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Cysteine and aspartic proteases cathepsins B and D determine the invasiveness of MCF10A neoT cells

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Background. *Lysosomal cathepsins B and D have been reported to play a role in various processes leading to progression of malignant disease. In ras-transformed MCF10A neoT cells both enzymes show similar vesicular distribution in perinuclear and peripheral cytoplasmic regions.*

Results. *The co-localization of cathepsins B and D in some vesicles as defined by confocal microscopy supports their co-ordinate activity in the proteolytic cascade. On the other hand, we showed that stefin A, an endogenous intracellular inhibitor of cysteine proteases, did not co-localize with cathepsin B and is presumably not involved in regulation of its enzymatic activity within the vesicles. Intracellular localization of both enzymes was confined to similar vesicles as the fluorescent degradation products of DQ-collagen IV either in individual cells or cell spheroids. The capability of these two enzymes to degrade collagen and other components of extracellular matrix is further supported by the results of Matrigel invasion assay. We showed that specific intracellular (CA-074 Me) and extracellular (CA-074) inhibitors of cathepsin B and pepstatin A, an inhibitor of cathepsin D, significantly reduced invasion of MCF10A neoT cells. Our results also show that in contrast to some other studies the activation peptide of pro-cathepsin D exhibited no mitogenic effect on MCF10A neoT, MCF-7 or HEK-293 cells.*

Conclusion. *We conclude that lysosomal cysteine proteases cathepsins B and D predominantly participate in degradation of extracellular matrix and facilitate invasion of tumour cells.*

Key words: tumor cells, cultured; cathepsin B; cathepsin D; neoplasms invasiveness

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Introduction

Dissemination of malignant cells of primary tumours to distant tissues and the formation of secondary tumours is the primary cause of treatment failure and death of cancer patients. Besides serine and metallo proteases, lysosomal cysteine proteases cathepsins B and L and aspartic protease cathepsin D have

been shown to participate in processes of tumour progression, including tumour cell growth, invasion and metastasis.[1-3] Alterations in expression, processing and subcellular distribution of these three cathepsins have been linked with more aggressive tumour behaviour, increased risk of relapse and shorter survival of cancer patients.[1, 4]

In numerous studies it has been reported that proteolytic activity of cysteine proteases is involved in degradation and remodelling of extracellular matrix (ECM), a crucial step in tumour cell invasion.[5] Degradation of ECM in living tumour cells was localized either extracellularly or intracellularly, following endocytosis of partially degraded ECM proteins. Recently, we have demonstrated that tumour cells can use both proteolytic pathways simultaneously.[6] Cathepsin B has been suggested to facilitate ECM degradation and subsequent tumour cell invasion directly by intra- or extracellular degradation of ECM components or indirectly, by activation of other enzymes of the proteolytic cascade mediating this process.[7]

In contrast to cathepsins B and L, which are believed to be mainly involved in the invasion of tumour cells, the importance of cathepsin D in cancer progression is less clear. In breast cancer catalytically inactive cathepsin D pro-peptide was proposed to act as a mitogen, promoting tumour cell proliferation.[8] Additionally, we showed that active enzyme can promote tumour invasion *in vitro*.[9] This is in line with clinical studies, revealing the correlation of the mature form of cathepsin D with poor prognosis of cancer patients.[10]

In this study, cathepsins B and D have been simultaneously evaluated in MCF10A neoT cells with regard to their impact on cell proliferation, degradation of ECM and tumour cell invasion. Additionally, the localization of these two cathepsins and stefin A was determined in this cell line by immunofluorescence labeling and confocal microscopy.

Materials and methods

Cell cultures

Ras-transformed human breast epithelial cell line MCF-10A neoT was obtained from Prof. B. F. Sloane (Wayne State University, Detroit, USA). Human cell lines HEK-293 and MCF-7 were obtained from the American Tissue Culture Collection. MCF-10A neoT cells originate from MCF-10, a human breast epithelial cell line derived from a patient with fibrocystic breast disease that underwent spontaneous immortalization in culture and grows attached as MCF-10A cell line. Co-transfection of MCF-10A cells with plasmid containing the neomycin resistance gene and human T24 mutated c-Ha-ras oncogene using the calcium phosphate method resulted in MCF-10A neoT cells.[11,12] MCF-10A neoT cells were cultured as monolayers in DMEM/F12 medium supplemented with Hepes, 5% FCS, antibiotics and growth factors. Human breast cancer cell line MCF-7 and transformed human embryonic kidney cell line HEK-293 were cultured in MEM supplemented with 2 mM L-glutamine, Earle's BSS and adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 % FCS. Cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Cysteine protease inhibitors

The natural reversible tight-binding protein inhibitor chicken cystatin[13] and synthetic irreversible epoxysuccinyl inhibitor E-64 (Sigma, St. Louis, USA) were used as general cysteine proteinase inhibitors. Cathepsin B was inhibited with selective membrane impermeable epoxide derivative CA-074 (Bachem, Bubendorf, Switzerland) and membrane-permeable pro-inhibitor CA-074Me (Sigma, St. Louis, USA). Cathepsin L was inhibited by CLIK-148[9] (a gift from Prof. Nobuhiko Katunuma, Tokushima Bunri University, Japan) and cathepsin D by pepstatin A (Sigma, St. Louis, USA).

Cell viability and proliferation assays

Cytotoxic and/or proliferative effects of cysteine protease inhibitors on MCF-10A neoT cells were tested as described.[14] Cells were added to a final concentration of 5 x 10^4 cells/200 μl per well of a 96 well microtiter plate. Appropriate concentrations of inhibitors or control media were added. Plates were incubated for 24 hours at 37°C and 5% CO_2. The medium was carefully removed, 200 μl of 0.5 mg/ml MTT (3-4,5-dimethylthiazol-2,5 diphenyl tetrazolium bromide, Sigma, St. Louis, USA) was added and incubated for three hours at 37°C and 5% CO_2. The medium was carefully removed and formazan crystals dissolved in 200 μl/well of isopropanol. Absorbance was measured on an ELISA microplate reader at 570 nm, reference filter 690 nm. All tests were performed in quadruplicate.

Cell invasion assays

Effects of protease inhibitors on invasion were tested using the modified method described by Holst-Hansen et al.15 Transwells (Costar Corning, New York, USA) with 12 mm polycarbonate filters, 12 μm pore size, were used. 25 μl of 100 μg/ml fibronectin were applied on the lower side of the filters, which were left for one hour in a laminar hood to dry. The upper side of the filters was coated with 100 μl of 1 mg/ml Matrigel (Becton Dickinson, San Diego, USA) and 100 μl of DMEM/F12 was added. The Matrigel was dried overnight at room temperature in a laminar hood and reconstituted with 200 μl of medium for one hour at 37°C. The upper compartments were filled with 0.5 ml of cell suspension, final concentration 4 x 10^5 cells/ml, containing the appropriate concentration of the inhibitor. The lower compartments were filled with 1.5 ml of medium containing the same concentration of the inhibitor. The plates were incubated for 24 hours at 37°C and 5% CO_2. MTT was added to a final concentration of 0.5 mg/ml to the upper and lower compartments and plates were incubated for an additional 3 hours. Media from both compartments were transferred separately to Eppendorf tubes, and centrifuged at 6200 rpm for 5 minutes. Supernatants were discarded and the formazan crystals which remained dissolved in 1 ml of isopropanol. The colour intensity of the dissolved formazan crystals was measured as described above. As controls, cells were incubated with medium containing the appropriate volumes of distilled water, ethyl acetate and DMSO, the solvents used for preparation of concentrated solutions of the inhibitors and monoclonal antibody. Invasion was recorded as the percentage of cells that penetrated the Matrigel coated filters compared to controls, and was calculated as OD_{lower} / OD_{lower}+OD_{upper} x 100. All tests were performed in triplicate.

Mitogenic effect of procathepsin D activation peptide

18 amino acids long cathepsin D activation peptide[8] (IAKGPVSKYSQAVPAVTE) as a part of cathepsin D pro-peptide, was synthesised by NeoSystems (Calgary, Canada) at 95% purity. The mitogenic effect of the peptide was tested as described for cytotoxicity and proliferation assays, with minor modifications. Cells were added to a final concentration of 5 x 10^3 cells/200 μl per well of a 96 well microtiter plate. The activation peptide was added in a range of 0.001-10 μM concentrations. Plates were incubated for the period of 1-5 days at 37°C and 5% CO_2 and analysed as described above by MTT assay.

Immunocytochemistry

Cathepsins B and D, and stefin A were localized in MCF-10A neoT cells fixed in ice-cold methanol and permeabilized in 0.1% saponin in PBS, pH 7.4. Non-specific staining was blocked with 0.2% BSA in PBS, pH 7.4. Primary antibodies used were rabbit anti-human cathepsin B polyclonal antibody, mouse

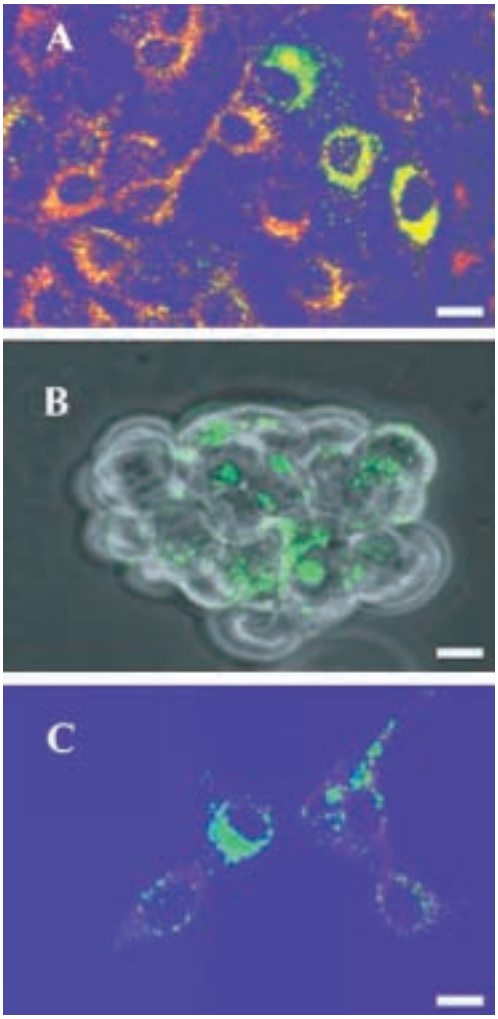

Figure 1. Immunolocalization studies in MCF-10A neoT cells. (A) Co-localization (yellow) of cathepsin B (red fluorescence) and cathepsin D (green fluorescence). (B) MCF-10A neoT spheroids of living cells. Green fluorescent degradation products are predominantly localised within the cells. (C) Localization of cathepsin B (green fluorescence) and stefin A (red fluorescence). Bars, 20 ?M.

anti-human cathepsin D D101 monoclonal antibody and mouse anti-human stefin A C5/2 monoclonal antibody (all KRKA d.d., Novo mesto, Slovenia). Antibodies recognize precursors, mature forms and enzyme/inhibitor complexes of all three antigens. Secondary antibodies were goat anti-rabbit labelled with Alexa Fluor 488 and goat anti-mouse labelled with Alexa Fluor 546 (Molecular Probes, Eugene, USA). Controls were run in the absence of primary antibodies, but in the presence of secondary antibody and pre-immune goat serum. Pro Long Antifade Kit was used for mounting coverslips on glass slides. A Zeiss LSM 510 confocal microscope was used to observe the cells.

Proteolysis assays

Pre-cooled glass coverslips were coated with 25 μg/ml of the quenched fluorescent substrate DQ-collagen IV (Molecular Probes, Eugene, USA) suspended in Matrigel, 10 mg/ml, for 10 minutes at 4 °C. Cells were plated and grown for a period of 72 hours. A Zeiss LSM 310 confocal microscope with 40 X water immersion objective was used to observe the cells or cell spheroids for fluorescent degradation products of DQ-collagen IV.

Results

Immunolocalization

Cathepsins B and D showed similar vesicular distribution in MCF-10A neoT cells, but the level of their expression varied between cells. They were localized both in the perinuclear and in the peripheral cytoplasmic region (Figure 1A) and both enzymes co-localized in some of the vesicles. Stefin A, an endogenous intracellular inhibitor of cysteine proteases, localized evenly in the cell cytoplasm, but no co-localization with cathepsin B was observed (Figure 1C).

Degradation of DQ-collagen IV by living MCF-10A neoT spheroids

The ability of MCF-10A neoT cells to degrade extracellular matrix was analysed by the novel confocal assay desribed by Sameni et al.[16] using quenched fluorescent substrate DQ-collagen IV. MCF-10A neoT cells plated on a DQ-collagen IV/Matrigel matrix formed sphe-

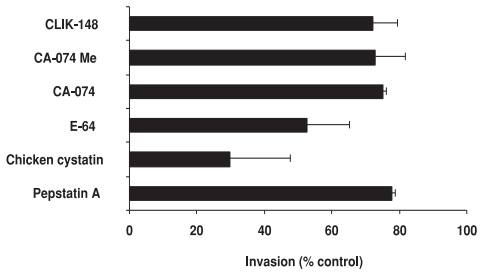

Figure 2. Effect of cysteine and aspartic protease inhibitors on Matrigel invasion by MCF-10A neoT cells. Concentration of chicken cystatin was 2 mM, all other inhibitors were used at 10 mM concentration. Data are represented as mean (S.D. of two independent determinations performed in triplicate.

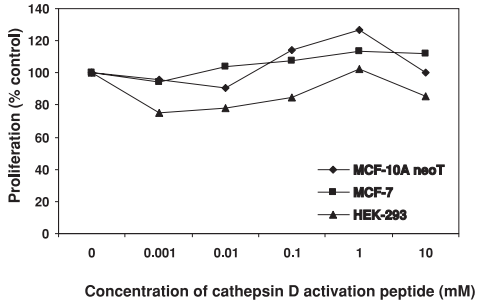

Figure 3. Effect of pro-cathepsin D activation peptide IAKGPVSKYSQAVPAVTE on the growth of cancer cell lines. After five days incubation the number of cells was determined by MTT method and compared to controls

roids after 72 hours. Fluorescent degradation products seen as green fluorescence were observed inside the cells forming the body of the spheroid (Figure 1B). They were localised in vesicular structures as observed in individual cells.[6] Individual spots of degradation were observed also in the pericellular regions.

Effect of protease inhibitors on the viability and proliferation of MCF-10A neoT cells

Prior to the invasion assays, the selected protease inhibitors were tested for their possible cytotoxic and proliferative effects. No effect on cell viability and/or proliferation was observed within the same concentration range as used in invasion assays (data not shown).

Effect of protease inhibitors on Matrigel invasion of MCF-10A neoT cells

The roles of cathepsins B and D in the invasion of MCF-10A neoT cells were determined with selected natural and synthetic inhibitors of cysteine and aspartic proteases. All inhibitors decreased cell invasion after 24 h (Figure 2). Inhibition of aspartic protease cathepsin D with 10 μM pepstatin A resulted in 22.1 ± 0.8 % decrease in invasion of MCF-10A neoT cells. The effect of pepstatin A was much lower compared to general cysteine protease inhibitors chicken cystatin (70.2 ± 17.8 % at 2 μM) and E-64 (47.5 ± 12.7 % at 10 μM). To assess the contribution of cathepsin B, CA-074, a specific inhibitor of extracellular cathepsin B and membrane-permeable analogue CA-074 Me were used. CA-074 and CA-074 Me reduced invasion by 24.9 ± 1.2 % and 27.0 ± 8.7 % at 10 μM, respectively. For comparison, 10 μM concentration of CLIK-148, a specific synthetic inhibitor of cathepsin L resulted in 27.7 ± 7.1 % decrease in cell invasion.

Effect of cathepsin D activation peptide on tumour cell proliferation

To investigate the mitogenic effect of the activation peptide of pro-cathepsin D, a corresponding synthetic peptide was used in MTT proliferation assay. In contrast to results published by Vetvicka *et al.*[8] and Fusek and Vetvicka[17], no significant mitogenic effect was observed on MCF-10A neoT and MCF-7 cell proliferation within the comparable concenration range after five days (Figure 3). Additionally, no proliferative activity was observed in human embryonic kidney cells HEK-293 used as negative control.

Discussion

Metastatic process depends on the ability of tumour cells to invade through ECM. This process is facilitated by proteolytic degradation of various ECM proteins, including different types of collagens, laminin and fibronectin by proteases. The common believe is, that this process takes place extracellularly at the invasive front of tumour cells. New techniques, like confocal laser scaning microscopy enable analysis of proteolytic degradation of quenched fluorescent protein substrates, like DQ-collagen IV, in living cells and the results show that cells differ in their sites of matrix remodeling, located either extracellularly or intracellularly.[5,16] We found that invasive MCF-10A neoT cells accumulate fluorescent products of digested DQ-collagen IV inside the cells, in vesicle-like structures.[6] This result was confirmed in this study by using 3-dimensional spheroid cultures of MCF-10A neoT cells. Again, positive vesicular staining was found in most of the cells forming the body of the spheroid. In addition, individual spots of pericellular fluorescence observed both in individual cells and in spheroid cultures suggest, that MCF-10A neoT cells can simultaneously use both pathways of ECM degradation.

Several studies have implicated cysteine proteases in invasion and tumour progression. Therefore, we tested the impact of general and specific cysteine protease inhibitors on invasiveness of MCF10A neoT cells in the concentration range not affecting cell viability and proliferation. Both general inhibitors, i.e. the reversible tight-binding protein inhibitor chicken cystatin[13] and the irreversible inhibitor E-64 were the most effective in Matrigel invasion assay, with 70.2 ± 17.8 % (2 μM concentration) and 47.5 ± 12.7 % (10 μM) inhibition of invasion, respectively. CA-074, an inhibitor of extracellular cathepsin B, decreased invasion by 24.9 ± 1.2 % at 10 μM, a result is comparable to the effect of intracellular cathepsin B inhibitor CA-074 Me (27.0 ± 8.7 %). We may expect that the inhibitor, capable to impair the activity of both, intracellular and extracellular fraction of cathepsin B would be even more effective to decrease tumour cell invasion. Further, we demonstrated that cathepsin L also participates in this process. However, none of the inhibitors completely blocked MCF-10A neoT cell invasion, suggesting the involvement of other, presumably serine and matrix metallo proteases in invasion process.

Our finding, that aspartic protease inhibitor pepstatin A also reduced Matrigel invasion of MCF-10A neoT cells, suggests that beside cysteine cathepsins B and L, active aspartic protease cathepsin D is implicated in invasion process as well. This is in contrast with reports of Johnson et al.[18] that the irreversible peptide inhibitor pepstatin A was ineffective in inhibiting MCF-7 tumour cell invasion *in vitro.* In our case treatment of MCF-10A neoT cells with 10 μM pepstatin A resulted in 22.1 ± 0.8 % decrease in invasion. Reduction of invasion was even higher when SQAPI-like natural protein inhibitor of aspartic proteases was used.[9]

Besides direct degradation of ECM, cathepsin D was suggested to act as an initiator protease upstream in a proteolytic cascade activating pro-cathepsin B7. Activated cathepsin B can further convert serine protease prourokinase type plasminogen activator (uPA) into active enzyme uPA, which, in turn, is able to activate plasmin and matrix metallo proteases. These enzymes can then actively degrade various ECM components. Our results support the involvement of cathepsin D in the proteolytic cascade. Cathepsin D was co-localized with cathepsin B in the same cytoplasmic vesicles in MCF10A neoT cells and is, therefore, able to activate procathepsin B. However, we have to be aware that antibody used for localization of cathepsin B recognises besides precursor also other forms of the enzyme[19] and that other assays and experiments, including specific fluorogenic sub-

strates are needed to determine the ratio between pro and active forms of cathepsin B and to assess the rate of its activation.

The effect of endogenous protease inhibitors on degradation of ECM in MCF10A neoT cells remains to be evaluated. Whereas no endogenous inhibitor of cathepsin D is known so far, the activity of cysteine proteases cathepsins B and L are regulated by their endogenous inhibitors, the cystatins.[1] In MCF10A neoT cells we localised an intracellular inhibitor stefin A, which was difusely distributed throughout cell cytoplasm. It was not co-localised with cathepsin B what indicates that at least inside the cells it is not important for regulation of cathepsin B dependent degradation of ECM. The results are similar to that of Calkins and Sloane[20] in hepatoma cells, reporting differetial intracellular distribution of cathepsins and stefins.

In conclusion, our results show that lysosomal cathepsins B and D, overexpressed in most of malignant tumours, predominantly participate in degradation of ECM and facilitate tumour cell invasion. Regulation of their enzymatic activities by exogenous inhibitors represents a new possibility for therapeutic intervention in cancer patients.

Aknowledgement

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