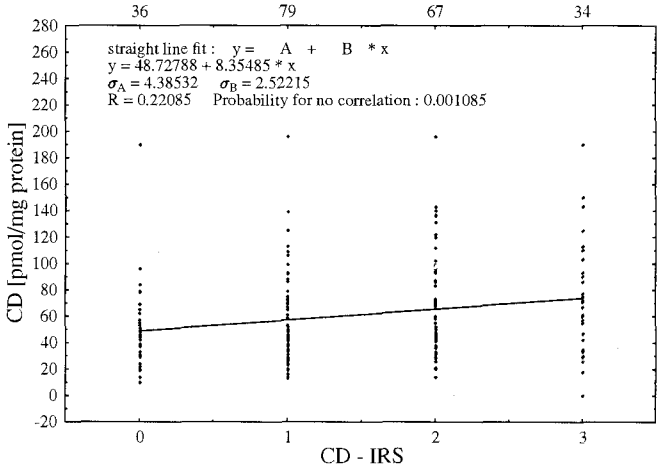

Fig. 3. Biochemical cathepsin-D (CD) values vs immunohistochemical CD values. Scatter plot and statistical analysis. Tumour cells, first evaluation

Table 2. Biochemical quantiles for different immunohistochemically determined (IRS) grades. CD, Cathepsin D; BC, biochemical assay; IH, immunohistochemical assay

	CD-IRS grade	Quantiles				
		Minimum	25%	50% (median)	75%	Maximum
Tumour cells	0	10	31	48	62	190
CD(BC) vs CD(IH)	1+	13	34	45	70	196
1st evaluation	2+	14	41	58	94	196
	3+	0	42	72	93	190
Tumour cells	0	14	35	46	65	190
CD(BC) vs CD(IH)	1+	10	30	45	69	196
2nd evaluation	2+	14	43	60	87	140
	3+	0	36	61	102	196
Macrophages	0	20	35	68	99	125
CD(BC) vs CD(IH)	1+	10	35	49	75	190
1st evaluation	2+	0	34	47	74	196
	3+	24	45	58	86	196
Macrophages	0	10	26	65	93	190
CD(BC) vs CD(IH)	1+	13	35	46	73	143
2nd evaluation	2+	0	34	48	72	190
	3+	14	44	58	86	196
Tumour cells + macrophages ×1	1+	10	24	37	57	190
CD(BC) vs CD(IH)	2+	14	39	46	70	139
1st evaluation	3+	13	34	50	79	143
	4+	14	43	69	86	196
	5+	0	44	61	98	122
	6+	29	33	57	86	150

Table 3. Relation of biochemical and immunohistochemical CD values (least squares fit to a straight line from Figs. 3–6). CD, Cathepsin D; BC, biochemical assay; IH, immunohistochemical assay; TC, tumour cells; MP, macrophages; SD, standard deviation; SL, straight line

Cell type	Statistical comparison	1st or 2nd evaluation	*y* Intercept A of the SL (A)	Slope B of the SL (*sigma* A)	SD of the *y* intercept A (B)	SD of the slope B (*sigma* B)	Linear correlation coefficient (*r*)	Probability of no correlation (PNC)	Number of cases
TC	CD(BC) vs CD(IH)	1st	48.72788	8.35485	4.38532	2.52215	0.22085	0.001085	216
TC	CD(BC) vs CD(IH)	2nd	49.69926	7.02017	4.41893	2.32391	0.20223	0.002828	216
MP	CD(BC) vs CD(IH)	1st	57.76250	1.81898	5.57456	2.89301	0.04294	0.530191	216
MP	CD(BC) vs CD(IH)	2nd	57.80823	1.68026	5.47327	2.65060	0.04329	0.526841	216
TC + MP × 1	CD(BC) vs CD(IH)	1st	44.14596	5.25613	6.39838	1.85892	0.18977	0.005137	216
TC + MP × 2	CD(BC) vs CD(IH)	1st	48.10736	2.60189	6.52256	1.23051	0.14306	0.035626	216

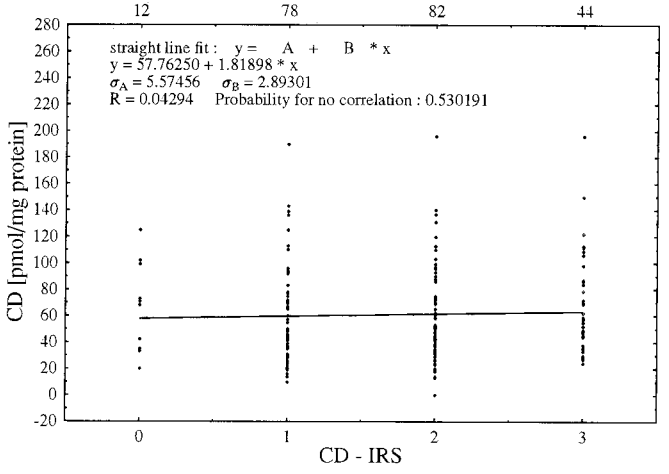

Fig. 4. Biochemical cathepsin-D (CD) values vs immunohistochemical CD values. Scatter plot and statistical analysis. Macrophages, first evaluation

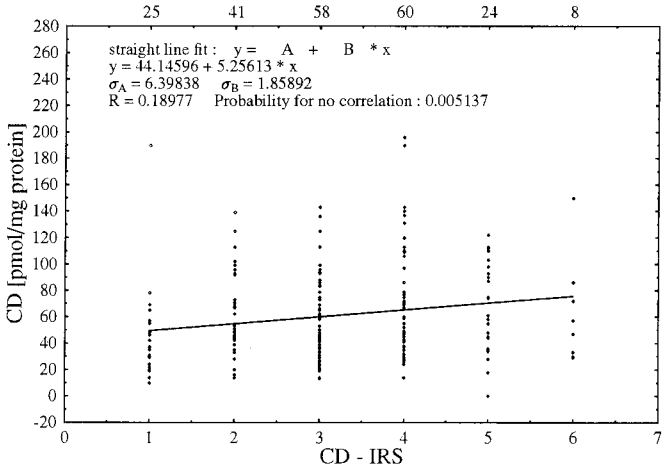

Fig. 5. Biochemical cathepsin-D (CD) values vs immunohistochemical CD values. Scatter plot and statistical analysis. Tumour cells + macrophages × 1, first evaluation

the same tumour was examined in step sections (Table 1). Agreement between the first and second immunohistochemical CD evaluation was satisfactory (Fig. 2). In 139 of 216 carcinomas (64.4%) the findings were entirely identical, while in 74 cases (34.3%) the difference was one point and in only 3 cases (1.9%), two points. The first evaluation rendered 16.7%, the second evaluation 18.1% negative cases without any visible staining of the tumour cells.

When the IRS values of the tumour cells were plotted against the biochemical findings (Fig. 3) no clear-cut correlation appeared to exist although a tendency towards higher biochemical values with increasing IRS was observed. This impression was substantiated by the quantiles, particularly the second and third quantile on the basis of the second evaluation (Table 2) and by the PNC far below the 5% level (Fig. 3; Table 3) while the *r* value was low. The results for the macrophages were much worse (Fig. 4; Tables 2, 3). None of the quantiles, *r* values or PNCs supported a possible correlation between the biochemical and immunohistochemical CD values. Consequently, the results for the combined IRS of tumour cells and macrophages × 1 and the tumour cells and macrophages × 2 were worse than those for

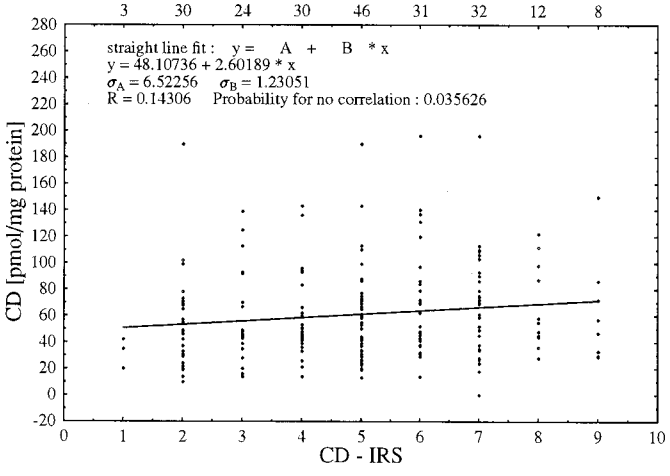

Fig. 6. Biochemical cathepsin-D (CD) values vs immunohistochemical CD values. Scatter plot and statistical analysis. Tumour cells + macrophages × 2, first evaluation

the tumour cells alone (Figs. 5, 6; Tables 2, 3). The PNC lay below the 5% level for both combinations but it was higher than for tumour cells alone and was worse when the IRS for the macrophages was doubled.

Discussion

The present findings point to a *statistical* correlation between the biochemical and immunohistochemical determinations of the CD content of breast cancer cells. Moreover, this correlation also existed when the biochemical values were compared with the combined IRS for tumour cells and macrophages but the PNCs were much higher. This observation is explained by the missing correlation between biochemical and immunohistochemical CD values in macrophages alone. It may be concluded from our results that the CD content of macrophages visualized by immunohistochemistry will have a negative influence upon the correlation of biochemical and immunohistochemical CD values. This assumption is supported by the observation that PNC will increase if the tumour cell IRS is combined with the macrophage IRS $\times 2$ instead of the macrophage IRS $\times 1$.

This observation is difficult to explain. We speculate, however, that the macrophages contain an epitope missing in tumour cells. As described above, the CIS kit for immunohistochemistry contains only one antibody while the biochemical kit contains two. If the tumour cells lacked the epitope recognized by the second antibody both methods – biochemistry and immunohistochemistry – would give comparable results based upon the demonstration of the first epitope. However, the macrophages might contain both epitopes, and then both methods would give different results, with a higher content of CD observed by biochemistry. If the macrophage IRS is doubled for the calculation of the combined tumour cell and macrophage IRS, PNC will also increase, and this assumption is consistent with our observations. It must be emphasized that this interpretation of our results is speculative.

The distribution of *individual* CD values over a wide range, as seen in Figs. 3 and 4, may be explained by insufficient biochemical and/or immunohistochemical data. Biochemical results are based upon one determination and it is well known from other studies, particularly for steroid hormone receptors, that re-examination of the same material by the same laboratory or by other laboratories may give differing results (Thorpe and Rose 1986). Similar findings may be expected for biochemical CD control examinations. The immunohistochemical results reported here are also based upon the evaluation of only one slide per tumour. Evaluation of step sections from the same tumour revealed remarkable intratumoral variation of the CD content (Table 1) and this may cause discrepancies.

The wide distribution of individual CD values prevents the definition of a threshold value between negative and positive tumours as far as immunohistochemistry is concerned. The biochemical threshold value has not yet been defined, and the literature reports values from 35 to 78 pmol/mg protein (Spyratos et al. 1989; Gonzalez-Gancedo et al. 1990; Romain et al. 1990; Tandon et al. 1990; Choné et al. 1991; Granata et al. 1991; Namer et al. 1991). Dianon distinguishes three categories: up to 30 pmol/mg protein (favourable prognosis), 31–60 pmol/mg protein (indifferent prognosis) and more than 60 pmol/mg protein (unfavourable prognosis). The CD IRS of an individual breast cancer may be high although the biochemical value is low and vice versa. Therefore, one method cannot be replaced by the other unless the differences have been explained by further studies and unless the prognostic value of the CD IRS has been determined by retrospective or prospective clinical studies. In our opinion, both the biochemical and immunohistochemical CD assay should be performed and reported to the clinician whenever possible.

We are familiar with only two immunohistochemical studies on the CD content of breast cancer tissue and its influence on the patients' prognosis (Henry et al. 1990; Domagala et al. 1992). According to Henry et al. (1990) CD immunohistochemistry could not confirm the results of biochemical studies. The authors on a study of 94 cases claim that CD-positive tumours have a better prognosis than those which are CD-negative, while the biochemical studies consistently report a negative correlation between CD content and prognosis. Henry et al. also describe a significant association between positive oestrogen receptor status and immunohistochemically detectable CD and refer to other results showing that CD is under oestrogen regulation in breast cancer cells (see also Introduction). They also report a particular prognostic advantage of CD expression in patients with oestrogen-receptor-positive tumours. In summary, they explain the improved prognosis of CD-positive tumours by the suggestion that oestrogen receptor and CD status are related and that what is found reflects a less aggressive tumour type. A recent study by Domagala et al. (1992) did not confirm a significant correlation between CD expression and overall survival for patients with node-negative or node-positive breast carcinomas. However, in node-negative cases, a tendency towards a better prognosis was seen in CD-positive, vimentin-negative tumours while the prognosis was worse in CD-positive, vimentin-positive tumours. In node-positive patients, CD was negatively correlated to prognosis. The number of cases in these subgroups was too low, however, to permit a conclusion and Domagala et al. did not examine the oestrogen receptor content of their cases. Their study is based on exactly the same number of cases (94) as that of Henry et al. (1990). In 188 of 216 cases of our present series, the biochemical oestrogen and progesterone receptor values were compared to the CD IRS. The PNCs were 0.95 for the oestrogen and 0.34 for the progesterone receptor; thus a correlation was not established.

A major problem in comparing the three immunohistochemical CD studies is that different antibodies were used. Henry et al. (1990) used a rabbit polyclonal CD antiserum at 1:300 dilution, possibly identical with the rabbit polyclonal antibody (diluted 1:100) used by Domagala et al. since both antibodies were obtained from England, while our studies are based upon the mouse monoclonal CIS antibody. The three studies also refer to unknown or different types of breast cancer. Henry et al. (1990) do not mention the histological types of their 94 cases while Domagala et al. (1992) only examined invasive ductal breast cancer (NOS), and our study

comprised a wide spectrum of different histological types. The present data do not allow us to evaluate the relationship of immunohistochemical CD content and prognosis. Further studies should be based upon a much larger number of cases using the same antibody.

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