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Participation of Intracellular Cysteine Proteinases, in Particular Cathepsin B, in Degradation of Collagen in Periosteal Tissue Explants

L. B. CREEMERS* †, K. A. HOEBEN†, D. C. JANSEN*, D. J. BUTTLE†, W. BEERTSEN* and V. EVERTS* †

* Department of Periodontology, Academic Center for Dentistry, Amsterdam (ACTA)

† Department of Cell Biology and Histology, Academic Medical Center, Amsterdam, The Netherlands and

‡ Department of Human Metabolism and Clinical Biochemistry, Institute for Bone and Joint Medicine, University of Sheffield, Sheffield, UK.

Abstract

The involvement of cysteine proteinases in the degradation of soft connective tissue collagen was studied in cultured periosteal explants. Using cysteine proteinase inhibitors that were active intracellularly or extracellularly (Ep453 and Ep475, respectively), it was shown that over-all collagen degradation, as measured by the release of hydroxyproline, decreased significantly on inhibition of the intracellular pool of cysteine proteinases by Ep453. This inhibitor also induced an accumulation of intracellular fibrillar collagen in fibroblasts, indicating a decreased degradation of phagocytosed collagen. The extracellular inhibitor, Ep475, had minor or no effects.

Histochemical analysis using a substrate for the cysteine proteinases cathepsins B and L revealed a high level of enzyme activity, which was completely blocked in explants preincubated with a selective intracellular inhibitor of cathepsin B, Ca074-Me. Moreover, the cathepsin B inhibitor strongly affected collagen degradation, decreasing the release of hydroxyproline and increasing the accumulation of phagocytosed collagen. These effects were comparable or slightly stronger than those found with the general intracellular inhibitor (Ep453). Taken together, these data strongly suggest that intracellular cysteine proteinases, in particular cathepsin B, play an important role in the digestion of soft connective tissue collagen.

Key words: collagen breakdown, cysteine proteinases, explant culture, histochemistry.

Introduction

For the past two decades, studies on the enzymatic degradation of collagen have focussed largely on the contribution of a single class of proteolytic enzymes, the

Abbreviations used: IMDM, Iscove's modified Dulbecco's medium.

matrix metalloproteinases. Another class of enzymes that is likely to take part in collagen resorption are the collagenolytic cysteine proteinases, such as the cathepsins B, L and K. These enzymes are capable of degrading fibrillar collagen at acidic pH and are primarily located in the lysosomes of cells (Kirschke et al., 1995). Their involvement in breakdown of collagenous proteins has been convincingly demonstrated in the digestion of bone matrix by osteoclasts (Everts et al., 1992; Hill et al., 1994). With respect to soft connective tissue degradation, however, their participation has remained obscure.

Because of the observation that fibroblasts of most connective tissues contain phagocytosed fibrillar collagen in their lysosomal apparatus (Dyer and Peppler, 1977; Svoboda et al., 1981; Asuwa, 1988), and the presence of collagenolytic enzymes in fibroblast lysosomes (Mort et al., 1981; Wang, 1982), an intracellular route of collagen resorption was advanced. This pathway has been proposed as of major importance to the physiological turnover of extracellular collagen (Ten Cate and Deporter, 1975; Everts et al., 1996). Support for the involvement of cysteine proteinases in intracellular collagen breakdown came from tissue culture studies in which a substantial accumulation of intracellular fibrillar collagen was found upon inhibition of the activity of these enzymes (Everts et al., 1985). Recently, their actual participation in the degradation of soft connective tissue was demonstrated by the suppression of the release of collagen degradation products by approximately 30% following cysteine proteinase inhibition (Creemers et al., in press). However, these data did not give any clue as to the site of activity of the cysteine proteinases. Despite their preferential localization in lysosomes, members of this enzyme class can be secreted by macrophages, osteoclasts, epithelial cells and fibroblasts (Burnett et al., 1995; Reddy et al., 1995), possibly exerting their action in an acidified pericellular microenvironment, or even at neutral pH (Buck et al., 1992; Dennison et al., 1992). Furthermore, it is not clear which of the collagenolytic cysteine proteinases is responsible for collagen breakdown in soft connective tissue. At least five members of this family, cathepsins B, L, N, S (Kirschke et al., 1995) and K (Bossard et al., 1996), are capable of collagen degradation. As the latter three enzymes (N, S and K) have a limited distribution throughout the body (Kirschke et al., 1995; Littlewood-Evans et al., 1997) and collagen breakdown takes place in all connective tissues, the ubiquitous cathepsins B and L are the more likely candidates.

The present study was undertaken to establish whether the contribution of cysteine proteinases to col-

lagen breakdown is to be attributed to intracellular or extracellular enzymes, and an attempt was made to identify the cysteine proteinase or proteinases involved. To this end, we studied in cultured periosteal explants the level of activity of cysteine proteinases, and the effect of various cysteine proteinase inhibitors on intracellular accumulation of collagen as well as on the release of collagen degradation products.

Materials and Methods

Materials

Iscove's modified Dulbecco's medium (IMDM), penicillin, streptomycin, amphotericin were obtained from Gibco (Gibco Lab., Grand Island, NY); bovine serum albumin (BSA, fraction V), leupeptin, 2-[N-morpholino]ethanesulfonic acid (MES), E-64 and bovine spleen cathepsin B (EC 3.4.22.1) were from Sigma (Sigma Chemical Co., St. Louis, MO). Z-Phe-Arg-MNA was purchased from Enzyme Systems Product (Livermore, CA), Z-Lys-ONp from Bachem (Bubendorf, Switzerland) and 2-hydroxy-5-nitrobenzaldehyde from Merck (Schuchardt, Germany). (4-Amidinophenyl)-methanesulfonylfluoride (APMSF) was from Boehringer Mannheim (Mannheim, Germany). The inhibitors Ep453 and Ep475 (Buttle et al., 1992a) were kind gifts of Dr M. Tamai (Taisho Pharmaceutical Co. Ltd, Tokyo, Japan), and CA074-Me was prepared as described previously (Buttle et al., 1992b). CA074-Me has the following rate constants (in $M^{-1}s^{-1}$) for the inactivation of various cysteine proteinases: 120,000 for cathepsin B; <10 for cathepsin H; 20 for cathepsin L; <10 for cathepsin S; and <10 for m-calpain (Buttle et al., 1992b). All other reagents were of analytical grade.

Tissue culture

Calvariae from rabbits (New Zealand White, aged 8 days) were dissected and cut into fragments of about $2 \times 2 \text{ mm}^2$, after which the periosteum was stripped off the convex and concave aspects. The tissue fragments were cultured in the absence of ascorbic acid in 24-well culture dishes in IMDM supplemented with 4 mg/ml BSA, amphotericin, streptomycin and penicillin as described previously (Everts et al., 1989). The cysteine proteinase inhibitors E-64 or leupeptin were added to the medium at a final concentration of 40 μM (Everts et al., 1985). Ep475 and Ep453, inhibitors of extracellular and intracellular cysteine proteinases, respectively,

and Ca074-Me, an intracellular inhibitor of cathepsin B, were dissolved in dimethylsulfoxide and used at 40 μ m. Dimethylsulfoxide was added to controls at equal concentrations, which never exceeded 0.1%. Media were collected after a culture period of 24 or 48 h and stored at -20°C until analyzed for hydroxyproline content (see "*Hydroxyproline assay*"). Tissue explants were either immersed in TissueTek II 4583 (Division Miles Laboratories Inc., Elkhart, IN) and frozen in liquid nitrogen (see "*Enzyme Histochemistry*") or processed for electron microscopy (see "*Electron Microscopy*").

Cell viability was monitored in conditioned medium by measuring the activity of the lysosomal enzyme β -glucuronidase towards the synthetic substrate 4-methylumbelliferone (Robins et al., 1968). This parameter was not affected by either of the inhibitors used (data not shown), nor did electron microscopic examination of tissue sections reveal morphological evidence of cytotoxic effects.

Hydroxyproline assay

The amount of hydroxyproline released in the culture medium was determined as described previously (Cremers et al., 1997). In short, conditioned medium was precipitated overnight at 4°C in 70% ethanol, and the precipitates were pelleted by centrifugation at 250g and washed in 70% ethanol. The supernatants were evaporated under vacuum, hydrolyzed for 3.5 h at 135°C in 6 N HCl, desiccated and dissolved in demineralized water. After a centrifugation step at 9,700g, the supernatant was dispensed into a 96-well microtiter plate. Buffer and chloramine-T reagent were added to each well, the final mixture containing 9.4 mg/ml chloramine-T in 6.7% n-propanol, 0.19 M citric acid, 0.33 M sodium acetate trihydrate, 0.33 M anhydrous sodium acetate, 0.08 M acetic acid and 0.32 M sodium hydroxide with a pH of 6.1. Following a 15 min incubation at ambient temperature, DMBA reagent (2 g dimethylaminobenzaldehyde dissolved in 1.25 ml n-propanol and 2.75 ml perchloric acid) was added 2:3 (DMBA:chloramine-T mixture) and mixed with a multi-channel pipette. The microplate was incubated for 25 min at 60°C in a water bath and subsequently cooled with tap water for 5 min. Extinction was read at 570 nm in a Bio-Rad microplate reader, and the hydroxyproline content of the samples was calculated using a standard curve constructed with unconditioned IMDM containing 4 mg/ml BSA and a range of hydroxyproline concentrations.

Electron microscopy

Explants were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for at least 3 h at room temperature. After washing in 0.1 M sodium cacodylate buffer, the specimens were postfixed with 1% OsO_4 in cacodylate buffer for 1 h at ambient temperature and dehydrated through a graded ethanol series. The explants were embedded in LX-112 (Ladd Res. Industries, Burlington, VT). Ultrathin sections were cut with a diamond knife and, after staining with uranyl acetate and lead citrate, examined in a Zeiss EM 10C electron microscope.

Morphometric analysis

Morphometric analysis was performed to establish the volume density of phagocytosed fibrillar collagen. To this end, micrographs were taken of 10 to 15 fibroblasts in one randomly selected ultrathin section per explant. Sections were screened in parallel rows with a fixed distance, and only fibroblasts of which (part of) the nucleus crossed the center of the screen were micrographed (Everts et al., 1989). Micrographs were printed at a final magnification of 19,500 $\times$, coded, randomized and subjected to a point-counting procedure.

Two types of collagen-containing vacuoles could be discerned: (1) membrane-bound profiles enclosing cross-banded collagen with the space between the membrane and the fibril fragments being filled with electron-dense material, and (2) profiles characterized by the presence of an electron translucent zone surrounding the fibril fragments. In previous studies it was shown that the first category of vacuoles are intracellular structures (Melcher and Chan, 1981) and contain lysosomal enzymes (Deporter and Ten Cate, 1973). Electron-translucent vacuoles most probably contain collagen that partly communicates with the extracellular space. Thus, only electron-dense vacuoles were considered to denote internalized collagen.

Cysteine proteinase assay

Cysteine proteinases were extracted by sonicating explants 3×5 sec in 200 μ l of a 100 mM phosphate buffer with 0.2% Triton X-100 and 2 mM EDTA (pH 6.0). The procedure was carried out on ice and repeated following over-night agitation at 4°C . Aliquots of 20 μ l were assayed for cysteine proteinase activity in a 100 mM phosphate buffer containing 0.61 mM Z-Lys-ONp, a synthetic substrate for cysteine and serine proteinases, 3.7 mM APMSF (inhibitor of serine proteinases), 2 mM EDTA and 2 mM dithiothreitol, pH 6.0. Negative con-

trols consisted of substrate without tissue extract. In addition, a number of tissue extracts were incubated in the presence of 60 μ M E-64.

Readings were taken after 30 min of incubation at 22 °C. The rate of substrate hydrolysis was determined using a Perkin-Elmer 650–40 fluorescence spectrophotometer (excitation at 405 nm and emission at 500 nm). Data of test samples are presented as extinction coefficients, with the extinction values of negative controls being subtracted.

Enzyme histochemistry

Explants incubated for 3 h in the presence or absence of 40 μ M Ca074-Me were embedded in Tissue Tek and frozen in liquid N₂. Cryosections were cut on a motor-driven Bright cryotome with automatic speed control at a cabinet temperature of –25 °C. Sections of 6 μ m were collected on glass slides and stored at –20 °C. For the demonstration of cathepsin B/L activity, sections were air-dried, and covered after addition of a medium containing 100 mM 2-[N-morpholino] ethanesulfonic acid buffer pH 6.0, 0.2 mg/ml Z-Phe-Arg-MNA, 1.3 mM EDTA, 10 mM dithiothreitol, 1 mM 2-hydroxy-5-nitrobenzaldehyde and 2.7 mM L-cysteine. Z-Phe-Arg-MNA was dissolved in dimethylformamide, of which the final concentration was 5%. Control sections were incubated in medium containing 50 μ M E-64. After 15–90 minutes of incubation, the sections were examined with a Leitz light microscope (Leica, Rijswijk, The Netherlands) equipped with a mercury epifluorescence lamp. Micrographs were made using Kodak Ektachrome 400 ASA film.

Statistical analysis

Data are presented as mean $\pm$ SEM of 6–10 explants per incubation unless stated otherwise. Statistical analysis was performed by Welch's t-test or by Mann-Whitney test, except for comparison of three or more groups, which were analyzed by nonparametric ANOVA and Dunn's multiple comparisons test.

Results

Cysteine proteinase involvement

Enzyme activity in tissue extracts. The activity of cysteine proteinases was demonstrated in tissue extracts of fresh explants and explants cultured for 24 and 48 h. No differences were observed between cultured and

fresh explants (Fig. 1). Extracts incubated with E-64 showed a low residual activity, indicating that the activity found originated from cysteine proteinase.

Fig. 1. Cysteine proteinase activity in tissue extracts from fresh explants ($t = 0$) and explants cultured for 24 h ($t = 24$) and 48 h ($t = 48$). Values represent the mean $\pm$ SEM of eight explants per time interval.

Fig. 2. Hydroxyproline release (ng/explant) by periosteal explants cultured for 24 and 48 h in the presence or absence of the proteinase inhibitor leupeptin (40 μ M). Values are expressed as the mean $\pm$ SEM of 32 to 40 explants. * $P < 0.005$.

Involvement in over-all collagen breakdown. Addition of the cysteine (and serine) proteinase inhibitor leupeptin to the culture medium of explants resulted in a significant decrease of collagen breakdown as assessed by the released amount of hydroxyproline (Fig. 2). The inhibition of release was approximately 75% after 24 h and 50% after 48 h.

Intracellular collagen accumulation. Microscopic examination of explants cultured for 24 h in the presence of leupeptin revealed an accumulation of intracellular fibrillar collagen (Fig. 3A) of more than two-fold the amount found in control explants (Fig. 3B). After 48 h, the difference was up to a ten-fold higher volume percentage of collagen.

Fig. 3A. Intracellular cross-banded collagen fibrils (arrows) in lysosomal vacuoles of periosteal fibroblasts of explants cultured for 48 h in (Aa) control medium and (Ab) medium containing the proteinase inhibitor leupeptin (40 μ M). Aa, Ab: magnification 24,000.

Fig. 3B. Volume density of collagen-containing vacuoles (ccv) of fibroblasts of explants cultured for 24 and 48 h in the presence or absence of leupeptin (40 μ M). Values represent the mean $\pm$ SEM of five to eight explants per incubation. * P < 0.05, ** P < 0.01.

Fig. 4A. Hydroxyproline release (%) by periosteal explants cultured for 24 and 48 h in the presence or absence of the extracellular (Ep475) and intracellular (Ep453) cysteine proteinase inhibitors (both 40 μ M). Values are expressed as the mean $\pm$ SEM of three to four experiments per incubation. * P < 0.05.

Extracellular vs. intracellular cysteine proteinases

In an attempt to discern the site of activity of cysteine proteinases, analogues of E-64 were used. Ep475 is active extracellularly, as it is not able to penetrate the plasma membrane, and Ep453 is active only after entry into the cell and release of an inactivating ethyl ester by cytoplasmic esterases (Buttle et al., 1992a).

Over-all collagen breakdown. Incubation with Ep475 (extracellular inhibitor) had no statistically significant effect on hydroxyproline release in any of the three experiments performed, although a slight inhibition of approximately 10% was found (Fig. 4A) after 48 h of culturing. The intracellular inhibitor Ep453 resulted in a decrease of collagen breakdown that was statistically significant in two out of three experiments after 24 h and in all four experiments after 48 h (P < 0.05–0.01). At both time intervals, the mean inhibition was approximately 40%.

Intracellular collagen accumulation. The extracellular inhibitor of cysteine proteinases, Ep475, did not have an effect on the accumulation of phagocytosed collagen (Fig. 4B). In contrast, Ep453 did induce a significant increase, leading to a volume density of internalized collagen that was twice that of the control values (P < 0.01).

Figure 4B. Volume density of collagen-containing vacuoles (%ccv) of fibroblasts of explants cultured with or without Ep453 (40 μ M) and Ep475 (40 μ M) for 24 h. Values are expressed as the mean $\pm$ SEM of five to six explants. * P < 0.01.

Cathepsin B involvement

By using an inhibitor selective for cathepsin B (Ca074-Me; Buttle et al., 1992b), the potential participation of this enzyme in collagen degradation was evaluated. This

inhibitor, like Ep453, is only active after intracellular hydrolysis of its ethyl ester and thus only affects intracellular cathepsin B activity. As cathepsin L, in addition to cathepsin B, is considered to be ubiquitously present, we also measured the activity of this enzyme. To this end a syn-

Fig. 5. Cathepsin B and L activity as revealed by enzyme histochemistry of cryosections of fresh periosteal explants. Reaction product (arrows) after 5A: 30, 5B: 60 and 5C: 90 min. Figure 5D. Lack of enzyme activity, even after 90 min of incubation, in sections of explants preincubated with Ca074-Me. Magnification: 500 $\times$.

Fig. 6A. Hydroxyproline release (ng/explant) by periosteal explants cultured for 24 and 48 h in the presence or absence of the intracellular inhibitor of cathepsin B, Ca074-Me (40 μ M). Values are representative for two experiments and are expressed as the mean $\pm$ SEM of ten explants. * $P < 0.05$.

Fig. 6B. Volume density of collagen-containing vacuoles (%ccv) of fibroblasts of explants cultured with or without Ca074-Me (40 μ M) for 24 h. Values are expressed as the mean $\pm$ SEM of six explants. * $P < 0.02$.

thetic substrate for both enzymes (Kamboj et al., 1993) was used in combination with a Ca074-Me preincubation.

Enzyme histochemical determination of cathepsin B activity. The activity of cathepsin B and cathepsin L was evaluated in explants that had been preincubated for 3 h in medium with or without Ca074-Me, to allow the inhibitor to enter the cells and become activated. Cryosections of these explants were incubated with a solution containing the substrate for cathepsin B and L. In sections of control explants, yellow fluorescent spots, indicating enzyme activity, were noted after 30 min (Fig. 5A), increasing in number after 60 min (Fig. 5B), until a strong signal – already displaying redistribution of crystals – was observed after 90 min (Fig. 5C). In contrast, the same procedure performed with Ca074-Me-incubated explants did not show any reaction product, not even after 90 min (Fig. 5D). Neither was any signal detected in the control sections concomitantly incubated with E-64 (not shown).

Over-all collagen degradation. To determine whether cathepsin B was responsible for some of the cysteine proteinase-mediated collagen degradation, the release of hydroxyproline in the presence of Ca074-Me was monitored. After 24 and 48 h of culturing, collagen degradation was significantly reduced by approximately 40% and 70% (both $P < 0.05$; Fig. 6A).

Intracellular collagen accumulation. Under the influence of the cathepsin B inhibitor, the volume percentage

of intracellular fibrillar collagen showed a six-fold increase ($P < 0.02$; Fig. 6B).

Discussion

Several authors have suggested that cysteine proteinases play a part in the digestion of collagen in soft connective tissue (Etherington, 1977; Wang, 1982). Support for this view was provided by the observation that fibrillar collagen accumulated in fibroblast lysosomes upon inhibition of this class of enzymes (Everts et al., 1985). This was considered to signify that intracellular breakdown of phagocytosed extracellular collagen was arrested due to the inhibition of the activity of lysosomal cysteine proteinases. Because the contribution of an extracellular pool of enzymes could not yet be excluded, the present study was undertaken to determine the site of their activity. By using cysteine proteinase inhibitors that were active either inside or outside the cell, we demonstrated that predominantly the intracellular pool of cysteine proteinases is involved.

Given their localization in the lysosomal apparatus of various cell types (Mort et al., 1981; Wang, 1982; Kirschke and Barrett, 1987), the cysteine proteinases involved in collagen degradation are likely to be of lysosomal origin. Although, to our knowledge, the actual presence of cysteine proteinases in collagen-containing vac-

uoles has never been demonstrated, the lysosomal origin of these vacuoles has been well established (see Everts et al., 1996). With the extracellular inhibitor, nevertheless, a slight effect on over-all collagen degradation was found at the 48 h time interval. Thus, the possibility cannot be entirely excluded that some collagen is degraded by cysteine proteinases outside the cell. If so, the latency of the effect may be explained by a slower tissue penetration of the extracellular, as opposed to the intracellular, inhibitor. Alternatively, the inhibitor may have been endocytosed, which enabled it to exert its activity intracellularly. This delay in the effect of the extracellular inhibitor seems consistent with previous data obtained in an experimental model of lens cataract, where, after prolonged culture periods (5 days), an accumulation of the extracellular inhibitor (Ep475) in the lens tissue was found, together with an effect on the cysteine proteinase-mediated degradation of crystalline (Azuma et al., 1992). However, studies on proteoglycan degradation in cartilage explants failed to show an effect of this compound (Buttle and Saklatvala, 1992). Penetration into the highly charged cartilage matrix may have been more difficult here, or the endocytotic activity of chondrocytes may be different.

Our observations lend strong support to the view that cathepsin B is (one of) the cysteine proteinase(s) involved in the degradation of connective tissue collagen (Van Noorden et al., 1987; Kirschke et al., 1995). Not only was its activity demonstrated, but also intracellular inhibition of the enzyme resulted in both an accumulation of phagocytosed fibrillar collagen and a decrease of over-all collagen degradation. These effects were similar to those generated by the intracellular general inhibitor of cysteine proteinases (Ep453). It thus appears that intracellular cathepsin B is a major cysteine proteinase involved in the degradation of collagen in soft connective tissue. Cathepsin L seemed not to be active in our model, despite its potent collagenolytic activity and its alleged widespread occurrence (Kirschke and Barrett, 1987). This finding is in agreement with data obtained with another soft connective tissue, the synovial membrane, where cathepsin L activity could not be demonstrated in fibroblasts (Van Noorden et al., 1988; Kiyoshima et al., 1994).

Cysteine proteinase-mediated degradation of extracellular matrix proteins appears to occur in many soft and hard connective tissues. For soft connective tissue, the fibroblast-mediated intracellular degradation of internalized collagen by this enzyme class has been suggested to represent a major pathway under steady-state conditions (Ten Cate and Deporter, 1975; Everts et al., 1996). While cathepsin B seems to be a major cysteine proteinase participating in (periosteal) soft connective tissue collagen

degradation, osteoclastic digestion of bone collagen depends on either cathepsin L (Kakegawa et al., 1993) or the recently cloned cysteine proteinase cathepsin K (Bossard et al., 1996; Inui et al., 1997). The breakdown of cartilage components may also involve cathepsin B (Van Noorden et al., 1988; Buttle and Saklatvala, 1992; Baici et al., 1995), for example in cytokine-stimulated breakdown, where it possibly acts in a proteolytic activation cascade (Buttle et al., in press). Finally, metastasis of several malignancies is associated with increased production and/or secretion of cathepsin B (Sloane and Honn, 1984; Moin et al., 1989; Keppler et al., 1994), possibly promoting extracellular matrix degradation needed for tumor cell dissemination. Under these circumstances, various cathepsin B isoforms have been detected (Page et al., 1992), some of which were shown to be active at neutral pH (Buck et al., 1992; Spiess et al., 1994).

In conclusion, the data presented in this study provide ample support for the role of intracellular cysteine proteinases, in particular cathepsin B, in the degradation of soft connective tissue collagen.

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- Dr. Vincent Everts, Dept. Cell Biology and Histology, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

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