Modulation of fibroblast activity by collagens
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CHAPTER 2

Collagen breakdown in soft connective tissue explants is associated with the level of active gelatinase A (MMP-2) but not with collagenase

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Abstract

Recent data suggest that gelatinase A (matrix metalloproteinase-2, MMP-2) plays an important role in the degradation of collagen of soft connective tissues. In an attempt to investigate its participation in more detail we assessed the digestion of collagen in cultured rabbit periosteal explants and compared this with the level of active MMP-2 and collagenases. The data demonstrated that both collagen degradation and MMP activity increased in time. Conditioned medium obtained from explants cultured for 72 hours showed that the level of active MMP-2 correlated with collagen degradation (r = 0.80, df = 23, p<0.0001). Such a relationship was not found for collagenase activity (r = -0.08, df = 21, NS). The possible involvement of MMP-2 in collagen degradation was investigated further by incubating explants with selective gelatinase inhibitors (CT1166, CT1399 and CT1746). In the presence of these compounds breakdown of collagen was almost completely abolished (>80%). Finally we assessed whether periosteal fibroblasts had the capacity to degrade collagen type I that conferred resistance to collagenase activity. Breakdown of this collagen did not differ from degradation of normal collagen. Taken together, our data provide support for the view that MMP-2 plays a crucial role in collagen degradation of soft connective tissue.

Introduction

Remodelling and turnover of soft connective tissues imply continuous synthesis and degradation of extracellular matrix components. Among the enzymes that are thought to be important in matrix breakdown are the matrix metalloproteinases, MMP-1 (interstitial collagenase) and MMP-2 (gelatinase A)\(^1\)\(^-\)\(^2\). Recent observations suggest that it is particularly MMP-2 that plays an essential role in this process. First, Liu and coworkers\(^3\) found that mutant mice whose collagen type I is resistant against collagenase activity, because the specific cleavage site for this enzyme is mutated, showed a relatively normal postnatal development. These findings suggest that during normal turnover and remodelling of one of the major collagens, collagenase is not a prerequisite. Second, in almost all non-inflamed (soft) connective tissues MMP-2, but not MMP-1, can readily be localised\(^4\)\(^-\)\(^7\). Third, MMP-2 proves to be constitutively expressed by connective tissue cells (e.g. fibroblasts), whereas MMP-1 is only expressed following appropriate stimulation\(^6\)\(^-\)\(^8\). Finally, the participation of MMP-2 in the degradation of fibrillar collagen in soft connective tissue was recently demonstrated in an \textit{in vitro} model system\(^4\).
If MMP-2 is indeed important in the breakdown of collagen, it may be hypothesised that the rate of degradation would relate to the level of activity of MMP-2. To investigate whether such a relationship exists, we used an in vitro model system consisting of periosteal tissue explants from rabbit calvariae and compared the level of active collagenases (MMP-1, MMP-8 and MMP-13) versus the level of active MMP-2 with the amount of digested collagen. In addition, the effect of selective gelatinase inhibitors on collagen breakdown was analysed. Finally, we tested to what extent type I collagen that is resistant to collagenase activity could be broken down by isolated periosteal fibroblasts.

Materials and methods

Materials

Iscove's modified Dulbecco's medium (IMDM), Hanks' balanced salt solution (HBSS), penicillin, streptomycin, amphotericin were purchased from Gibco (Gibco Lab., Grand Island, NY). Bovine serum albumin (BSA, fraction V), sheep IgG and fluorescein isothiocyanate (FITC)-labelled donkey-anti-[sheep IgG] were from Sigma (Sigma Chemical Co., St. Louis, MO). Multi-well culture dishes were from Costar (Cambridge, MA), gelatin (from porcine skin, 250 bloom) from Fluka (Fluka Chemie, Buchs, Germany) and 40% acrylamide/Bis. 37.5:1 from Bio-Rad (Hercules, CA). The synthetic MMP-inhibitors CT1166 (N1-[1-(S)-(morpholinosulphonylaminoethylcarbonyl)-2-cyclohexyl-ethyl]-N4-hydroxy-2-(R)[3-(4-methylphenyl)propyl] succinamide), CT1399 (N4-hydroxy-N1-[1-(S-morpholinosulphonylaminoethylaminocarbonyl)-2-cyclohexyl-ethyl]-2-(R)-(4-chlorophenylpropyl)succinamide) and CT1746 (N1-[2-(S)-(3,3-dimethylbutanamidyl)]-N4-hydroxy-2-(R)-[3-(4-chlorophenyl)-propyl]succinamide) were kindly provided by Drs. T. Crabbe and R. Morphy at the Departments of Biology and Chemistry (Celltech Therapeutics Ltd., Slough, UK). The Kᵢ-values of CT1166 and CT1399 are identical for MMP-2, -9, -3, -1 and -7: 0.01, 0.016, 2.75, 385, and 6400 nM, respectively. The Kᵢ-values of CT1746 for the mentioned MMPs are: 0.04, 0.17, 10.9, 122, and 6400 nM, respectively. Tissue-Tek 4583 was from Miles Scientific (Div. of Miles Lab. Inc., Napperville,IL). Recombinant human MMP-2, a sheep polyclonal antibody (IgG fraction) to rabbit MMP-2 and MMP-9 and a sheep polyclonal antibody (IgG fraction) to rabbit MMP-1 were kindly donated by Drs. R. Hembry and G. Murphy (School of Biological Sciences, University of East Anglia, Norwich, UK) and Dr. J.J. Reynolds (Dept. of Orthodontics and Paediatric Dentistry, United Medical and Dental School, London, UK).
Tissue culture

Periosteal tissue of rabbit calvariae was dissected and cultured in IMDM as described previously\textsuperscript{12}. For immunolocalization a number of cultured and non-cultured explants were immersed in Tissue-Tek 4583 and immediately frozen in liquid nitrogen and stored at -80°C. The other explants and conditioned media were frozen at -20°C. The conditioned media were analysed for the activity of MMPs and hydroxyproline content. Explants were extracted as described by Eeckhout et al.\textsuperscript{13}. Briefly, they were taken up in 200 µl extraction-buffer (10 mM sodium cacodylate, 1 M NaCl, 0.01% Triton X-100 and 1 µM ZnCl\textsubscript{2}, pH 6.0), sonicated (3 x 5 sec), incubated overnight at 4°C, followed by a second sonification step (3 x 5 sec) and the extracts were analysed for the activity of MMPs. In a preliminary series of experiments this extraction method was compared with the one presented by Woessner\textsuperscript{14}. Our data indicated that extraction of periosteal tissue explants with the Eeckhout method yielded a similar level of MMPs as with the Woessner method (data not shown).

The synthetic MMP-inhibitors (CT1166, CT1399 and CT1746) were added to a number of explants at final concentrations of 0.1 or 0.2 µM. The inhibitors were dissolved in dimethyl-sulfoxide (DMSO). DMSO was added to controls in equal amounts, its concentration not exceeding 0.1%.

Coating of the culture plates

Native collagen type I of the tail of a mouse that has a mutated collagenase cleavage site\textsuperscript{8} and of a normal littermate (kindly donated by Dr. R. Jaenisch, Whitehead Institute for Biomedical Research, Cambridge, MA) was isolated as described by Bazin and Delauny\textsuperscript{15}. The collagen was dissolved in 0.01 M acetic acid in a concentration of 50 µg/ml. 24-well culture plates were coated by incubating the wells with 500 µl diluted collagen. The plates were air-dried in a laminar flow cabinet at ambient temperature and subsequently sterilised by UV light for 20 min. The wells were washed with HBSS before the cells were seeded.

Cell isolation and culture

Periosteal explants were washed in IMDM supplemented with 10% FCS and amphotericin (2.5 mg/ml), streptomycin (1 mg/ml) and penicillin (1000 U/ml). The tissue samples were placed in 6-well culture plates and incubated in IMDM supplemented with 10% FCS and antibiotics in a
humidified atmosphere containing 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) (pH 7.3), transferred to 25 cm² culture flasks and designated as 'passage one' (P1). For the experiments, cultures between P3 and P8 were used. Four days prior to the onset of the experiment the medium of the cells was changed with IMDM supplemented with 5% FCS and antibiotics, which was changed after three days with IMDM supplemented with 4 mg/ml BSA and antibiotics. The isolated cells (3 x 10⁴ cells/well) were incubated with 300 μl IMDM (+ 4 mg/ml BSA) in collagen-coated 24-well culture plates in a humidified atmosphere containing 5% CO₂ in air at 37°C.

**Immunolocalization**

Tissue-Tek embedded explants were cut (6 μm section thickness) on a motor-driven Bright cryotome at -28°C. Immunolocalization of the MMPs was performed as described previously.¹⁰ The primary antibodies were sheep anti-MMP-2/MMP-9 IgG and sheep anti-MMP-1 IgG, non-immune sheep IgG was used as control. All antibodies were applied at a final concentration of 80 μg/ml IgG. The second antibody was an FITC-labelled anti-sheep antibody. The nuclei of the cells were stained by propidium iodide (50 ng/ml). The sections were covered with Vectashield (Vector Laboratories, Burlingame, CA) and examined with a Leica microscope equipped with filter settings for epifluorescence. Micrographs were made with Kodak 400 ASA films, upgraded to 1600 ASA.

**Zymographic analysis**

Aliquots of 5 μl of conditioned medium or tissue extract were 1:1 diluted with sample buffer (0.1 M Tris-HCl, 4% SDS, 20% glycerol, 0.005% BFB, 10 mM EDTA) and electrophoresed through a 10% polyacrylamide gel containing 2% gelatin or 1% casein, as described by Heussen and Dowdle.¹⁷ After electrophoresis, gels were washed and incubated for 18 h at 37°C. Gels were stained with 0.1% Coomassie Brilliant Blue and destained with 7% acetic acid and 5% methanol.

In order to quantify MMP-2 activity, densitometric analysis was performed by assessing the ratio between MMP-2 and a standard concentration of recombinant MMP-2. This analysis was carried out using a Quantimet 500 (Leica, Cambridge Ltd., UK) image analysis system and the Qwin v02.00B software program. In preliminary experiments we established that a linear relationship exists from 0 up to 3 mU recombinant MMP-2. Zymographic analyses of the conditioned media and tissue extracts were performed within this range. The data were expressed...
as units or as arbitrary densities.

Collagenase bioassay

Conditioned media and the extracted explants were analysed for the presence of active collagenases using fibrillar \([^{14}C]\)-labelled collagen as described previously\(^{18,19}\). The data were expressed as units collagenase per explant. One unit of collagenase was defined as the amount of enzyme needed to digest 1 \(\mu\)g collagen per minute at 35\(^\circ\)C.

Hydroxyproline assay

The amount of hydroxyproline released in medium of cultured explants was established and used as a parameter of collagen degradation. In previous studies it was shown that, under the culture conditions employed, hydroxyproline in the culture media reflects degradation of collagen and not the release of newly synthesised collagen\(^4\). Hydroxyproline analysis was performed by using a microassay developed by Creemers et al.\(^{20}\).

Statistical analysis

Statistical analysis included correlation analysis (linear regression) and Mann-Whitney tests (hydroxyproline data). \(P<0.05\) (two-tailed) was assumed to indicate statistical significance.

Results

Presence and activity of MMPs in periosteal explants

Immunolocalization

Immunolocalization of gelatinases (MMP-2 and MMP-9) revealed their presence in all explants. The signal increased during culturing, showing the highest level of immunostaining at 72 h. Most of the fluorescence was associated with the fibroblasts (Fig. 1A); very little of it with the extracellular matrix. MMP-1 proved to be undetectable in consecutive tissue sections (Fig. 1B).

Zymographic analysis

Gelatin zymographic analysis of conditioned media and tissue extracts demonstrated three lytic
bands of 92, 72 and 66 kDa (Fig. 2), respectively. These bands were identified as gelatinases by

the use of activity blocking anti-MMP-2/MMP-9 antibodies (not shown). The culture media and
tissue extracts, collected after 24, 48 and 72 h, demonstrated a time-dependent increase in the
levels of both inactive (72 kDa) and active (66 kDa) MMP-2. Such a time-dependent shift was
not found for MMP-9. All media contained a relatively high level of MMP-9, whereas in the tissue
extracts hardly any MMP-9 was found (Fig. 3). In contrast herewith, MMP-2 was abundantly
present in both media and extracts. Casein zymography, used to detect MMP-3, MMP-7 and
MMP-10, did not reveal any activity in media or tissue extracts (data not shown).
Collagenase release

With respect to collagenase (MMP-1, -8 and/or -13) we observed that, during the first 48 h of culturing, the activity in conditioned medium was below the detection limit. At the 72 h time-point some activity was noted (6.2 ± 1.3 mU/explant). No collagenase activity was detected in the extracted explants.

Collagen degradation

Media collected at the 24, 48 and 72 h time intervals revealed a time-dependent increase of its hydroxyproline content, which was most pronounced between 48 and 72 h (Fig. 4). In media of tissue explants cultured for 72 h with MMP-inhibitors (0.1 μM CT1166, 0.2 μM CT1399 and 0.2μM CT1746) a virtually completely inhibited digestion of collagen was noted (> 80%; Fig. 5).

Fig. 3. Densitometric analysis of MMP-9 and MMP-2 (pro-and active forms in conditioned media and tissue extracts of explants cultured for 72 h. The values are presented as % density per band (66 or 72 kDa) of total density (total gelatinolytic activity) per explamt (mean ± SD of 12 experiments).

Is there a relation between enzyme activity and collagen degradation?

The observed increase in the level of MMPs correlated positively with an increased hydroxyproline release. It was especially MMP-2 that corresponded to the released amount of hydroxyproline, and a statistically significant positive correlation was apparent (Fig. 6A). Such a relationship was not found with collagenase (Fig. 6B).
Fig. 4. Time-dependent increase of the level of hydroxyproline content of media each 24 h during a 72 h culture period. Values are expressed as the mean ± SD of 10 explants per time interval.

Fig. 5 Hydroxyproline release by periosteal explants cultured for 72 h in the absence (control) or presence of 0.1 µM CT1166, 0.2 µM CT1399, or 0.2 µM CT1746. Values are expressed as mean % ± SD of at least 8 explants per incubation. *: p < 0.001 compared to the control.
Fig. 6. (A) Linear regression analysis of the level of active MMP-2 and the amount of hydroxyproline released in the medium by the same explant ($r = 0.81; p<0.0001, df = 23$); (B) Linear regression analysis of the level of active collagenase and the released amount of hydroxyproline ($r = -0.08, p = 0.72, df = 21$).
Degradation of collagenase resistant collagen by isolated periosteal cells

Since the presented data suggested that collagenase is not prerequisite for periosteal cells to digest collagen, we attempted to investigate this further by making use of type I collagen that was isolated from a mouse strain having a mutation in the collagen type I molecule that confers resistance to collagenase activity. Isolated periosteal fibroblasts were seeded on this collagen as well as on collagen obtained from a normal littermate. Analysis of the level of hydroxyproline released in the medium revealed no differences (Fig. 7). Zymographic analysis of the culture media demonstrated that cells cultured on either collagen secreted similar levels of inactive and active MMP-2 (Fig. 8).

![Graph showing hydroxyproline release](image)

Fig. 7. The amount of hydroxyproline released in medium of periosteal cells cultured for 24 and 48 hours on normal type I collagen and type I collagen with a mutated collagenase cleavage site. Values are expressed as mean ± SD of 10 wells per coating.

![Gelatin zymogram](image)

Fig. 8. Gelatin zymogram showing MMP-2 activity in conditioned media of periosteal cells cultured on type I collagen (lane 1) or on type I collagen with a mutated collagenase cleavage site (lane 2). Note that the level of MMP-2 is similar for both conditions.
Discussion

The present study demonstrated that collagen degradation of soft connective tissue explants correlated positively with the level of active MMP-2, whereas such a relationship was not found with active collagenases. Although this relationship suggests that MMP-2 is important for the digestion of collagen in the soft connective tissue model used, the lack of a relation with collagenases does not exclude a priori a role of MMP-1 in this process. The low levels of collagenase activity in the conditioned media can be explained by assuming that most of the enzymes were bound to their natural inhibitor, TIMP-1\textsuperscript{22}. However, this does not seem very likely since we have shown previously that, in contrast to calvarial bone, isolated periosteal tissue produces extremely low levels of this inhibitor\textsuperscript{23,24}. Moreover, incubations carried out with synthetic MMP-inhibitors, of which two were used at a concentration far below the $K_i$-value for MMP-1, almost completely prevented degradation. Apart from MMP-1, it is also possible that MMP-13 (collagenase-3) participated in the breakdown. After all low concentrations of CT1166 appear to inhibit the activity of this enzyme (Drs. J.M. Délaissé and M. Ferreras, personal communication). Yet, involvement of this enzyme is not very likely since no correlation was found between collagenase activity (which also includes the activity of MMP-13) and collagen breakdown of the tissue explants. Finally, fibroblasts isolated from the periosteum proved to have the capacity to digest collagenase-resistant type I collagen to the same extent as non-mutated (normal) collagen. Although this finding supports the view that collagenase is not a prerequisite for collagen digestion, it does not provide direct evidence for MMP-2 involvement. After all, Aimes and Quigly\textsuperscript{25} demonstrated that cleavage of collagen by MMP-2 also occurs at the "34-34" site, the site that was mutated in the collagen used in the present study. However, we analysed digestion of this collagen not by incubating it with purified enzyme but by seeding periosteal fibroblasts on it. In this far more complex system we assume that membrane-bound enzymes, such as MMP-2\textsuperscript{4}, in conjunction with plasma membrane-associated proton pumps\textsuperscript{16}, result in the degradation of the collagen fibril.

The effects of the inhibitors on the collagen breakdown and the positive correlation of MMP-2 activity and collagen breakdown support the notion that MMP-2 is involved in degradation of collagen. However, the question still remains whether other MMPs, such as MMP-3 and MMP-9 which are also inhibited by the synthetic inhibitors, participated. Casein zymography did not reveal any activity in conditioned media or tissue extracts, indicating that MMP-3 was below the detection limit. The presence of MMP-9, on the other hand, was
convincingly demonstrated in conditioned media. The level of activity of this enzyme did not show any relationship with the time-dependent increase of collagen breakdown, thus making its participation doubtful. Apart from these enzymes, it is feasible that members of the recently described group of MT-MMPs (MMP-14-17)\textsuperscript{26-29} were involved in the degradation of collagen. It is of interest to note that at least one of these enzymes (MT1-MMP) not only appears to have the capacity to activate membrane-bound MMP-2 but also to digest various extracellular matrix components\textsuperscript{30,31}.

Our results, in conjunction with other studies\textsuperscript{4,5,25}, suggest that (membrane bound) MMP-2, either or not with MT-MMPs, is important in the degradation of soft connective tissue collagen. MMP-1, hitherto considered crucial in this process, does not seem to play a critical role.

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