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Original Paper

Cathepsins B, H, L and Cysteine Protease Inhibitors in Malignant Prostate Cell Lines, Primary Cultured Prostatic Cells and Prostatic Tissue

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Elevated activities of cysteine proteinases, the cathepsins B, H, L (CB, CH, CL) and diminished cysteine protease inhibitors (CPI) have been demonstrated in a variety of tumours and have been suggested to contribute to invasion and metastasis. The situation for prostate cancer is still unknown. In this study, using fluorimetric assays, the catalytic activities of CB, CH, CL were measured in prostatic tissue samples after radical prostatectomy, adenomectomy, transurethral resection of the prostate, in cell cultures grown from cancerous and non-cancerous parts of human prostate after prostatectomy and in the cell lines LNCaP, DU 145 and PC 3. CPIs were determined using heat activation before testing their inhibitory activity against purified CB. Comparing matched pairs of normal and cancerous tissue samples from the prostate, significantly decreased levels of CB, CL in malignant parts of the prostate were found. In contrast, primary cell cultures from cancerous samples showed elevated levels of CB, CH, CL and increased ratios of cathepsins to CPI compared with cell cultures from normal prostate. Established cell lines showed a similar distribution pattern of each cathepsin, DU 145 containing the highest levels, followed by LNCaP and PC 3. Our results suggest that elevated cathepsin levels and consequently increased ratios of cathepsins to CPI in primary cell cultures from cancerous versus non-cancerous parts of the prostate may be indicative of a cellular proteolytic imbalance in prostatic cancer cells. In this respect, primary cell culture experiments should be preferred to determinations in tissue samples. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

RECENT STUDIES have shown that tumour growth, invasion and metastasis are strongly correlated to the behaviour of proteolytic enzymes [1]. Cellular proteases are generally subdivided into four groups: serine, cysteine, aspartic and matrix metalloproteinases. Cathepsins B, H and L (CB, CH, CL) are lysosomal proteolytic enzymes belonging to the cysteine protease family. They are widely distributed in almost all mammalian cells, being mainly responsible for intracellular protein turnover [2]. It has been demonstrated that cathepsins cannot only degrade components of the extracellular matrix, such as laminin, collagen and elastin structures of basement membranes, but also activate other proteolytic enzyme systems [3].

In vivo, the activity of cysteine proteases is controlled by specific endogenous inhibitors, e.g. stefins and cystatins [4]. The catalytic cathepsin activity is regulated by the balance between the amount of active enzyme and the amount of the inhibitor. Imbalance between proteases and their inhibitors is believed to promote tumour progression [5].

Recent studies have shown that cytosols of tumour tissues contain higher levels of CB, CH, CL than normal adjacent tissue. Higher enzyme activity correlates with tumour progression and shortened patient survival [6, 7]. This has been demonstrated in lung, head and neck, colon and other cancers [7–9].

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There are a few data on these components in prostate carcinomas and normal human prostatic tissue samples. To our knowledge only CB, CL and CD have been examined in prostatic tissue so far [10–13]. The results of CB were mainly based on immunohistochemical data but not on quantitative enzyme determinations [12, 13]. These studies did not include the relationships of cathepsins to the cysteine protease inhibitors (CPI). In addition, no systematic studies have been performed until now on whether the androgen-responsive LNCaP cells and the androgen-non-responsive cells PC 3 and DU 145 used as established cell lines in human prostate cancer research behave like human prostate cells in culture or tissue concerning the cathepsins [14]. Thus, the aim of this study was to compare CB, CH, CL and CPI in both tumour and normal prostatic tissues, in established malignant cell lines (LNCaP, DU 145, PC 3) and in primary cell cultures.

MATERIALS AND METHODS

Reagents and materials

For the enzyme assays, the substrates benzyloxycarbonyl-L-arginyl-L-arginyl-7-amino-4-methylcoumarine (Z-Arg-Arg-AMC) for CB; L-arginine 7-amido-4-methylcoumarine (Arg-N Mec) for CH; benzyloxycarbonyl-L-phenylalanyl-L-arginyl-4-methylcoumarine (Z-Phe-Arg-AMC) for CL; L-*trans*-epoxysuccinylleucylamido (4-guanidino)butane (E-64) as inhibitor for all cathepsins; amino-methylcoumarine as standard and CB (Cat. no. C 0150) were obtained from Sigma (St. Louis, Missouri, U.S.A.). Benzyloxycarbonyl-L-phenylalanyl-L-phenylalanyl-diazomethylketon (Z-Phe-Phe-CHN_2) was purchased from Bachem (Heidelberg, Germany). All other chemicals of highest commercially available purity were obtained from Sigma or Boehringer GmbH (Mannheim, Germany).

Culture media RPMI-1640 and KSFM (keratinocyte serum-free medium; cat. no. 1700534; with 5 μg/l epidermal growth factor and 50 mg/l bovine pituitary extract) as well as trypsin ethylene diamine tetra acetic acid (EDTA) solution (0.05%, 0.02%) and Dulbecco's phosphate buffered saline solution (DPBS; cat. no. 14190) were obtained from Gibco Life Technologies (Eggenstein, Germany). KSFM medium was supplemented with bovine serum albumin (final concentration 250 mg/l; Behring AG, Marburg, Germany), transferrin (10 mg/l; Boehringer), dihydrotestosterone (5 μg/l; Sigma), non-essential amino acid solution (1%; Gibco, cat. no. 11140-35) and penicillin/streptomycin (125 kU and 125 mg/l; Gibco) and used as primary prostate cell culture (PPCC) medium. Triton X-100, collagenase type IA (245 units/mg) and fetal calf serum were purchased from Sigma; hyaluronidase (1000 units/mg) from Boehringer; the cell attachment matrix (ECL) consisting of enactin, collagen IV and laminin from Biozol (Echting, Germany). Falcon-Primaria culture flasks from Becton Dickinson (Heidelberg, Germany) and plastic culture flasks from Costar (Cambridge, Massachusetts, U.S.A.) were used.

Tissue samples and cell cultures

Prostate tissue samples were obtained from 36 patients (age range 44–81 years), who were receiving treatment at the Department of Urology at the Charité. The use of this human tissue for research purposes was approved by the Ethical Committee of the University Hospital Charité, Berlin.

15 patients (mean age 63 years) suffering from cancer of the prostate underwent radical prostatectomy. The stage was assigned to each patient according to the TNM system and the histological grade of the cancer was classified as grade 1, 2 and 3 [15]. Samples of tumour tissue (PC) and normal adjacent tissue (PN) were taken from the same organ so that matched pairs were used for tissue investigation and for growing primary cell cultures (further referred to as PCC and PCN). Biopsies performed for the confirmation of prostate cancer were performed at least 6 weeks prior to radical prostatectomy. There were no clinical signs of prostatitis when the prostatectomy was carried out. Thus, there was no evidence that a preoperative biopsy affected the results in the normal and malignant parts of the prostate. Samples of benign prostatic hyperplasia (BPH) were obtained from 15 patients, of which 9 (mean age 71 years) were treated by adenomectomy (Ad) and 6 (mean age 69 years) by transurethral resection of the prostate (TURP). Six tissue samples were obtained from patients (mean age 60 years) undergoing cystoprostatectomy for bladder cancer (Cy). Small pieces of tissue were dissected immediately after removal of the prostate. The cut edges within the prostate were inked so that the dissected pieces could be easily assigned to the adjacent prostate tissue examined histopathologically [16]. Histological analysis of all tissue pieces used was carefully performed by a clinical pathologist to ensure that the material used for tissue investigation and culture was either malignant or non-malignant tissue. Specimens were frozen and stored in liquid nitrogen until preparation or were immediately used for growing cell cultures.

The cultures of human prostatic epithelial cells were established either from the cancerous or from the non-cancerous parts of the same prostate that had been surgically removed by radical prostatectomy. The protocol for cell culture corresponded to an approach previously described [17, 18]. A cell suspension was prepared by finely chopping the prostatic portions with a scalpel and incubating 100 mg of tissue in 2 ml PPCC medium together with 2 mg collagenase and 2 mg hyaluronidase on a rotator at 37°C for 16 h. The suspension was forced through a 100 μm Nylon sieve and washed with the same volume of PPCC medium. The combined filtrate fraction was centrifuged at 270 ***g*** for 5 min. The pellet was resuspended in 6 ml PPCC medium and again centrifuged. The pellet thereof was resuspended in 4 ml PPCC medium, supplemented with 50 μg/ml ECL attachment matrix solution and maintained in 25 cm^2 Falcon-Primaria culture flasks. The cells were incubated in a humidified 5% CO_2/95% air mixture at 37°C and were fed fresh PPCC medium without ECL attachment matrix solution twice weekly until the cultures became confluent. The culture medium was removed and the monolayers were washed twice with 2 ml of PBS solution at room temperature and detached using trypsin/EDTA solution (0.05%, 0.02%) under microscopic control within 2–5 min. The cells were subcultured at a split ratio of 1:2 or 1:3. These conditions led to epithelial cells with the previously described characteristics [16]. The collagenase digestion of tissue pieces and the use of the special growth medium KSFM are useful methods to prevent fibroblasts or other non-epithelial cells from growing [16]. Immunohistochemical evaluation of cytokeratin expression and prostate-specific antigen were performed to confirm the epithelial nature and the prostatic origin of the cells. For this study, cells were used at third passages. Both malignant and non-malignant prostatic tissue samples were always investigated in pairs (see Results).

PC 3, DU 145 and LNCaP cells obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) [19–21] were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin and streptomycin as mentioned above. The PC 3 and DU 145 cells were cultured in plastic culture flasks from Costar, the LNCaP cells in Falcon-Primaria culture flasks.

Preparation of tissue homogenates and cell lysates

Tissue samples (~30–50 mg) were thawed, cut into small pieces and homogenised in 10 mM sodium phosphate buffer, pH 7.46, containing 0.25% Triton X-100 and 10 mM $CaCl_2$ with a Wheaton Homogenizer. These homogenates were centrifuged (Eppendorf Gerätebau, Hamburg, Germany) at 23 100 ***g*** for 15 min. After repeating this procedure both supernatants were amalgamated and used for measuring enzyme activity. All operations were carried out at 4°C. Adding the corresponding cathepsin values measured in the supernatants after a 4-fold extraction and considering these data as 100%, that two-step extraction procedure resulted in a recovery rate between 94 and 98% for the cathepsins studied. In cell culture experiments, the culture medium was removed, the monolayers were washed twice with DPBS solution and the cells were detached as described above. Then, the cells were pelleted by centrifugation and homogenised as described previously. The supernatants were either immediately analysed after preparation or stored at − 80°C for no longer than 1 week until analysis.

Enzyme measurements and other assays

CB, CH and CL were measured according to the fluorimetric methods of Barrett and Kirschke [22]. Assays were performed using a thermomixer Eppendorf 5435 (Eppendorf Gerätebau) at 30°C by adding 75 µl assay buffer to 150 µl of diluted sample into 1.5 ml Eppendorf tubes. Final concentrations were 100 mM phosphate buffer, pH 6.0, 1 mM disodium EDTA, 2 mM cysteine base for CB, 100 mM phosphate buffer, pH 6.8, 1 mM disodium EDTA, 10 mM cysteine base for CH and 100 mM acetate buffer, pH 5.5, 1 mM disodium EDTA, 2 mM dithiothreitol for CL. Samples were allowed to incubate for approximately 2 min before adding 75 µl of substrate solutions followed by mixing. Substrate stock solutions for CB (Z-Arg-Arg-AMC), CH (Arg-N Mec) and CL (Z-Phe-Arg-AMC) were dissolved in dimethylsulphoxide to 1 mM and stored at − 20°C. For use, the substrate stock solutions were freshly diluted to 20 µM with water (final substrate concentration in the reaction mixture 5 µM). The reaction was stopped after exactly 30 min by adding 300 µl of 100 mM chloroacetate buffer, pH 4.3. Control assays were performed for each enzyme sample assay. For that purpose, CPI E-64 [23] was added in a final concentration of 5 µM prior to the addition of the sample and the reaction mixture was then similarly treated as described above.

Fluorescence was measured in a spectrofluorophotometer (Shimadzu RF-1501, Kyoto, Japan) connected to a PC, excitation wavelength 370 nm and emission wavelength 460 nm. The fluorometer was zeroed against water and readings were standardised with 0.5 µM amino-methylcoumarine. Differences between sample assays and E-64 controls represented CB, respectively CH activities [24]. CL was measured in the presence and absence of the selective CL inhibitor Z-Phe-Phe-CHN_2 (0.5 µM final concentration) [24, 25]. All enzyme activities were expressed in units (1 U = 1 µmol substrate turnover per minute). Precision controls were performed with control materials prepared from rat liver homogenate. Intraserial and interserial precisions for CB, CH and CL were between 1.8 and 4.3%, and 5.3% and 9.2%.

The determination of CPI dissociation from complexes with proteases is necessary and was achieved by heat activation of 100 µl of sample at 100°C for 10 min [24]. Samples were then centrifuged at 19 000 ***g*** for 10 min. The supernatants were used to determine CPI activity. Inhibition was tested against purified CB from human placenta (Sigma). We prepared the samples before performing the assay procedure as described above. The 50 µl sample was added to 20 µl of CB assay buffer before adding 4 µl of CB stock solution. Following mixing, prepared samples were left for 30 min at room temperature before adding 396 µl of 0.1% Brij 35 solution. The following assay is identical to the one for CB except that the reaction was stopped after 60 min. CB was titrated with different concentrations of E-64 to determine the active concentration of CB. The amount of CPI was calculated from the standard curve. CPI was expressed in inhibitory units (1IU = inhibition of 1 U of CB activity).

Protein concentration was measured using the Coomassie Brilliant Blue method with bovine serum albumin as the standard [26].

Statistical analyses

Statistical calculations (Student's *t*-test of paired and unpaired data; variance analysis (ANOVA); correlation coefficient according to Spearman, r_s) were analysed using the statistical software packages Statgraphics, version 5.1 (Statistical Graphics Corp., Rockville, Maryland, U.S.A.) and GraphPad Prism (GraphPad Software Inc., San Diego, California, U.S.A.). Statistical differences of at least $P < 0.05$ were considered statistically significant.

RESULTS

Enzyme activities were related to the total protein content in the samples as well as to wet weight (tissue samples) and the number of cells (cell lines). Since we found a close correlation between the enzyme activities related to total protein and the other two reference bases ($r_s = 0.845$ and $r_s = 0.792$), we performed evaluation per g protein.

Figure 1 shows CB, CH, CL and CPI values for tissue samples. The results of PN showed significantly higher activity of CB, CL and CPI compared with adjacent PC. Comparing the other three groups, samples obtained by Ad contained the highest amount of cathepsins and CPI, followed by samples obtained from Cy, whereas TURP samples generally had the lowest activity of all. No differences in CH levels were found between all tissue specimens investigated (Figure 1b). Significantly reduced CPI levels were found in malignant prostate tissue specimens compared with the non-malignant prostatic tissue samples. The enzyme levels found in primary (PCN, PCC) and established (LNCaP, DU 145, PC 3) cell lines are shown in Figure 2. Primary cultures of prostatic epithelial cells obtained from malignant samples (PCC) of five different patients showed significantly higher activity levels of CB, CH and CL than cultures from non-cancerous prostatic tissue samples (PCN). The mean fold increase amounted to approximately 2.1, 0.35 and 1.2, respectively. For CPI this was not statistically significant (Figure 2d). There was no correlation between the cathepsin levels and the stage and grade of the tumour (data not shown).

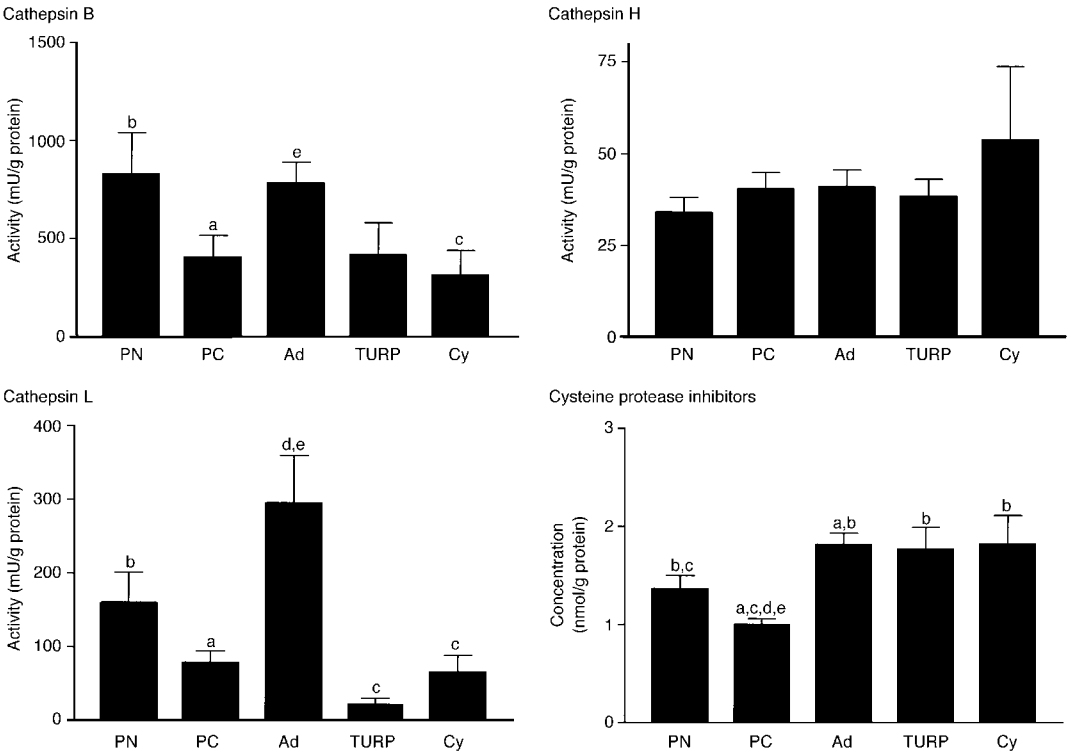

Figure 1. Cathepsins B, H, L and cysteine protease inhibitors (CPI) in tissue samples from the non-cancerous (PN) and cancerous (PC) part of the same prostate, obtained after radical prostatectomy ($n = 10$), adenomectomy (Ad; $n = 9$), transurethral resection of the prostate (TURP; $n = 6$) and cystoprostatectomy (Cy; $n = 6$). Values are given as arithmetic means ± standard error of the mean (SEM). Analyses of variance (ANOVA) showed significant differences between the groups studied for cathepsins B, L and CPI. Letters above each column indicate significant differences (according to Student's *t*-test for paired and unpaired data) between the group and PN, a; PC, b; Ad, c; TURP, d; and Cy, e.

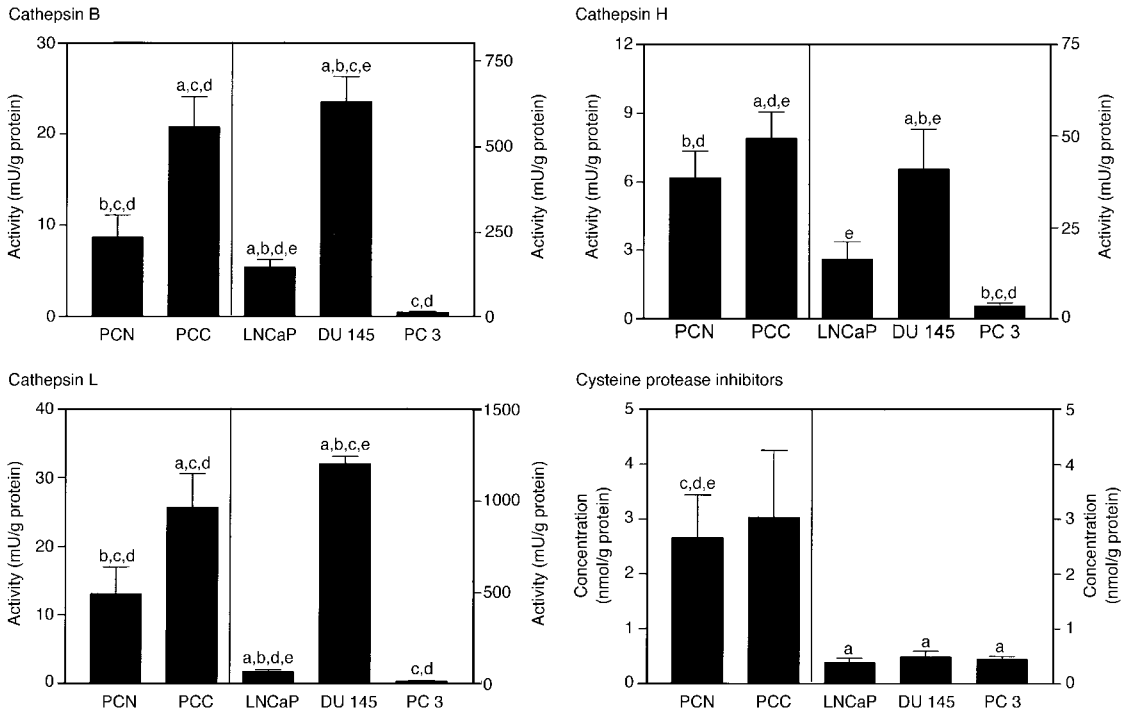

Figure 2. Cathepsins B, H, L and cysteine protease inhibitors (CPI) in human prostatic epithelial cells established from the non-cancerous (PCN) and cancerous (PCC) part of the same prostate (obtained from 5 patients) and in the three established cell lines LNCaP, DU 145 and PC 3 (five samples each). Values are given as arithmetic means ± standard error of the mean (SEM). Analyses of variance (ANOVA) showed significant differences between the groups studied for cathepsins B, H, L and CPI. Letters above each column indicate significant differences (according to Student's *t*-test for paired and unpaired data) between the group and PCN, a; PCC, b; LNCaP, c; DU 145, d; and PC 3, e.

When the ratios of CB, CH or CL to CPI were calculated, significantly higher values were found in malignant cell cultures than in the corresponding normal cell culture (7.89 ± 3.99 versus 5.29 ± 4.5 for CB to CPI; 4.29 ± 3.02 versus 3.76 ± 3.03 for CH to CPI; 14.8 ± 10.5 versus 8.53 ± 9.07 mU/nIU for CL to CPI and all values together: 8.95 ± 7.68 versus 5.86 ± 6.02, respectively). This imbalance was mainly caused by increased cathepsin levels since the CPI values, as shown above, were similar in cells from malignant and non-malignant tissue.

LNCaP, DU 145 and PC 3 cell lines showed significant differences to each other in cathepsin activity levels but not in CPI concentration (Figure 2). DU 145 cells contained the highest amount of each cathepsin, followed by LNCaP and PC 3.

DISCUSSION

CB, CH, CL and their endogenous inhibitors (CPIs) are believed to play an important role in the processes of tumour invasion and metastasis [27]. It has been demonstrated that cathepsins are able to degrade structures of the extracellular matrix and the basement membrane. Release of proteolytic enzymes from tumour cells can contribute to the detachment of cells from the primary tumour and lead to metastatic spread by degrading components of the venule wall. However, our results of CB, CH and CL measured in various benign and malignant prostatic tissue samples are not uniform and, moreover, they are partly in contrast to the data obtained from primary tissue cultures. Thus, our data have to be discussed with regard to three points: the acquisition of the prostatic tissue samples, the malignant and non-malignant source of the specimens and the differences observed between tissue samples, primary tissue cultures and cell lines DU 145, PC 3 and LNCaP.

We found different cathepsin levels in the prostatic tissue samples, dependent on the procedure of tissue acquisition. For example, CL (Figure 1c) was significantly lower in tissue material obtained from TURP than in that from Ad, although a similar pattern might be expected. Enzyme activities are probably damaged in tissue specimens by heat when the cauterising knife is used for the TURP, but differently for the various cathepsins. In addition, differences in CB and CL found in prostatic tissue from Ad as compared with that from Cy indicate that samples from BPH had higher values than those from normal prostatic specimens. These changes have not been described until now, but the findings support the view that proteases are obviously involved in processes that commonly occur with aging in the prostate [28]. Consequently, when comparing enzyme activities in cancerous and noncancerous tissue samples, it is necessary to determine enzyme activities in matched pairs of cancerous and adjacent, normal tissue specimens. We found higher CB, CL and CPI values in normal than in matched cancerous samples whereas CH was unchanged. These results are partly striking since elevated levels of various cathepsins have been generally reported in tumour tissues compared with normal tissues for gastric [29], colorectal [7, 30], lung [8], head and neck [9, 31], breast [6, 24], thyroid [32] and melanoma cancer [33]. However, Kos and colleagues [31] observed a lower concentration of CH in tumour tissue and Kirschke and associates [34] found CB, CH and CL levels in kidney tumour tissue to be lower than in normal samples.

All three cathepsins CB, CH and CL together with CPIs have been measured in only a few studies and the results are contradictory. Lah and colleagues [24] reported lower CPI activity in two-thirds of their 50 tumour samples compared with normal breast tissue. Sheahan and associates [30] found equal levels of CPIs in matched pairs of colorectal tissue samples.

It was striking that, in contrast to the behaviour of the cathepsins in the tissue specimens, all three cathepsins were significantly elevated in primary tissue cultures grown from cancerous tissue samples compared with the matched noncancerous tissue cultures. However, the apparent discrepancy between tissue specimens and tissue cultures can be explained in view of the histology of the prostate. The prostate consists of the three major histological components: the stroma, epithelium and luminal space. The largest component is the stroma. Bartsch and colleagues [35] found a 5:1 ratio between stromal and epithelial components and Marks and associates [36] a ratio of 3.9:1 with a high range between 1.6:1 and 7.6:1. The ratio increases in the case of BPH because the epithelial component decreases and the stromal component increases. Consequently, cathepsin activities in tissue samples are obviously determined by these components and their changes. It is possible that the change of the total cathepsin activities determined in prostatic tissue is more influenced by the changed proportion of stromal cells, such as smooth muscle cells and fibroblasts, than by the changed cathepsin activities observed in prostatic epithelial cells. These stromal cells are known to express cathepsins [37]. However, there are no quantitative cathepsin data available for these stromal components in the prostate.

By raising primary cell cultures from tissue samples, the results for enzyme activities characteristic of cancerous and noncancerous cells seem to be more precise and valid. Similar conclusions were made by other authors and short-term cultures were recommended for studies such as these [38]. Other alternatives such as microdissection by scraping or membrane-mounted native tissue, *in situ* polymerase chain reaction (PCR), antibody based tumour cell selection and flow cytometry are techniques to procure selected cell populations for demonstrating tumour-specific changes [39]. Recently, the pros and cons for each method with regard to the contamination by other cells, speed, cost, accuracy and reproducibility have been compared [39]. It was concluded that a final decision on the optimal method is not possible at this stage and the method selected rather depends on purpose and field of application. However, short-term cultures make it possible to study tumour-specific alterations (e.g. different gene expressions) under the influence of other components (cytokines; contribution of other cells by co-culture experiments) and offer, therefore, the advantage of 'dynamic' experiments.

Since we carefully characterised the tissue specimens, we excluded the problem of heterogeneity of the prostate cancer [16, 18], thus ensuring that we were investigating pure primary cultures from cancerous and corresponding noncancerous tissue samples. All three cathepsins were elevated in cells from the cancerous part of the prostate, whereas the CPI concentrations were not different (Figure 2). However, the ratios of cathepsins to the CPI were similarly increased. These data show a changed proteolytic balance in cancerous cells. It is assumed that the balance between proteases and their inhibitors has more importance for the invasive and metastatic properties of the cells than the concentrations of the cathepsins or other proteases and their endogenous

inhibitors alone [1]. We found a similar imbalance in the case of metalloproteinases in relation to their corresponding tissue inhibitors in prostate cancer [40]. The present data of cathepsins support our view that cancerous prostatic cells are characterised by an imbalance of proteolytic activities. Whether the levels of the cathepsin activities are able to indicate the invasive and/or metastatic properties of these cells remains to be shown in future studies.

The distribution pattern of CB, CH and CL has not been previously systematically studied in the three cell lines DU 145, PC 3 and LNCaP. Therefore, our results are not directly comparable with other data. Enzyme levels in these cell lines clearly differ from concentrations found in primary cell cultures. However, a similar distribution for each cathepsin was observed in all three cell lines. DU 145 contained the highest activity followed by LNCaP and PC 3. Weiss and colleagues [14] studied CB expression in DU 145 and LNCaP cells, and observed that DU 145 expressed an increased amount of CB compared with LNCaP, which our findings confirmed. LNCaP cells are androgen-responsive, PC 3 and DU 145 cells are androgen-non-responsive. Androgen-non-responsive cells occur under the environmental pressure of androgen-withdrawal therapy and are considered indicative of an advanced stage of the prostate cancer. Thus, these cell lines are often used to show differences between androgen-responsive and non-responsive properties of the prostate cancer. For example, the androgen-non-responsive PC 3 cells show a strong invasive potential compared with the androgen-responsive LNCaP cells [41]. However, our comparative data between the three cell lines and the primary cell cultures show no relationship between enzymatic activity and androgen responsiveness. It must be taken into account that the changed cathepsin values in these cell lines could result from the influence of specific cell culture conditions and are, therefore, not related to the original characteristics in primary cells. In our opinion these cell lines are, therefore, not suitable for studies on the relationship between proteases and the development of prostate carcinoma.

In summary, changed cathepsin levels and ratios of cathepsins to CPI in primary cell cultures from cancerous versus non-cancerous parts of the prostate may be indicative of a deregulated proteolytic balance in prostatic cancer cells. In this respect, primary cell culture experiments should be preferred to determinations in tissue samples and cell lines DU 145, PC 3 and LNCaP. We consider these data as the basis for further studies in this field.

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