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Intracellular Proteolytic Activity of Cathepsin B Is Associated With Capillary-Like Tube Formation by Endothelial Cells In Vitro

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Abstract The lysosomal cysteine protease cathepsin B is implicated in degradation of extracellular matrix (ECM), a crucial step in a variety of physiological and pathological processes, including tumor dissemination and angiogenesis. In this study, we analyzed the contribution of extracellular and intracellular cathepsin B activity on the formation of capillary-like tubular structures by human umbilical vein endothelial cells (HUVECs) grown on Matrigel matrix, using general and specific cysteine protease inhibitors. We demonstrated, by confocal assay using quenched fluorescent protein substrate DQ-collagen IV, that endothelial cells degrade ECM both intracellularly and pericellularly. Intracellular cathepsin B activity detected by degradation of Z-Arg-Arg cresyl violet substrate was co-localized with the products of DQ-collagen IV degradation in the perinuclear region and in the capillary-like tubular structures. Treatment of cells with membrane-permeable CA-074 Me effectively abolished intracellular cathepsin B activity, and resulted in reduced tube length ($32.3 \pm 9.4\%$ at 10 μM), total tubule area ($49.6 \pm 12.4\%$ at 10 μM), and the number of branch points of tubules ($47.5 \pm 7.7\%$ at 10 μM) in a dose-dependent manner. In contrast, CA-074 (0.1–10 μM), a membrane-impermeable cathepsin B specific inhibitor, general cysteine protease inhibitors chicken cystatin (5 μM) and E-64 (10 μM), and the metalloprotease inhibitor Minocycline (10 μM) showed no significant inhibitory effect in our angiogenesis model. These results show that, besides multiple regulatory molecules, intracellular cathepsin B also contributes to the neovascularization process and should be considered as a potential therapeutic target. J. Cell. Biochem. 97: 1230–1240, 2006.

Key words: angiogenesis; cathepsin B; collagen IV; human umbilical vein endothelial cells; proteolysis

The formation of new blood vessels from pre-existing ones is referred to as angiogenesis. It is the predominant mechanism of blood vessel formation in the adult, in which activated endothelial cells of existing blood vessels degrade and remodel the underlying basement membrane and extracellular matrix (ECM), proliferate, migrate and invade the surrounding perivascular stroma, and form tube-like structures that give rise to new blood vessels [Liotta et al., 1991; Folkman, 2003]. Angiogenesis occurs under normal physiological conditions such as wound repair, the menstrual cycle and embryogenesis, and in pathological events, including diabetic retinopathy, rheumatoid arthritis, and cancer [Carmeliet, 2003]. The architecture of vessels in pathological tissue usually differs from that found in healthy tissue. In tumors, vessels are irregular, heterogeneous and leaky. The latter characteristic may provide the structural basis for selective impairment of tumor vessels by inhibitors of angiogenesis [Hashizume et al., 2000; Cao, 2004]. In the case of tumor induced angiogenesis, endothelial cells invade surrounding tissue in a process similar to that observed for tumor cells [Paku, 1998]. The vascular stage of tumors is recognized by exponential growth, invasion of surrounding tissue, and metastatic spread.

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A variety of proteases, including metallo-, serine, and cysteine proteases, have been implicated in angiogenesis [Menashi et al., 1993; Pepper, 2001]. Their proteolytic activity facilitates degradation and remodeling of ECM proteins, a process that not only destroys the matrix barrier, but also results in the release and/or formation of pro- and anti-angiogenic factors and exposure of cryptic protein sequences that play a functional role in angiogenesis [Ferreras et al., 2000; Xu et al., 2001]. A precise balance between enzyme activity and inhibition is necessary for normal capillary formation [Pepper and Montesano, 1990].

Lysosomal cysteine protease cathepsin B is one of the enzymes associated with angiogenesis. The proteolytic activity of cathepsin B facilitates direct degradation of various ECM proteins, including laminin, fibronectin, tenascin C, and type IV collagen [Buck et al., 1992; Mai et al., 2002]. The latter is a major component of ECM and the vascular basement membrane [Hudson et al., 1993]. The indirect role of cathepsin B involves the activation of other enzymes of the proteolytic cascade mediating ECM degradation, like metalloproteases and urokinase plasminogen activator (uPA) [Schmitt et al., 1997]. Cathepsin B exhibits broad substrate specificity at both acidic and neutral pH [Keppler and Sloane, 1996]; it is normally present in the lysosomes of various cell types, including endothelial cells [Kos and Lah, 1998]. In normal cells, the enzyme is localized predominantly in the perinuclear region, while in tumor and transformed cells, it is translocated from the perinuclear to the peripheral cytoplasmic and plasma membrane region [Sloane et al., 1994]. In addition, cathepsin B has been found in the extracellular environment of tumor cells, both in a soluble form and bound to the plasma membrane or ECM proteins [Linebaugh et al., 1999; Sinha et al., 2001]. Recent studies have shown that, in tumor cells, both extracellular and intracellular cathepsin B are involved in ECM degradation [Sameni et al., 2000; Premzl et al., 2003].

Strojnik et al. (1999) reported that the occurrence of cathepsin B localized in endothelial cells of brain tumors correlated with poor survival in patients with brain cancer and can therefore be used as a prognostic factor. Cathepsin B is involved in tumor angiogenesis in patients with gliomas [Mikkelsen et al., 1995] and, in glioma cell lines, angiogenesis is inhibited by inhibition of cathepsin B gene expression [Lakka et al., 2004; Yanamandra et al., 2004]. The proposed role of cathepsin B in angiogenesis has been, in addition to ECM degradation, the degradation of tissue inhibitors of metalloproteases, TIMP-1, and TIMP-2, leading to increased activity of MMPs and subsequently enhancing angiogenesis [Kostoulas et al., 1999].

The aim of the present study was to examine intracellular and extracellular cathepsin B for pro-angiogenic activity in the capillary-like tube formation assay, using human umbilical vein endothelial cells (HUVECs) and cysteine protease inhibitors. Additionally, we determined the enzyme's protein and activity levels and monitored intracellular proteolysis in living HUVECs.

MATERIALS AND METHODS

Cell Culture and Reagents

HUVECs were obtained from American Type Culture Collection. They were maintained in M-199 from Gibco (Eggenstein, Germany), supplemented with 10% fetal bovine serum purchased from Hyclone (Logan, UT), 15 ng/ml epidermal growth factor, 15 ng/ml basic fibroblast growth factor (bFGF), both obtained from Sigma (St. Louis, MO), 100 μg/ml penicillin, and 200 μg/ml streptomycin, both from Krka, d.d. (Novo mesto, Slovenia). In in vitro assays, cells were grown in the presence of fetal bovine serum depleted of cysteine protease inhibitors as described previously [Kos et al., 1992; Premzl et al., 2001].

Protease Inhibitors

Chicken cystatin, a reversible tight binding protein inhibitor [Kos et al., 1992], and the irreversible epoxysuccinyl inhibitor E-64 purchased from Sigma were used as general cysteine protease inhibitors. Cathepsin B was inhibited with selective membrane-impermeable epoxide derivative CA-074 from Bachem AG (Bubendorf, Switzerland) and its membrane-permeable analog CA-074 Me obtained from Peptide Institute, Inc. (Osaka, Japan). CA-074 Me is a pro-inhibitor, which, after internalization and conversion by non-specific esterases to CA-074, inactivates intracellular cathepsin B [Buttle et al., 1992]. Minocycline was purchased from Calbiochem (San Diego, CA), a tetracycline derivative, inhibits collagenase activity and

angiogenesis [Tamargo et al., 1991] and was used to inhibit MMP-3.

Immunocytochemistry

Cathepsin B was localized in HUVECs fixed in 4% paraformaldehyde and permeabilised by 0.01% Triton X-100 in 4% paraformaldehyde. Non-specific staining was blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4. Rabbit anti-human cathepsin B polyclonal antibody was obtained from Krka, d.d. and mouse anti-β-tubulin monoclonal antibody from Sigma were used as primary antibodies. Secondary antibodies were goat anti-rabbit labeled with Alexa Fluor 488 and goat anti-mouse labeled with Alexa Fluor 546, both purchased by Molecular Probes (Eugene, OR). Controls were run without primary antibodies but in the presence of secondary antibodies and pre-immune goat serum. Pro Long Antifade kit from Molecular Probes was used for mounting cover slips on glass slides. Fluorescence labeling was analyzed with a Zeiss LSM 510 confocal microscope. Alexa Fluor 488 and Alexa Fluor 546 were excited using argon (488 nm) and He/Ne (543 nm) lasers. The emission signal was filtered using LP 505–530 nm (green fluorescence) and LP 560 nm (red fluorescence) light filters.

Proteolysis Assays

To examine the proteolytic activity of HUVECs a pre-cooled cover glass of a LabTek chambered cover glass system from Nalge Nunc Int. (Naperville, IL) was coated with 25 μg/ml of quenched fluorescent substrate DQ-collagen IV from Molecular Probes suspended in Matrigel from Becton Dickinson (Bedford, MA) at 10 mg/ml, for 10 min at 4°C. Prior to seeding the cells, DQ-collagen IV/Matrigel matrix was allowed to polymerize for 1 h at 37°C. Cells (5,000/chamber) were plated and grown for the same period (24 h) as in angiogenesis assays. A Zeiss LSM 510 confocal microscope, objective 63×, numerical aperture 1.3, was used to observe the cells and capillary-like tubular structures for degradation products of DQ-collagen IV.

The activity of cathepsin B in living cells was localized by a specific fluorogenic substrate, Z-Arg-Arg cresyl violet, kindly provided by Prof. Dr. Cornelius Van Noorden, University of Amsterdam, Netherlands. Enzymatic cleavage of one or both arginine residues by cathepsin B converts the substrate molecule into a red fluorescent product [Van Noorden et al., 1998]. The substrate easily penetrates cell membrane, and thereby enables the localization of enzyme's activity in living cells with the use of fluorescent microscopy. For the experiments, cells were cultured in a LabTek chambered cover glass either pre-coated with Matrigel (10 mg/ml), DQ-collagen IV/Matrigel matrix or without coating for 24 h in the presence of cathepsin B specific inhibitors CA-074 Me, CA-074, and E-64 or vehicle only controls. After the incubation period, culture medium was removed and substituted with 10 μM solution of the Z-Arg-Arg cresyl violet in fresh serum-free culture medium. Cells were monitored for the red fluorescent product after 15 min using a 516–555 nm single band filter for excitation and a 574–648 nm barrier filter.

Cell Viability Assays

Cytotoxic effects of cysteine and metalloprotease inhibitors on HUVECs were tested using the MTT method as described previously [Holst-Hansen and Brünner, 1998]. Cells (5×10^5/well) were plated to a 96-well plate and appropriate concentrations of inhibitors or vehicle only control media were added. Plates were incubated for 24 h at 37°C and 5% CO_2. The medium was carefully removed, and 200 μl of 0.5 mg/ml MTT from Sigma added and incubated for an additional 3 h at 37°C with 5% CO_2. The medium was again removed and the formazan crystals dissolved in 200 μl/well of isopropanol. Absorbance was measured on an ELISA microplate reader at 570 nm, reference filter 690 nm. The viabilities, corrected for control values, were expressed as a percentage of the viability of the corresponding vehicle-only control. All tests were performed in quadruplicate.

Capillary-Like Tube Formation Assay

The effect of protease inhibitors on endothelial cell tube formation was assessed as follows. Two hundred microliter of Matrigel (10 mg/ml) was applied to pre-cooled 48-well plates, incubated for 10 min at 4°C and then allowed to polymerize for 1 h at 37°C. Cells were suspended in M-199 medium supplemented with 0.1 μM phorbol 12-myristate 13-acetate (PMA) from Sigma, 30 ng/ml bFGF, antibiotics and 10% fetal bovine serum (the latter being depleted of cysteine protease inhibitors by affinity chromatography on a CM papain-Sepharose column),

and containing the appropriate concentration of inhibitor. Five hundred microliter (1×10^5 cells/well) were added to each well. As controls, cells were incubated with vehicle-only control medium containing the appropriate volumes of distilled water, ethyl acetate, and dimethyl sulfoxide (DMSO), the solvents used for preparing concentrated solutions of the inhibitors. After 24 h incubation at 37°C and 5% CO_2, cells were viewed using a phase-contrast IMT-2 inverted research Olympus microscope. The length of capillary-like tubular structures was measured in five randomly chosen view fields in each well, using a microscope ruler. The effects of inhibitors were expressed as the length relative to that in each vehicle-only control. All tests were performed in triplicate and repeated twice.

Additionally, the effects of inhibitors on total tubule area and the degree of branching were assessed by image analysis. Tubules were visualized by staining with hematoxylin and eosin [Donovan et al., 2001]. Five randomly chosen view fields were photographed in each of triplicate wells both of treated samples and corresponding controls. Images were captured at 40× magnification using Olympus C3040 digital camera attached to a microscope, and saved as TIFF images. Images were analyzed using the free software program Scion Image that was available and downloaded from the web site of the Scion Corporation (http://www.scioncorp.com). Images were imported into Scion Image and converted to a binary format. The binary threshold function was adjusted to obtain the best contrast of tube-like structures with the background. Tubule area was calculated as the total number of pixels in thresholded images and the degree of branching was assessed by manually counting the branch points.

Measurement of Cathepsin B Activity

Inhibition of intracellular and extracellular cathepsin B was examined in HUVECs cultured in the presence of CA-074, CA-074 Me or E-64, under the same conditions as in tube formation assay. As controls, cells were incubated with vehicle-only control medium containing the appropriate volumes of ethyl acetate, DMSO, and distilled water, respectively. Media and cells were harvested and processed as described [Qian et al., 1989; Achkar et al., 1990]. Cathepsin B activity was determined fluorimetrically using Z-Arg-Arg-AMC (Bachem) after 30 min of pre-incubation with dithiothreitol and EDTA [Qian et al., 1989]. Latent cathepsin B in the medium was measured after activation by limited proteolysis with pepsin A obtained from Sigma as described by Qian et al. (1989). Values were normalized for cell protein and are the mean ± SE of two measurements.

Determination of Protein Concentration

Protein concentrations in samples were determined by the Bradford method using the Bio-Rad protein assay dye reagent from Bio-Rad Laboratories (Hercules, CA) and BSA from Sigma as a standard, according to the instructions of the manufacturer.

Quantification of Cathepsin B Protein by ELISA

HUVECs were grown in 75 cm^2 cell culture flasks in the serum-containing medium. After cells reached 70%–80% confluence, the medium was removed and cells grown in serum-free culture conditions for additional 24 h. At the end of this incubation period, the medium was collected, centrifuged 5 min at 27,000*g* at 4°C and the pellet discarded. The adherent cells were washed twice with PBS, pH 7.2, lysed with 2 ml of 400 mM phosphate buffer, pH 6.0, supplemented with 75 mM NaCl, 4 mM EDTA-Na_2, and 0.25% Triton X-100 and detached with a cell scraper. The lysate was stored overnight at −70°C. Cells were pelleted by centrifugation for 15 min at 14,500*g* at 4°C. The supernatant (cytosolic fraction) was collected and the pellet (membrane fraction) resuspended in lysis buffer, pH 6.0. Proteins were extracted with 10% trichloroacetic acid, lyophilized, resuspended in 100 μl of PBS, pH 6.0, and stored at −20°C for subsequent analysis. Intracellular and secreted cathepsin B was quantified by direct ELISA, using rabbit polyclonal anti-human cathepsin B antibodies obtained from Krka, d.d., 5 μg/ml, and secondary sheep anti-cathepsin B antibodies conjugated with horseradish peroxidase (1:5,500). The primary antibody used for ELISA recognizes pro-enzyme, the mature form of the enzyme, as well as enzyme-inhibitor complex [Kos et al., 1995]. Cathepsin B determined in ng/ml was standardized as ng/mg of total protein in samples.

Statistical Analysis

SPSS PC software, Release 6.0 (SPSS, Inc., Chicago, IL) was used for statistical analysis.

P values were calculated by Mann–Whitney test, based on comparisons of treated cells with appropriate control samples tested at the same time. P-value 0.05 or less was considered significant.

RESULTS

Effect of Protease Inhibitors on the Viability of HUVECs

The selected cysteine and metallo-protease inhibitors showed no significant effect on the viability of HUVECs within the same concentration range as used in the capillary-like tube formation assay (Fig. 1). Cell viability after 24 h incubation with general cysteine protease inhibitors E-64 (10 μM) and chicken cystatin (5 μM) was $101.7 \pm 0.5\%$ and $79.9 \pm 0.6\%$, respectively. The effects of cathepsin B specific inhibitors 10 μM CA-074 ($96.9 \pm 1.2\%$ viability) and its membrane-permeable analog CA-074 Me ($101.7 \pm 0.4\%$ viability) was negligible, as was that of Minocycline ($102.3 \pm 2.6\%$) at 10 μM.

Effect of Protease Inhibitors on HUVECs Tube Formation on Matrigel

HUVECs incubated on Matrigel aligned and organized into capillary-like tubes interconnected in mesh-like structure (Fig. 2A). The effect of protease inhibitors on the integrity of these structures was observed only when cells were treated with higher concentrations of CA-074 Me as illustrated in Figure 2B.

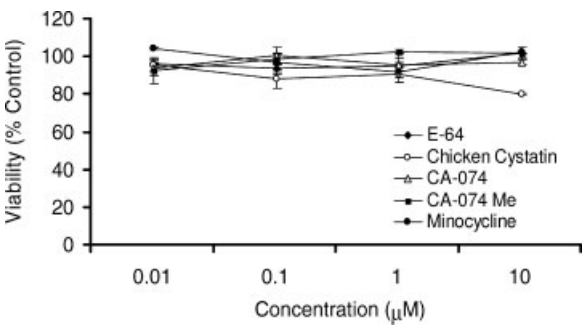

Fig. 1. Effects of protease inhibitors on the viability of HUVECs. Cells were plated in wells of a microtiter plate and incubated for 24 h at 37°C and 5% CO_2 in the presence of general cysteine protease inhibitors chicken cystatin and E-64, specific inhibitors of extracellular (CA-074), and intracellular (CA-074 Me) cathepsin B and metaLoprotease inhibitor Minocycline. As controls, cells were incubated with medium containing the appropriate volumes of solvents used for the preparation of stock solutions of the inhibitors. Measurements were corrected for the O.D. of the background and viability is presented as a percentage of that of the control. Mean value ± 2 SD (standard deviation) of quadruplicates are shown.

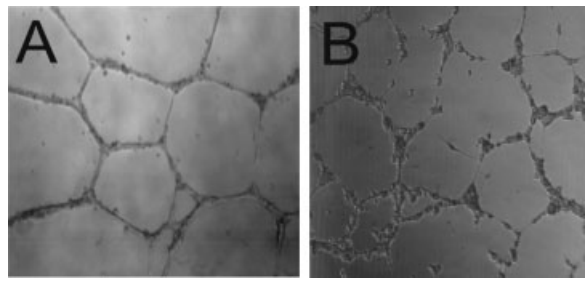

Fig. 2. Formation of capillary-like tubular structures by HUVECs. **A**: Typical appearance of capillary-like tubular structures formed by HUVECs incubated for 24 h at 37°C on Matrigel. **B**: Intracellular cathepsin B inhibitor CA-074 Me (10 μM) affected the integrity of capillary-like tubes after 24 h incubation. Magnification 60×.

A dose-response was observed in cells treated with CA-074 Me in the 0.1–10 μM concentration range (Fig. 3A), but only cells treated with 1 μM and 10 μM CA-074 Me had a tube length significantly shorter than in the corresponding vehicle only controls of CA-074 Me ($19.8 \pm 4.3\%$, $P < 0.05$ and $32.3 \pm 9.4\%$, $P < 0.05$, respectively). In comparison, 0.1 μM CA-074 Me led only to $7.6 \pm 3.9\%$ shorter tube length. All other inhibitors showed no significant inhibition in the length of the capillary-like tubes. Thus, no dose-response was observed for membrane-impermeable analog CA-074 in the same concentration range as used for CA-074 Me. At 10 μM, CA-074 was the most effective with a tube length reduction by $12.6 \pm 3.8\%$. The general cysteine protease inhibitors chicken cystatin (5 μM) and E-64 (10 μM) led to only $6.6 \pm 8.5\%$ and $10.9 \pm 3.8\%$ shorter tube length, respectively. Minocycline showed a similar inhibitory effect with $13.8 \pm 0.7\%$ decrease in tube length at 10 μM concentration.

Treatment of cells with CA-074 Me also affected the total area of tubules that covered Matrigel, which was significantly reduced by $41.5 \pm 20.9\%$ at 1 μM ($P < 0.05$) and by $50.4 \pm 12.4\%$ at 10 μM ($P < 0.01$) CA-074 Me compared with the appropriate controls. No alterations in total tubule area were observed for non-methylated membrane-impermeable analog CA-074 ($103.2 \pm 20.3\%$ at 0.1 μM, $100.1 \pm 12.9\%$ at 1 μM, and $107.5 \pm 15.2\%$ at 10 μM), while the treatment with chicken cystatin (5 μM), E-64 (10 μM), and Minocycline (10 μM)

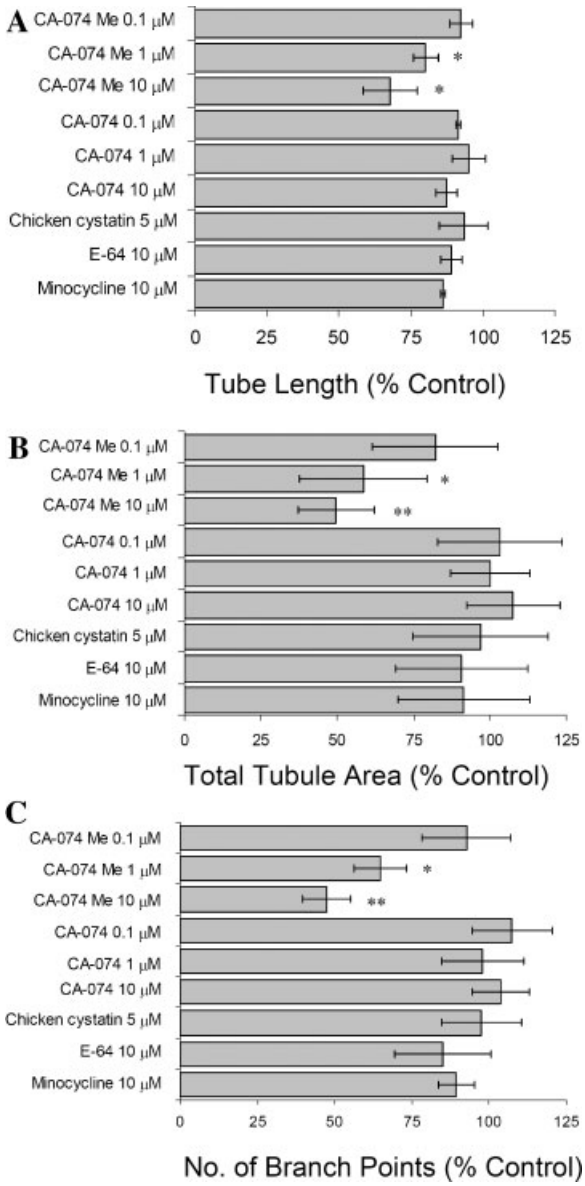

Fig. 3. Effects of protease inhibitors on HUVEC tube formation on Matrigel. Cells were seeded on top of Matrigel and incubated for 24 h in the presence of 0.1–10 μM membrane-permeable cathepsin B-specific inhibitor CA-074Me, its membrane-impermeable analog CA-074 (0.1–10 μM), general cysteine protease inhibitors chicken cystatin (5 μM), and E-64 (10 μM), and metalloprotease inhibitor Minocycline (10 μM). In controls, appropriate volumes of distilled water, ethyl acetate, and DMSO, the solvents used for the preparation of stock solutions of inhibitors, were used. The tubule parameters, including the tubule length (**A**), total tubule area (**B**), and the number of branch points (**C**) were determined as described under Materials and Methods and the results for individual parameters are presented as a percentage of values for the corresponding vehicle-only control. Values are mean ± 2 SD from two separate experiments performed in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ with respect to the corresponding vehicle-only controls.

decreased tubule area in Matrigel assay for less than 10% (Fig. 3B).

Assessment of the degree of branching showed the progressive reduction in the number of branch points by treatment of cells with increasing concentration of intracellular cathepsin B inhibitor CA-074 Me (7.4 ± 14.5%, 35.3 ± 8.5% and 52.5 ± 7.7% for 0.1 μM, 1 μM, and 10 μM inhibitor, respectively) as shown in Figure 3C. The effects of the same concentrations of CA-074 (0.1–10 μM), general cysteine protease inhibitors chicken cystatin (5 μM) and E-64 (10 μM), and MMP-3 inhibitor Minocycline (10 μM) were comparable to the number of branches in the corresponding vehicle-only controls (Fig. 3C).

Localization of Cathepsin B Protein

Alexa Fluor 488 labeled intracellular cathepsin B was localized in vesicle-like structures in the perinuclear region of HUVECs (Fig. 4A). Cathepsin B protein was also secreted from HUVECs, as determined by ELISA of samples of conditioned media. The relative concentration of intracellular cathepsin B was significantly higher (97.1 ng/mg protein) than the relative concentration of secreted enzyme (28.1 ng/mg protein).

Effects of Cysteine Protease Inhibitors on Cathepsin B Activity

To distinguish between extracellular and intracellular forms of cathepsin B, the same groups of inhibitors were used as above. Cathepsin B activity in cell lysates was inhibited more strongly by 10 μM CA-074 Me (activity: 2.25 ± 0.29 nmol/min/mg) than by 10 μM CA-074 (146.82 ± 31.70 nmol/min/mg) or by the same concentration of general cysteine protease inhibitor E-64 (102.71 ± 13.02 nmol/min/mg). Compared to the vehicle-only control, this corresponds to 99.4%, 40.4%, and 63.2% inhibition, respectively. No active cathepsin B was detected in conditioned medium before the activation with pepsin A. All three inhibitors showed nearly complete inhibition of the secreted, pepsin A-activated cathepsin B in conditioned cell culture medium (0.42 ± 0.02 nmol/min/mg by CA-074 Me, 0.42 ± 0.03 nmol/min/mg by CA-074, and 0.30 ± 0.05 nmol/min/ mg by E-64). The corresponding control values were 8.08 ± 0.36 nmol/min/mg, 9.61 ± 0.82 nmol/min/mg, and 8.57 ± 0.49 nmol/min/mg, respectively.

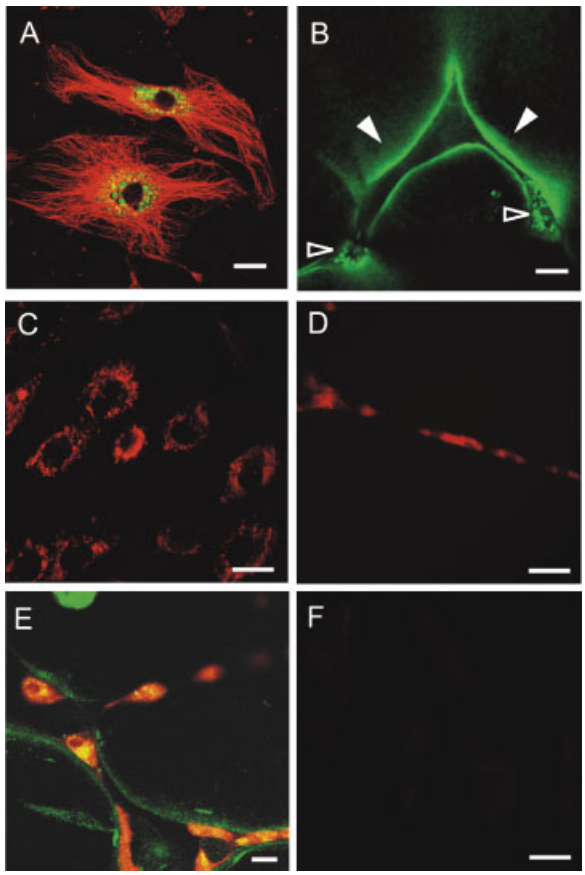

Fig. 4. **A**: Intracellular localization of cathepsin B in HUVECs. Cathepsin B was labeled with rabbit anti-human cathepsin B polyclonal antibodies and detected with goat anti-rabbit Alexa Fluor 488 (green fluorescence) secondary antibodies. Microtubules are marked with mouse anti-β-tubulin monoclonal antibody and detected with goat anti-mouse antibody labeled with Alexa Fluor 546 (red fluorescence). Immunofluorescence staining for intracellular cathepsin B (green fluorescence) shows it to be localized vesicularly in the perinuclear region. **B**: Degradation of DQ-collagen IV by living HUVECs forming capillary-like structures on Matrigel. Green fluorescent degradation products of DQ-collagen IV (25 μg/ml) were localized primarily in the pericellular region along tubular structures. White arrows indicate the diffusion of DQ-collagen IV degradation products from the cell membrane. Additionally, intracellular fluorescence was observed in the perinuclear regions (hollow arrows). **C**: Cathepsin B activity in living HUVECs was imaged by degradation of Z-Arg-Arg cresyl violet substrate. Red fluorescence (degraded substrate) is localized predominantly in the perinuclear region. **D**: Active cathepsin B was observed also in capillary-like tube structures, but not in the pericellular region. **E**: Co-localization of proteolytically active cathepsin B and degraded DQ-collagen IV in HUVECs forming capillary-like structures on Matrigel. Yellow color indicates co-localization of intracellularly active cathepsin B detected by degradation of Z-Arg-Arg-cresyl violet (red fluorescence) with degraded DQ-collagen IV (green fluorescence) internalized in living cells. Image is representative of two independent experiments. **F**: Preincubation of HUVECs with 10 μM CA-074 Me abolished intracellular cathepsin B activity. All images are representative of at least two independent experiments. Bars, 20 μm.

Proteolytic Activity of Living HUVECs

HUVECs were evaluated for their ability to degrade ECM, using the confocal assay described by Sameni et al. [2000]. Fluorescent degradation products of DQ-collagen IV were observed primarily pericellularly along the tubular structures. In addition, intracellular fluorescence was detected in the vesicles in the perinuclear regions (Fig. 4B).

Localization of proteolyticaly active cathepsin B in HUVECs was established by the enzyme-specific fluorogenic Z-Arg-Arg cresyl violet substrate. The substrate was very rapidly internalized and degraded in the cells grown on the bottom of cover glass chambers or those grown on Matrigel, as observed by red fluorescence (Fig. 4C). Granular distribution of fluorescence in the perinuclear region (Fig. 4C) matched the cathepsin B antibody staining profile (Fig. 4A). Active cathepsin B was also detected in capillary-like tube structures formed by cells grown on Matrigel matrix (Fig. 4D), but not in the pericellular region. Cathepsin B activity in tube structures determined by Z-Arg-Arg-cresyl violet co-localized with the DQ-collagen IV degradation products (Fig. 4E).

Z-Arg-Arg cresyl violet was also used to establish the effects of selected inhibitors on intracellular cathepsin B by fluorescence microscopy. Incubation of cells with 10 μM concentrations of E-64 and CA-074 under the same experimental conditions as in tube formation assay resulted in no detectable change in fluorescence compared to controls (not shown). In contrast, presence of CA-074 Me in the 0.1–10 μM concentration range in culture medium completely abolished the fluorescence, showing that intracellular cathepsin B was strongly inhibited (Fig. 4F).

DISCUSSION

Angiogenesis is vital and highly regulated process in a variety of normal physiological conditions and its dysregulation is associated with pathological disorders including tumor growth and dissemination of tumor cells during the metastatic process. It is facilitated by degradation of ECM proteins and subsequent remodeling of ECM by various proteases [Pepper, 2001]. Using fluorescence quenched DQ-collagen IV as a substrate and confocal laser scanning microscopy, we visualized the

proteolytic activity of living HUVECs forming capillary-like tubular structures on Matrigel. We showed that degradation of DQ-collagen IV in HUVECs occurred predominantly in the pericellular region at regions of cell-matrix contacts. However, green fluorescent products of digested DQ-collagen IV also accumulated in vesicular structures in the perinuclear cytoplasmic region, possibly as a result of phagocytosis of DQ-collagen IV, partially digested extracellularly. Observed degradation of ECM proteins by proteases might provide sprouting endothelial cells with a less rigid microenvironment favoring the tube formation and capillary maturation [Deroanne et al., 2001].

Collagen IV, one of the major ECM components, is degraded by various proteases, including cathepsin B [Buck et al., 1992], and collagen fragments like tumstatin, canstatin, and arresten have been shown to regulate angiogenesis [Colorado et al., 2000; Kamphaus et al., 2000; Hamano et al., 2003]. Proteolytic cleavage of collagen IV by MMPs exposes a cryptic site within the triple helical structure of the molecule, whose presence is required for angiogenesis and tumor growth in vivo [Xu et al., 2001; Hangai et al., 2002]. Both, new cryptic sites and changed structure of ECM proteins can also induce endothelial cell migration [Hangai et al., 2002; Sottile, 2004]. Besides degrading ECM, cathepsin B can also regulate the bioavailability of angiogenic factors, such as angiogenesis inhibitor endostatin, critical for the proper development of the vascular structure [Ferreras et al., 2000; Koblinski et al., 2000]. At the same time other proteases, like collagenase and plasmin, may contribute to the release of angiogenesis inducing factors, such as bFGF, from ECM [Whitelock et al., 1996]. Thus, the release of pro- and anti-angiogenic factors from ECM and their modulation by proteolysis is important for the complex homeostatic regulation of normal tissue angiogenesis [Hanahan and Weinberg, 2000]. Disbalance in this processes results in the pathological forms of new blood vessel formation such as tumor angiogenesis.

Cathepsin B is normally localized in lysosomes in the perinuclear region, and the vesicular distribution of the enzyme in this region was demonstrated by staining HUVECs with cathepsin B-specific antibodies. Intracellular protein profile matched the enzyme activity visualized by red fluorescence of degraded cathepsin B specific Z-Arg-Arg cresyl violet substrate and DQ-collagen IV degradation products, suggesting that at least some of the enzyme may facilitate further intracellular degradation of ECM substrates like collagen IV already partially degraded pericellularly and then internalized. This is further supported by the co-localization of cathepsin B activity and DQ-collagen IV degradation products in capillary-like tube structures observed with the confocal microscopy. Measurements of cathepsin B protein and activity levels in conditioned media showed that HUVECs also secrete the enzyme, predominantly in its pro-enzyme form, which could be activated, either autocatalytically or by other proteases. However, no pericellular or cell membrane associated cathepsin B activity was observed when Z-Arg-Arg cresyl violet was added to the cells. The lack of membrane-associated cathepsin B activity in HUVECs is rather surprising since it has been demonstrated that extracellular cathepsin B can interact with annexin II tetramer and co-localize with MMPs and serine proteases to caveolae on the membrane of tumor cells [Mai et al., 2000], leading to downstream regulation of the proteolytic cascade. Annexin II tetramer has also been found on the extracellular surface of endothelial cells, including HUVECs [Kassam et al., 1998], and it has been shown to stimulate t-PA dependent plasminogen activation [Kassam et al., 1998], which, in turn, can activate pro-cathepsin B in vitro [Dalet-Fumeron et al., 1996]. In addition to serine proteases t-PA and u-PA, metalloprotease MMP-2 [Puyraimond et al., 2001] has also been shown to be localized to caveolae of endothelial cells, but no co-localization of cathepsin B has so far been reported. It is still possible that potentially degraded Z-Arg-Arg cresyl violet substrate diffused from cell membrane, as observed for DQ-collagen IV (Fig. 4B), and became invisible by our detection method.

Using non-toxic concentrations of cathepsin B-specific inhibitors, and general cysteine protease inhibitors, we studied the effect of intracellular and extracellular cathepsin B on the formation of capillary-like tubular structures in an in vitro model, representing a faithful reproduction of the in vivo situation [Auerbach et al., 2003]. Treatment with membrane-permeable CA-074 Me led to a significant decrease in a dose-dependent manner not only in tube length ($32.3 \pm 9.3\%$ at 10 μM), but also resulted in a decrease of the total area of tubules formed on

Matrigel ($50.4 \pm 12.4\%$ at 10 μM) and the number of branch points formed between tubules ($52.5 \pm 7.7\%$ at 10 μM). The observed effects of CA-074 Me were described as reduced "integrity" of the capillary-like tubes and may result from accumulation of internalized collagen fibrils in the lysosomal apparatus as observed for other cysteine protease inhibitors, capable of being internalized [Everts et al., 1996]. On the other hand, the inhibition of extracellular cathepsin B activity by membrane-impermeable epoxide derivative CA-074 showed no dose-response and resulted in much weaker effect, causing only $12.6 \pm 3.8\%$ reduction in the length of the tubes at 10 μM. In addition, no significant effects were detected in other two parameters measured that include the total area of tubules and the number of branch points. Similarly, general cysteine protease inhibitors chicken cystatin and E-64 also led to limited effects in our angiogenesis model.

Previously, we and others have shown that, in tumor cells, CA-074, although negatively charged at physiological pH, is slowly internalized, probably by endocytosis, and may interact with intracellular cathepsin B. The same was observed in this study with endothelial cells, not only for CA-074, but also for E-64, although both inhibitors exhibited significantly lower inhibition of intracellular cathepsin B activity than membrane-permeable CA-074 Me. Nevertheless, the inhibition of intracellular cathepsin B by CA-074 and E-64 was not detected by the use of cathepsin B specific Z-Arg-Arg cresyl violet substrate and fluorescence microscopy. Thus, this study suggests that in our angiogenesis model predominantly intracellular fraction of endothelial cell-associated cathepsin B participates in the formation of capillary-like tubes. Our results are in accordance with recently observed active role of cathepsin B on in vitro and in vivo angiogenic processes [Gondi et al., 2004; Kruszewski et al., 2004]. However, the observed effect of inhibiting cathepsin B on in vitro angiogenesis is less marked than its effect on tumor cell invasion in vitro [Premzl et al., 2003]. In a very recent study by Im et al. (2005), it was suggested that cathepsin B can regulate the intrinsic angiogenic threshold of endothelial cells by modulating vascular endothelial growth factor (VEGF) activity or the amount of anti-angiogenic protein endostatin, and, therefore, its higher levels may reduce angiogenesis. In our study, we used bFGF (in addition to PMA) to induce tube formation and observed pro-angiogenic activity of intracellular cathepsin B. It is possible that pro- and anti-angiogenic activity of cathepsin B depends on the level of various endothelial growth factors and that a net equilibrium between these factors and catepsin B activity contributes to the so called angiogenic switch, which describes the transition of non-vascular tumor to the invasive vascularized stage.

In conclusion, our results show that intracellular cathepsin B seems to play an important role in capillary-like tube formation by HUVECs in Matrigel in vitro model. Its contribution to the processes of angiogenesis, particularly towards ECM degradation, designates it as an additional potential target for the regulation of undesired neovascularization.

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