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Differential regulation of MMP-13 (collagenase-3) and MMP-3 (stromelysin-1) in mouse calvariae[1]

Chantal Peeters-Joris [a,*], Khalil Hammani [2,a], Christian F. Singer [3,b]

[a] *Laboratoire de Chimie Physiologique (Connective Tissue Group), Université de Louvain and C. de Duve Institute of Cellular Pathology, Brussels, Belgium*

[b] *Cell Biology Unit, Université de Louvain and C. de Duve Institute of Cellular Pathology, Brussels, Belgium*

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Abstract

Bone resorption in mice involves the degradation of extracellular matrix. Whereas several proteases seem to be implicated in this process, it becomes increasingly clear that matrix metalloproteinases (MMPs), amongst them especially MMP-13 and MMP-3, play an essential role. We have purified MMP-13 and MMP-3 from mouse calvariae-conditioned media by differential fractionation and analyzed their collagenolytic, caseinolytic, gelatinolytic and proteoglycanolytic activities. It could be shown that in mouse calvariae-conditioned media most of the measured enzyme activities were due to MMP-13, although zymographies revealed that MMP-3, MMP-2, MMP-9 as well as TIMPs were present too. MMP-13 and MMP-3 proteins were detected and their enzyme activities were neutralized by specific polyclonal antisera. Furthermore, it was demonstrated that in cultures of mouse calvariae the production of MMP-13 was induced by the potent MMP-stimulator heparin and by parathyroid hormone (PTH), whereas the levels of MMP-3 remained unchanged. Although PTH-induced bone resorption was inhibited by calcitonin treatment, MMP-13 mRNA and protein expression were not significantly altered by this hormone. Together with previous observations, these results indicate that PTH regulates bone resorption through MMP-13, but not by MMP-3, and that its reversion by calcitonin involves neither of the two enzymes.

Keywords: Metalloproteinase; Tissue inhibitor of metalloproteinase; Expression; Parathyroid hormone; Heparin; Antibody

1. Introduction

Bone resorption involves the removal of minerals and the degradation of organic matrix constituents. This physiopathological process requires the co-operation between osteoblasts and osteoclasts and

Abbreviations: APMA, 4-aminophenylmercuric acetate; CM, mouse calvariae-conditioned medium; DTT, dithiothreitol; MMP, metalloproteinase; MMP-2, (72 kDa) gelatinase A; MMP-3, stromelysin-1; MMP-9, (92 kDa) gelatinase B; MMP-13, collagenase-3; PGC, proteoglycan aggregate; SDS, sodium dodecyl sulfate; TIMP, tissue inhibitor of metalloproteinases

* Corresponding author. Department of Biology, University of Louvain, Canoy, Place Croix du Sud 5, B-1348 Louvain-la-Neuve, Belgium. Fax: +32 (10) 473515; E-mail: peeters@bchm.ucl.ac.be

[1] Dedicated to Professor Gilbert Vaes.

[2] Present address: Department of Biology, University Moulay Ismail, Boutalamine B.P. 509, Errachidia, Morocco.

[3] Present address: Department of Special Gynaecology, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

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results in the secretion of lysosomal cysteine proteinases and of collagenase [1]. The rodent-type collagenase, collagenase-3 or MMP-13, belongs to the family of matrix metalloproteinases (MMPs), a group of endopeptidases together able to degrade most of the structural protein constituents of the extracellular matrix [2–4]. MMPs form a group of zinc-dependent endopeptidases that share homologous protein sequences. They are active at neutral pH in the presence of bivalent metal ions and are inhibited by tissue inhibitors of metalloproteinases (TIMPs). Secreted as proenzymes, they are activated through limited proteolytic cleavage, which probably occurs by proteinases such as plasmin [5], stromelysin-1 [6] or by the recently discovered membrane-type MMPs [7]. In vitro activation of the latent enzymes can be achieved through cleavage with trypsin or through induction of conformational changes by reagents such as organomercurials or detergents.

Among the MMPs, collagenases are exceptional in their ability to degrade native collagen, the major protein in the extracellular matrix, by cleaving its triple helix into characteristic 1/4 and 3/4 fragments. Furthermore, parathyroid hormone (PTH), a physiological inducer of bone resorption, has been found to stimulate MMP-13 protein in rodent bones with consequences on the condition of extracellular matrix [8]. Proteoglycans are another important constituent of the extracellular matrix. MMP-3 is an enzyme which not only cleaves the protein core of proteoglycans [9], but also is a potent activator of other MMPs [6]. We have therefore purified MMP-13 and MMP-3 from mouse calvariae-conditioned media (CM) and analyzed the proteolytic activities of both enzymes in collagenolytic, caseinolytic, gelatinolytic and proteoglycanolytic assays as well as in gelatin and casein zymographies. Purified MMP-13 and MMP-3 were also used to raise specific polyclonal antisera adequate for the immunodetection of mouse MMP-13 and MMP-3 protein by Western blotting or ELISAs. We have then used mouse calvariae to study the effect of various concentrations of heparin, a potent MMP-stimulator, and of the physiological hormone PTH on MMP-13 and MMP-3 gene and protein expression.

Part of this work has been presented as preliminary communication [10].

2. Materials and methods

2.1. Materials

Mouse MMP-13 [11] and MMP-3 [12] cDNAs, cloned into pBluescript KS^+, were a gift of Dr. Y. Eeckhout. Purified bovine TIMP was a gift of Drs. T. Cartwright and D. Faucher (Rhône Poulenc Santé, Vitry-sur-Seine, France). NMRI (Naval Medical Research Institute) mice derived from the UCL animal house (Brussels, Belgium). Polyclonal antisera were raised in goats at the Centre d'Economie Rurale (Marloie, Belgium). Manufacturers for specific reagents and equipment are listed hereafter. Ambion (Austin, TX, USA): mouse GAPDH cDNA. Amersham (Little Chalfont, UK): biotinylated donkey anti-goat IgGs and biotinylated peroxidase streptavidin complex. Amicon (Beverly, MA, USA): PM 10 and PM 30 Diaflo Ultrafilters, Centricon and Centriprep concentrators. Bachem (Bubendorf, Switzerland): bovine parathyroid hormone (1–34). Bio-Rad (Hercules, CA, USA): molecular weight standards and Prep Cell model 491. Boehringer (Mannheim, Germany): streptavidin-β-galactosidase and methylumbelliferyl-β-galactopyranoside. Gibco-BRL (Paisley, UK): basal medium (Eagle), Medium 199. Nunc (Roskilde, Denmark): flat and conical bottom microtiter plates, ELISA plates and culture material. Pharmacia (Uppsala, Sweden): DEAE Sepharose CL-6B, heparin–Sepharose CL-6B, chelating Sepharose 6B and PD10 column. Promega (Madison, WI, USA): in vitro transcription kit. Sigma (St. Louis, MO, USA): APMA, salmon calcitonin, fluorescamin, heparin, indomethacin and *N*-hydroxysuccimidobiotin. Worthington (Freehold, NJ, USA): trypsin and soybean trypsin inhibitor. All other reagents were analytical grade chemicals from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Bone organ cultures

Frontal and parietal calvariae of 4–5-day-old NMRI mouse were maintained in serum-free basal Eagle's medium (1 ml/calvarium), supplemented with 100 U/ml penicillin and 78 U/ml streptomycin, at 5% CO_2 for 1–4 days at 37°C either in the absence or presence of 300 μg/ml heparin as previously de-

scribed [13]. Conditioned media were then collected, centrifuged, stabilized by 5 mM $CaCl_2$, 0.05% Triton X-100 and 0.02% azide in either 50 mM Tris-HCl pH 7.5 or 50 mM cacodylate pH 7 buffer, and frozen. In the PTH-induced bone resorption experiments, calvariae were maintained in the absence or presence of PTH in serum-free Medium 199 (2 ml/calvarium), supplemented with 5 μg/ml indomethacin, 200 μg/ml glutamine and 30 μg/ml ascorbic acid, for 6–72 h at 37°C as previously described [8]. PTH-induced bone resorption was inhibited by addition of 0.9 U/ml calcitonin. Calvarial MMPs were extracted as previously described [14] and Ca^{2+} release into the medium was measured by atomic absorption spectrophotometry.

2.3. Culture medium fractionation

Two hundred to 500 ml of mouse calvariae-conditioned media, produced in the presence of 300 μg/ml heparin for 4 days, was fractionated as described below. The purification processes represent a final development of previously described procedures [15,16]. The media were first concentrated 10× on an Amicon PM 10 or PM 30 membrane, dialyzed twice against 10 vol of CCBT (50 mM Na-cacodylate buffer pH 7 containing 5 mM $CaCl_2$, 0.05% Brij-35 and 0.03% toluol) and centrifuged for 20 min at 41 000×*g*. The supernatants were applied on a DEAE Sepharose CL-6B column (15×1.6 cm), previously equilibrated with CCBT. The flow through medium was fraction C1. The column was rinsed with 60 ml CCBT yielding fraction C2. To purify MMP-13, the DEAE Sepharose CL-6B column was first washed with 60 ml of 0.1 M NaCl CCBT (fraction C3). Fraction C4 was obtained by eluting with 60 ml 0.2 M NaCl CCBT and by a subsequent passage through a heparin–Sepharose CL-6B column (20×1.6 cm) previously equilibrated with 0.1 M NaCl CCBT. A rinse with 80 ml of 0.6 M NaCl CCBT yielded fraction C5. The column was then eluted with 80 ml 1 M NaCl CCBT and the eluate was passed through a chelating Sepharose 6B column (5×1.6 cm) previously saturated with $ZnCl_2$ and equilibrated with 0.6 M CBBT: the flow through was fraction C6. The column was then eluted with 48 ml of 1 M NaCl CCBT containing 50 mM imidazole; the resulting eluate was fractionated in 6 ml fractions (fractions C7–C15). MMP-3 was purified from fractions C1 and C2. Both fractions were combined and applied to a heparin–Sepharose CL-6B column (8×2.6 cm) previously equilibrated with CCBT; the flow through eluate was termed fraction S1. The column was rinsed with 90 ml of CCBT (fraction S2), then with 90 ml 0.1 M NaCl CCBT (fraction S3), and subsequently eluted with 120 ml CCBT 0.2 M NaCl. A further passage through a chelating Sepharose 6B column (10×1 cm), previously saturated with $ZnCl_2$ and equilibrated with 0.1 M NaCl CCBT, resulted in fraction S4. Finally, the column was eluted with 0.2 M NaCl CCBT containing 50 mM imidazole, and the eluate was separated in 6 ml fractions (fractions S5–S10). The purification steps are schematized in Fig. 1. All chromatographic procedures were carried out at 4°C at a flow rate of 18 ml/min. When the initial sample was applied on the DEAE Sepharose CL-6B column and when eluates were passed through two columns the flow rate was reduced to 7.2 ml/min. Protein concentrations were measured with fluorescamin [17].

2.4. Enzyme assays

Total lytic activities were measured on collagen, casein and gelatin after activation by 0.25–0.4 mM APMA for 2 h at 25°C, or by 2.5–10 μg/ml trypsin for 10 min at 25°C, or at 37°C where indicated, as previously described [18]. Collagenolytic activities were measured by incubating the samples with ^{3}H-acetylated acid-soluble guinea pig skin or rat tail collagen at 25°C in a soluble collagen assay [18] or at 35°C in a diffuse-fibril assay [19]. Caseinolytic activities were assayed by incubating the samples with ^{3}H-methylated casein at 37°C [20] and gelatinolytic activities by incubating them with ^{3}H-acetylated gelatin at 25°C [21]. In all assays, 1 U corresponded to the amount of enzyme that degrades 1 μg of substrate per minute. Proteoglycanolytic activities were measured by the ability to degrade [^{3}H]proteoglycan aggregates (PGCs) as previously described [22]. Alternatively, [^{3}H]PGCs, equivalent to 3.75 mg/ml uronic acid in 5 mM Tris-HCl pH 7.5, were copolymerised with 5% acrylamide between two mini gel glass plates and 5 mm diameter 0.75 mm thick discs were punched out of the gel. The discs were rinsed

four times with 0.15 M NaCl, 0.02% azide. To measure proteoglycanolytic activity, each disc was incubated with the sample under mild agitation in a total volume 100 μl of 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 0.1 M NaCl, 0.05% Triton X-100, 0.02% azide for up to 9 h at 37°C. Under these conditions, PGCs degradation was linear in function of time and logarithmic in function of sample concentration for at least 9 h of incubation and for up to 40% of substrate degradation (data not shown). One unit was defined as the amount of enzyme degrading 10% of substrate in 1 h.

2.5. Zymographies and preparative electrophoresis

Gelatin, casein and reverse (gelatin) zymographies were performed as described elsewhere [21], except that mouse calvariae-conditioned culture medium was used in the overnight reverse (gelatin) zymographies incubation buffer. The samples analyzed in reverse zymographies were 10× more concentrated than in the corresponding zymographies. Where indicated, zymographies were carried out under reducing conditions by addition of 50 mM DTT in the loading buffer. Preparative electrophoresis was performed by using a Prep Cell with 20 ml of 10% polyacrylamide separating gel and 5 ml of 4% polyacrylamide stacking gel. The elution buffer was SDS-free and flow rate was 18 ml/min. Fractions containing either pure MMP-13 or MMP-3 were combined and, in order to eliminate the contaminating SDS, were dialyzed 3× against 5 mM Tris-HCl buffer pH 7.5 containing 0.005% Triton X-100 and 0.03% toluol and 3× against PBS containing 0.05% Brij-35. The dialysate was concentrated in a Centripep.

2.6. Production, purification and labeling of antibodies

Polyclonal sera against mouse MMP-13 or MMP-3 were produced by immunizing goats with 50–100 μg of the purified proteins obtained by Prep Cell electrophoresis. IgGs were purified by combined Rivanol fractionation [23] and ammonium sulfate fractionation. Protein concentrations were measured according to Lowry [24]. *N*-Hydroxysuccimidobiotin was used to biotin label the IgGs [25].

2.7. Western blotting

Samples were concentrated 10× with a Centricon and subjected to Western blotting [26] after SDS-polyacrylamide gel electrophoresis under non-reducing conditions. PBS containing 5% skimmed milk powder, 0.05% Tween-20 and 0.02% azide was used as blocking solution. The washing buffer was PBS, 0.05% skimmed milk powder, 0.05% Tween-80, 0.02% azide. Amounts of 0.5–10 μg/ml of anti-mouse MMP-13 or anti-mouse MMP-3 IgGs were used as primary antibody. Immune complexes were detected by biotinylated donkey anti-goat IgGs. The secondary antibodies were visualized by using a biotinylated peroxidase streptavidin complex with 4-chloro-1-naphthol as substrate according to the manufacturer's instructions.

2.8. ELISAs

Direct competitive ELISAs were developed for total MMP-3 and MMP-13. For the MMP-3 ELISAs, 96-well microtiter plates were coated with 100 μl of fractions C1+C2 adjusted to 0.6 μg/ml total protein in PBS. For the MMP-13 ELISAs, a fraction C4 was used that had been passed through a PBS, 0.02% azide equilibrated PD10 column, instead of a heparin–Sepharose CL-6B column, to eliminate interfering Brij-35; microtiter plates were coated with 100 μl of 2 μg/ml total protein eluate. The coated plates were left overnight at 4°C and washed 3× with the washing solution (PBS, 0.5% skimmed milk powder, 1 mM EDTA, 0.05% Tween-80, 0.02% azide). The protein binding sites were then saturated with 100 μl of PBS, containing 5% skimmed milk powder, 1 mM EDTA, 0.05% Tween-80 and 0.02% azide, for 1 h at 35°C, and rinsed 3×. Samples and standards were treated with 0.4 mM APMA for 2 h at 25°C and serial dilutions were incubated for 2 h at 35°C with an equal volume of either 10 μg/ml biotinylated anti-mouse MMP-13 IgGs or 20 μg/ml biotinylated anti-mouse MMP-3 IgGs; 100 μl was added per well and incubated for 2 h at 25°C. After three washes, 100 μl of 1/2000 streptavidin-β-galactosidase was added per well and the plates were incubated for 1 h at 35°C. After another washing cycle, 100 μl of 10 mM K-phosphate buffer pH 7, containing 0.3 mM methylumbelliferyl-β-galactopyranoside, 0.15 M NaCl,

2 mM $MgCl_2$ and 0.1% BSA, was added and the plates were incubated at 35°C. Resulting fluorescence was measured by a Cytofluor fluorimeter after different time intervals for up to 1 h. Under these conditions, both immunoassays were capable of detecting at least 4 ng of enzyme and were not influenced by the presence of TIMPs (data not shown).

2.9. RNA extraction and RNase protection assay

Total RNA was extracted from frozen calvariae as previously described [27]. The quantity of total RNAs was determined by spectrophotometry and quality was assessed by denaturing gel electrophoresis. MMP-13 and MMP-3 mRNAs were detected by RNase protection assays [28]. ^{32}P-Labeled probes were synthesized by in vitro transcription from MMP-13 and MMP-3 cDNA templates according to the manufacturer instructions. An amount of 20 μg of total RNA was hybridized to the probe and subjected to RNase protection assays as previously described [29]. Mouse GAPDH was used as loading control. Probably due to incomplete in vitro transcription of the MMP-3 cDNA, specific hybridization to mRNA resulted in a 'ladder-type' appearance of the protected fragment. Hybridization of MMP-3 alone confirmed that the ladder represented a specific signal (data not shown). The gels were scanned on an Apple color scanner using Ofoto software and the images analysis were performed using NIH Image 1.62.

3. Results and discussion

3.1. Purification of mouse MMP-13 and MMP-3

Purification of MMP-13 or of MMP-3 was performed as described in Section 2. The progressive enrichment of the fractions in MMP-13 or MMP-3 was assessed by collagenolytic, caseinolytic, gelatinolytic and proteoglycanolytic assays after APMA or trypsin activation, and by gelatin and casein zymographies. TIMPs were detected by reverse (gelatin) zymography.

The results of the fractionation of conditioned media are shown in Fig. 2. The vast majority of proteins (94%) was eliminated in fractions C1–C6 (Fig. 2A).

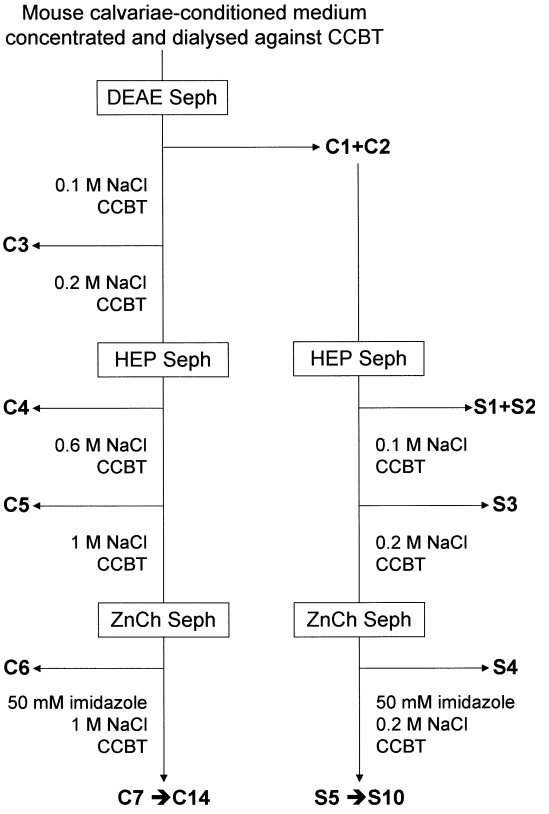

Fig. 1. Schematic presentation of the chromatographic conditions used to fractionate mouse calvariae-conditioned media. CCBT = 50 mM Na-cacodylate buffer pH 7 containing 5 mM $CaCl_2$, 0.05% Brij-35 and 0.03% toluol; DEAE Seph = DEAE Sepharose; HEP Seph = heparin Sepharose; ZnCh Seph = Zn^{2+}-saturated chelating Sepharose.

Although fractions 9 and 10 contained only 4% of the total protein in conditioned medium, they represented more than 90% of the overall collagenolytic activity (Fig. 2B after APMA activation, Fig. 2C after trypsin activation). Caseinolytic (Fig. 2E) and gelatinolytic (Fig. 2G) activity profiles after trypsin activation corresponded well with the collagenolytic activity profile. However, after APMA activation, but not after trypsin activation, caseinolytic activity was detected in fraction C2 (Fig. 2D) and gelatinolytic activity in fraction C3 (Fig. 2F). Whereas the source of the caseinolytic activity in C2 was not yet identified here, gelatinolytic activity of fraction C3 probably contained MMP-2, which degrades gelatin and is only activatable by APMA, but not by trypsin [30]. The caseinolytic activity after APMA activation (Fig. 2D) had the same profile as the proteoglycano-

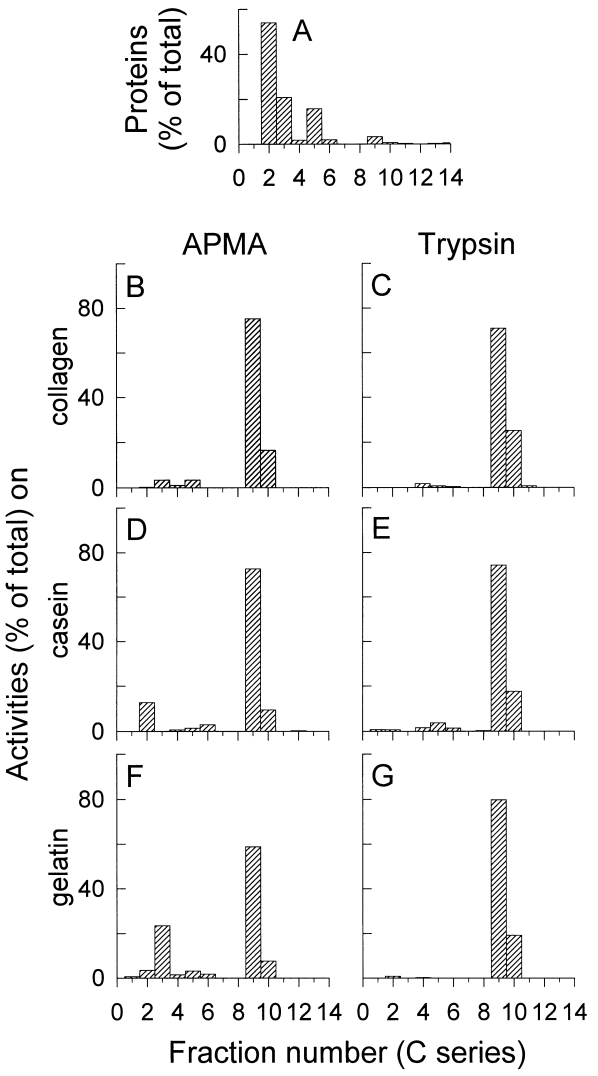

Fig. 2. Fractionation of mouse calvariae-conditioned media. Two hundred mouse calvariae were maintained in basal Eagle's medium for 4 days in the presence of 300 μg/ml heparin. The resulting conditioned media were concentrated 10× and fractionated according to the schema in Fig. 1. Enzyme activities were measured in collagenolytic (B,C), caseinolytic (D,E), and gelatinolytic (F,G) assays, after activation with either 0.4 mM APMA for 2 h at 25°C (B,D,F), or 5 μg/ml trypsin for 10 min at 25°C (C,E,G). Protein content (A) and enzyme activities (B–G) in fractions C1–C14 are presented as percent of the sum recovered in fractions C1–C14. 100% = 9.47 mg in A, 709 U in B, 1348 U in C, 219 U in D, 428 U in E, 1223 U in F and 1962 U in G.

lytic activity after APMA activation (data not shown), suggesting that the proteins present in C2 and C9+C10 have proteoglycanolytic and caseinolytic activities alike.

Zymographic analysis of fractions C1–C14 is shown in Fig. 3. Gelatinolytic activity of the conditioned medium (CM) was mainly recovered in fraction C3 at 68 and 62 kDa, corresponding to the unreduced state of latent and active MMP-2 [31], and in fraction C5 at 92 kDa, corresponding to MMP-9 (Fig. 3A). MMP-13 was mainly present in fractions C9 and C10 and appeared as two doublets in the gelatin zymography. The upper doublet of 61 and 55 kDa corresponded to the latent enzyme and the lower one of 46 and 40 kDa was caused by the active enzyme. Each doublet was present in the glycosylated and the non-glycosylated forms. Due to their close molecular weights, the more luminous MMP-2 doublet overlapped the latent MMP-13 doublet in the gelatin zymographies of the unfractionated media (CM). However, when 50 mM DTT was added to the loading buffer, the MMP-2, MMP-9 and TIMP signals disappeared thus allowing for enhanced visualization of MMP-13 activity (Fig. 3B). This specific effect of DTT on MMP-2 and MMP-9, but not on MMP-13, suggests the existence of disulfide bridge(s) in the cysteine-rich fibronectin-like domain which is exclusively present in gelatinase molecules and is responsible for their binding to gelatin. Fig. 3C shows that caseinolytic activity was present in fractions C2, C9 and C10. The activity profiles in fractions C9 and C10 were identical with the two doublets seen in the gelatin zymographies in Fig. 3A,B. They also correlated with the profiles observed for C9 and C10 in Fig. 2D,E, suggesting that MMP-13 has caseinolytic activity. The reverse (gelatin) zymographies indicate that TIMPs were present in fractions C2 and C3, and as traces also in fractions C9 and C10 (Fig. 3D).

Overall, the fractionation of mouse calvariae-conditioned media revealed that MMP-13 accounted for all the collagenolytic activity and for most of the caseinolytic and gelatinolytic activities measured in this tissue. The discrepancy observed for the MMP-2 containing C3 fraction between its relatively low gelatinolytic activity measured in the enzyme assay (Fig. 2F) and its strong signal observed on the gelatin zymography (Fig. 3A) is likely to be due to the presence of TIMPs.

In Fig. 3C a band of 57 kDa was observed in fraction C2 and, although inconsistently, in fraction C1. Since the size of mouse MMP-3 is 57 kDa and since MMP-3 is known to posses proteoglycanolytic

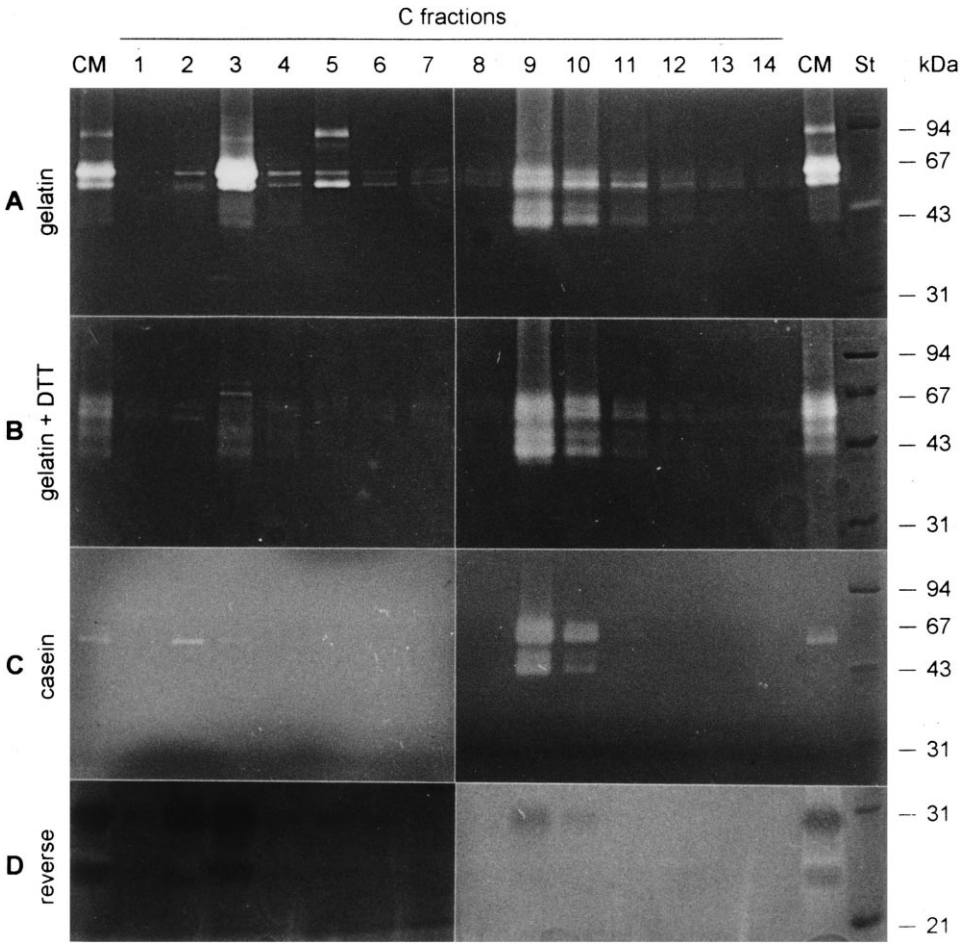

Fig. 3. Zymographic analysis of mouse calvariae-conditioned media. Unfractionated mouse calvariae-conditioned media (CM) and fractions C1–C14 were subjected to SDS-polyacrylamide electrophoresis containing gelatin (A,B,D) or casein (C). Prior to migration, 1 vol. of loading buffer without (A,C,D) or with 50 mM DTT (B) was added and 15 μl was loaded onto the gel. Reverse (gelatin) zymographies are shown in D. St = molecular weight standards.

activity, fractions C1 and C2 were combined and provided the starting material for the MMP-3 purification. The resulting fractions S1–S10 were subjected to caseinolytic and proteoglycanolytic assays (Fig. 4). Whereas 90% of the proteins of the starting material was eluted in S1–S3 (Fig. 4D), most caseinolytic activity (Fig. 4A after APMA activation, Fig. 4B after trypsin activation) and proteoglycanolytic activity (Fig. 4C) were recovered in fraction S8. No significant collagenolytic or gelatinolytic activities were detected in any of the fractions (data not shown).

Fractions S1–S10 were then subjected to gelatin, casein and reverse (gelatin) zymographies to further characterize the enzyme activities present (Fig. 5). In fraction S8, a strong single band of gelatinolytic (Fig. 5A) and caseinolytic (Fig. 5B) activities was visible at approximately 57 kDa, thus corresponding to MMP-3. It is also likely that MMP-3 was responsible for the strong caseinolytic and proteoglycanolytic activities of fraction S8 in Fig. 4A–C. Furthermore, a band of 51 kDa was visible in fractions S1 and S2 on the gelatin and casein zymographies whose identity is not clear. The reverse (gelatin) zymography in Fig. 5C shows that the bulk of TIMPs had already been eliminated from the S fractions by the passage through the heparin–Sepharose column.

Although the purified MMP-13 and MMP-3 appeared pure as judged by silver-stained SDS-PAGE (data not shown), reversed (gelatin) zymographies still showed contamination with traces of TIMPs (Fig. 3D, C9 and C10, and Fig. 5C, S8). These pro-

teins were eliminated from the end products by continuous elution electrophoresis with the 'Prep Cell'. Fig. 6 shows a representative reverse (gelatin) zymography of fractions C9+C10 and subsequent fractions in which residual TIMPs were progressively eliminated. TIMPs appeared in fractions 24–29, whereas MMP-13 was present in fractions 32–55 (only critical fractions 24–35 are shown). TIMPs were eliminated from the purified MMP-3 fraction (S8) by using the same procedure (data not shown).

3.2. Characterization of polyclonal sera against MMP-13 and MMP-3

The highly purified and TIMP-depleted MMP-13 and MMP-3 fractions were used to raise polyclonal antisera as detailed in Section 2. Fig. 7 shows a Western blot where conditioned medium, purified MMP-13 and purified MMP-3 were subjected to Western blotting and incubated with either anti-mouse MMP-13 or anti-mouse MMP-3 IgGs. Both IgGs preparations reacted with MMP-13 and MMP-3 present in the conditioned medium (CM), and each IgGs preparation detected only the purified MMP they had been raised against. No cross reactivity was observed and no bands at the size of TIMP-1 or TIMP-2 (21 and 28 kDa) were visible. Whereas the addition of anti-mouse MMP-13 IgGs to conditioned medium resulted in one double band, incubation with purified MMP-13 yielded two doublets and a band at 25 kDa, suggesting that MMP-13 had been partly autoactivated during the purification process and with time. Furthermore, neither anti-mouse MMP-13 nor anti-mouse MMP-3 IgGs crossreacted with partially purified mouse fractions of TIMPs or

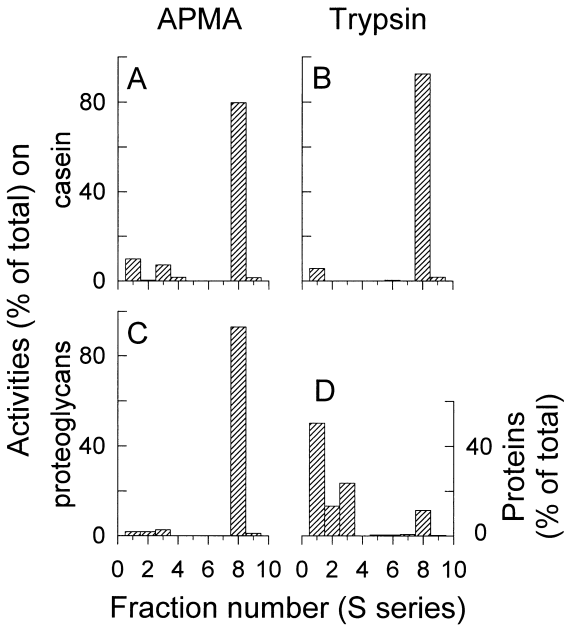

Fig. 4. Caseinolytic and proteoglycanolytic activities of MMP-3 enriched fractions. Four hundred and twenty-six mouse calvariae were maintained in basal Eagle's medium for 4 days in the presence of 300 μg/ml heparin. The resulting conditioned media were concentrated 10× and fractionated according to the schema in Fig. 1. Fractions C1 and C2 were combined and further processed to purify MMP-13. Enzyme activities (A–C) and protein content (D) in fractions S1–S10 are presented in percent of the sum recovered in the fractions S1–S9. Caseinolytic (A,B) and proteoglycanolytic (C) activities were measured after activation by 0.25 mM APMA for 2 h at 25°C (A,C) or by 5 μg/ml trypsin for 10 min at 37°C (B). Proteoglycanolytic activities were measured on PGCs entrapped in polyacrylamide discs as described in Section 2. 100% = 226 U in A, 304 U in B, 36.1 U in C and 2.62 mg in D.

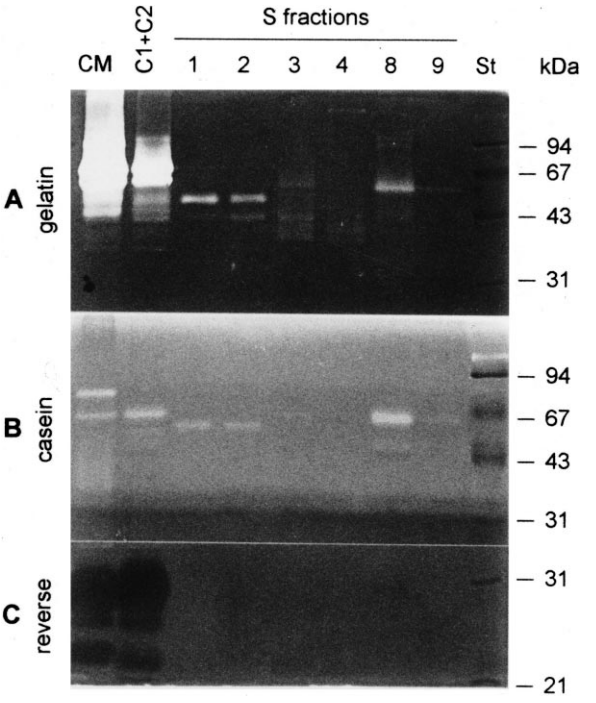

Fig. 5. Zymographic analysis of fractions S1–S9. Mouse calvariae-conditioned medium (CM), fractions C1+C2 and the S fractions were submitted to SDS-polyacrylamide electrophoresis containing gelatin (A,C) or casein (B). Fractions S1–S4, S8 and S9 are shown. Samples were diluted with 1 vol. of loading buffer without DTT. Reverse (gelatin) zymography is shown in C. St = molecular weight standards.

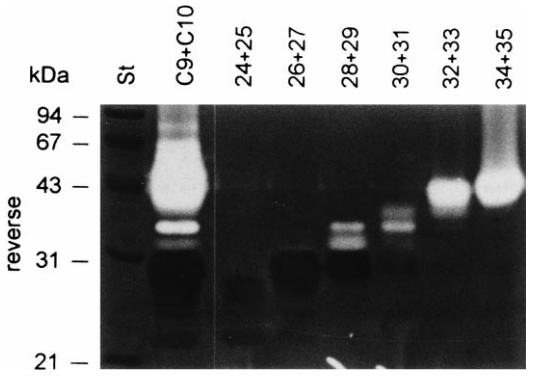

Fig. 6. Elimination of contaminating TIMPs by continuous elution electrophoresis. MMP-13 enriched fractions C9 (4 ml) and C10 (2 ml) were combined, concentrated 5× and diluted with 1 vol. loading buffer. An amount of 2 ml, containing 560 μg total proteins, was then layered onto a SDS-polyacrylamide column as detailed in Section 2. About 100 fractions of 3 ml each were collected. Fractions eluted before the loading buffer dye were discarded. Subsequent fractions were paired and analyzed by reverse (gelatin) zymography. Fractions 24–35 are shown. St = molecular weight standards.

MMP-2 (data not shown). The capacity of the antisera to neutralize the enzymatic activities of MMP-13 and MMP-3 was verified by immunoinhibition and immunoprecipitation experiments (data not shown).

3.3. *Heparin differentially regulates MMP-13 and MMP-3 expression in mouse calvariae-conditioned medium*

Heparin is a potent inducer of MMP-13 production by mouse bones in vitro [13]. To investigate the regulation of MMP-13 and MMP-3 expression, mouse calvariae were cultured in the absence or presence of increasing concentration of heparin for 2 or 4 days (Fig. 8). Gelatin zymographies performed on conditioned media show that in the absence of heparin the 46 and 40 kDa forms of MMP-13 were undetectable but appeared when 33–300 μg/ml heparin was present in the culture media (Fig. 8A). Conversely, the 68 and 62 kDa forms of MMP-2 and 92 kDa MMP-9 were already present in untreated media and remained unchanged upon the addition of heparin (Fig. 8A). Fig. 8B shows that the 57 and 60 kDa MMP-3 casein activities remained essentially unchanged when heparin was added to the cultures. Under the same experimental conditions, the secretion of TIMPs was similarly not affected by the presence of heparin (Fig. 8C). The differential expression pattern of MMP-13 and MMP-3 was even more evident in Western blots (Fig. 8D): whereas in the absence of heparin MMP-13 was not detectable in medium conditioned for 2 days, a faint signal was observed after 4 days. Addition of increasing concentration of heparin considerably augmented the signals. By contrast, MMP-3 was already immunodetected in untreated medium after 2 days and was not markedly altered by heparin addition (Fig. 8E).

The results of Western blots were furthermore confirmed by MMP-3 ELISAs, in which the protein amounts remained similarly unchanged regardless whether heparin was present or not, and whether calvariae were cultured for 24 or 48 h. By contrast, MMP-13 markedly augmented upon addition of heparin (Table 1).

To investigate MMP-13 and MMP-3 mRNA expression in response to heparin, RNase protection assays were performed on total RNAs from mouse calvariae that had been treated without or with 300 μg/ml heparin for 6–24 h. Whereas MMP-3 mRNA levels were equal in untreated and treated calvariae, MMP-13 mRNA expression was considerably upregulated in the presence of heparin after 24 h. However, in the absence of heparin MMP-13 mRNA appeared to increase at 24 h when compared to 6 h (Fig. 8F).

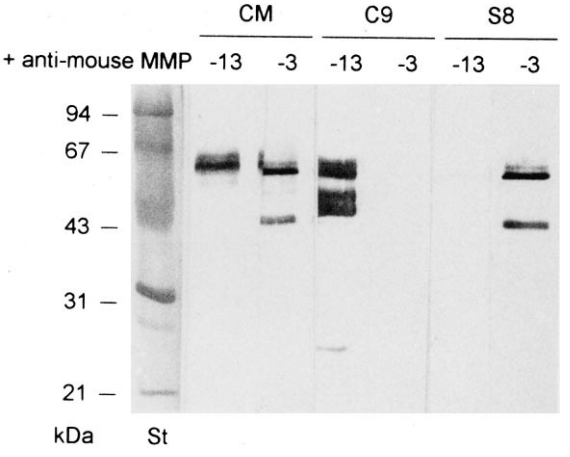

Fig. 7. Specificity of the anti-mouse MMP-13 and anti-mouse MMP-3 IgGs. 10× concentrated mouse calvariae-conditioned medium (CM), fractions C9, containing purified MMP-13, and fraction S8, containing purified MMP-3, were subjected to Western blotting and incubated with either 2.5 μg/ml anti-mouse MMP-13 IgGs (-13) or 0.6 μg/ml anti-mouse MMP-3 IgGs (-3). St = molecular weight standards.

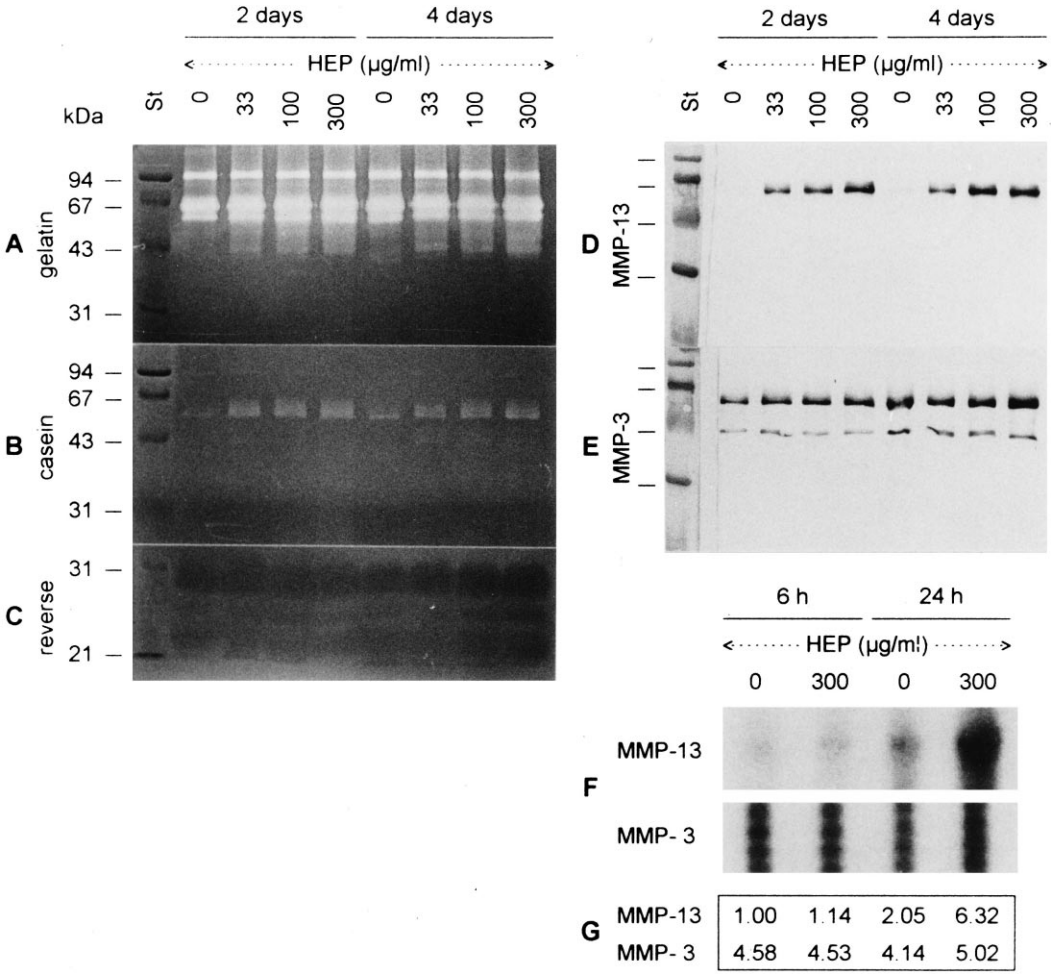

Fig. 8. MMP-13 and MMP-3 mRNA and protein expression in response to heparin. Ten mouse calvariae per experimental condition were maintained in basal Eagle's medium in the absence or presence of 33–300 μg/ml heparin for 2 or 4 days. Conditioned media were diluted with 1 vol. of loading buffer and 15 μl was subjected either to gelatin (A), casein (B) and reverse (gelatin) (C) zymographies or to Western blots incubated with 1 μg/ml anti-mouse MMP-13 (D) or MMP-3 (E) IgGs according to Section 2. RNase protection assays (F) were performed on total RNAs from 10 calvariae per experimental condition, maintained for 6 or 24 h in the absence or presence of 300 μg/ml heparin. Signal intensities, expressed in arbitrary units, are shown below the gels (G). St = molecular weight standards.

3.4. *PTH stimulates MMP-13, but not MMP-3, mRNA and protein expression in mouse calvariae extracts*

PTH is a physiological inducer of bone resorption and has previously been shown to stimulate MMP-13 protein expression in bones [8]. Calcitonin, on the other hand, counteracts PTH by inhibiting osteoclasts activity [32]. We have treated mouse calvariae with PTH in the absence or presence of calcitonin and compared the resulting collagenolytic activity with that of caseinolytic and gelatinolytic activities in the extracts (Fig. 9). Whereas collagenolytic and gelatinolytic activities significantly augmented with increasing PTH concentrations and reached a plateau at 10^{-8} M PTH (Figs. 9A and 9B, casein degradation did not change in response to PTH. The augmentation of Ca^{2+} release by increasing PTH (Fig. 9C) concentrations indicated that bone resorption had occurred (Fig. 9D). Addition of 0.9 U/ml calcitonin resulted in a significant decrease of extractable collagenolytic activity and in most cases also of gelatinolytic activity, whereas caseinolytic activity remained unchanged. Ca^{2+} release into the medium was

Table 1
Effect of heparin on the MMPs concentrations in the medium

Conditions		Enzyme assays (U/ml)			ELISAs (μg/ml)	
		COL	CAS	GEL	MMP-13	MMP-3
24 h	C	0.73	0.00	0.22	1.729	1.525
	+HEP	2.63	0.00	1.95	9.437	1.931
72 h	C	1.36	0.27	2.42	1.789	2.195
	+HEP	8.76	1.07	8.06	12.411	3.175

40 calvariae per experimental condition were maintained in cultured for 24 or 72 h without (C) or with 300 μg/ml heparin (+HEP). At the end of each culture, conditioned media were collected. All assays were performed after activation by 0.4 mM APMA for 2 h at 25°C. COL = collagenolytic activity measured with the diffuse fibril assay; CAS = caseinolytic activity; GEL = gelatinolytic activity.

equally decreased indicating that bone resorption had been inhibited by calcitonin. The observation that calcitonin decreased the PTH-induced collagenolytic activity somewhat contrasts with the results of Delaissé et al. [8] who described that calcitonin does not appreciably alter the effect of PTH. However, whereas Delaissé et al. used fetal mice calvariae, our experiments were performed with calvariae from newborn mice. As previously observed, culturing calvariae slightly, but significantly, increased collagenolytic and caseinolytic activities ($P < 0.05$ in Fig. 9A, $P < 0.017$ in Fig. 9C); still, gelatinolytic activities were equal in non-cultured calvariae and cultured calvariae (Fig. 9B).

Whereas the collagenolytic activity in Fig. 9A was specific for MMP-13, the gelatinolytic activity seen in Fig. 9B and the caseinolytic activity in Fig. 9C could also be partially due to other enzymes such as MMP-2, MMP-9 or MMP-3. We therefore further characterized the extracts from PTH-treated calvariae and performed reverse (gelatin) zymographies and casein zymographies after treatment of the samples with loading buffer without or with 50 mM DTT (Fig. 10). The reverse (gelatin) zymography in Fig. 10A shows in the absence of DTT that the levels of MMP-2 (68 and 62 kDa) and of the active form of MMP-9 (84 kDa) were increased in the presence of 5.10^{-8} M PTH, regardless on whether 0.9 U/ml calcitonin was present or not. Similarly, TIMP-1 protein was low in non-cultured and cultured calvariae, and increased upon addition of PTH in the absence or presence of calcitonin. To enhance the visibility of MMP-13, MMP-2, MMP-9 and TIMP-1 were inactivated by treatment with 50 mM DTT. Such treatment revealed that both the latent and the active forms of MMP-13 augmented after treatment with PTH in the absence or presence of calcitonin. Casein zymography (Fig. 10B) showed that MMP-3 activity was either absent or detected only at very low levels. Interestingly, in non-cultured and to a lesser extent in calvariae cultured without any addition, a caseinolytic activity was observed at 26 kDa. This 26 kDa activity was inhibited if the sample had been treated with loading buffer containing 50 mM DTT. By con-

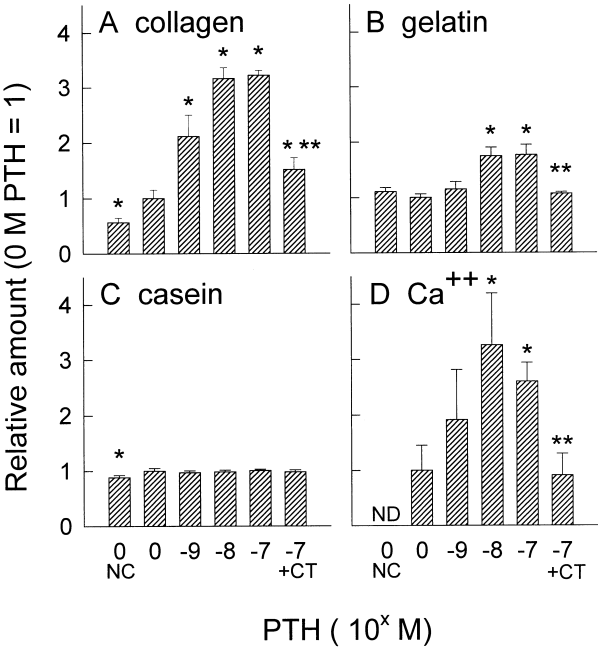

Fig. 9. PTH-induced bone resorption parallels MMP-13 but not MMP-3 production in mouse calvariae. Three calvariae per experimental condition were incubated for 3 days in the absence or presence of 10^{-9}–10^{-7} M PTH or 10^{-7} M PTH plus 0.9 U/ml of calcitonin and media were changed daily. Ca^{2+} concentrations were measured in each conditioned medium and the cumulative values over 3 days are shown in relation to non PTH-treated cultures (0 M PTH = 1) (D). At the end of the culture, calvariae were extracted as detailed in Section 2. Collagenolytic (A), gelatinolytic (B) and caseinolytic (C) activities were measured in the four sequential extracts and the sum of all four is shown in relation to the non PTH-treated culture (0 M PTH = 1). Values are means/calvarium ± standard deviation. 1 = 0.992 U in A, 0.528 U in B, 1.787 U in C and 25.2 μg in D. 0 NC = non-cultured calvariae; -7+CT = 10^{-7} M PTH plus 0.9 U/ml of calcitonin; ND = not done; *Significant vs. 0 M PTH ($P < 0.05$ or less). **Significant vs. 10^{-7} M PTH ($P < 0.01$ or less).

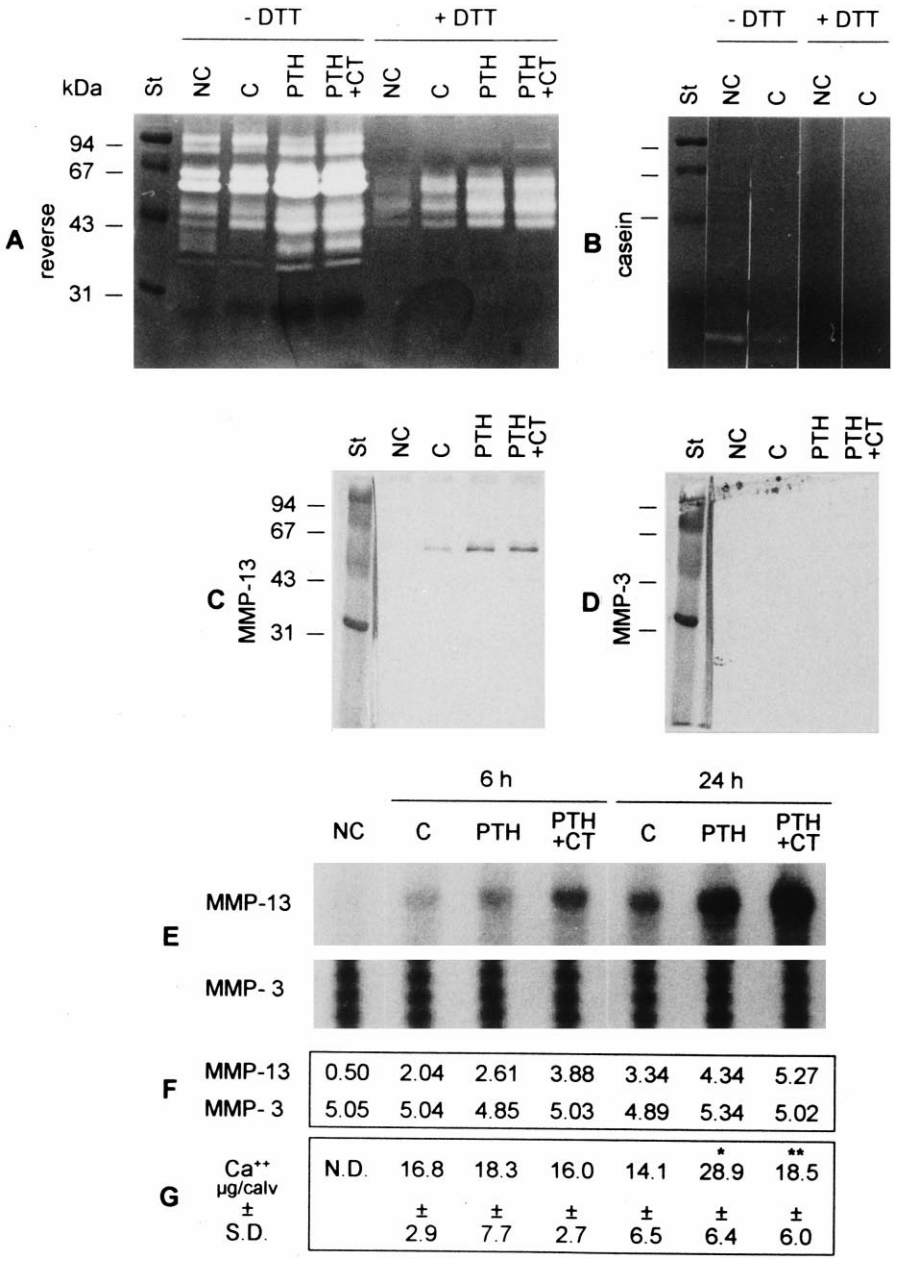

Fig. 10. PTH regulates MMP-13 but not MMP-3 mRNA and protein expression in mouse calvariae. Six calvariae per experimental condition were incubated for 2 days without (C) or with 5.10^{-8} M PTH in the absence (PTH) or presence of 0.9 U/ml calcitonin (PTH+CT). Media were changed after 24 h. Cultured as well as non-cultured (NC) calvariae were then extracted twice and concentrated 10×. Both extracts were combined, diluted with 1 vol. of loading buffer in the absence (-DTT) or presence (+DTT) of 50 mM DTT and 15 µl was submitted to reverse (gelatin) (A) or casein zymography (B). Western blots of the same samples were incubated with 5 µg/ml anti-mouse MMP-13 IgGs (C) and 5 µg/ml anti-mouse MMP-3 IgGs (D). RNase protection assays (E) were performed on total RNAs extracted from 10 calvariae per experimental condition either non-cultured (NC) or maintained in culture without (C) or with 5.10^{-8} M PTH in the absence (PTH) or presence of 0.9 U/ml calcitonin (PTH+CT) for 6 h and 24 h. Arbitrary values of signal intensities (F) as well as Ca^{2+} concentrations in the media (G) are given below the corresponding RNase protection assay. µg/calv = µg/calvarium. *Significant vs. incubation condition without PTH ($P < 0.0002$). **Significant vs. culture with PTH alone ($P < 0.003$). St = molecular weight standards.

trast, the activity was not inhibited by 10 μM E-64, 1 mM AEBSF (aminoethyl benzenesulfonylfluoride), 1 μM pepstatin, 10 mM 1,10-phenanthroline, or in the absence of Ca^{2+} and Zn^{2+}. Moreover, the activity on casein zymographies was stronger at pH 7.5 than at pH 5 and was absent at pH 10.3. Anti-mouse MMP-3 IgGs did not react with proteins in the 26 kDa region on the Western blots. The significance of this activity is not clear and we are currently further characterizing this protein.

Fig. 10C and D show Western blots of non-cultured and cultured calvariae in the absence or presence of PTH. MMP-13 levels were marginal in non-cultured calvariae, became clearly visible in cultured calvariae and were even higher in the presence of PTH; addition of calcitonin did not alter PTH-induced MMP-13 protein expression (Fig. 10C). MMP-3 in Fig. 10D was not immunodetectable in Western blots of extracts. In corresponding conditioned media, MMP-3 was clearly detectable by Western blotting, suggesting that the enzyme had already left the bone (data not shown).

The discrepancy between the lack of a calcitonin effect on MMP-13 expression in reverse (gelatin) zymography (Fig. 10A) and in Western blots (Fig. 10C), and the pronounced effects of this hormone detected as lower activities in the corresponding collagenolytic and gelatinolytic assays (Figs. 9A and 9B), is likely to be due to subtle increased TIMP concentrations. However, this still has to be investigated.

To analyze mRNA expression response to PTH, RNase protection assays were performed after 6 and 24 h of calvariae cultures. MMP-3 mRNA levels remained unchanged, regardless on whether calvariae were non-cultured or cultured under various conditions. By contrast, but in accordance with the findings by Henriet et al. [33], MMP-13 mRNA expression was at the level of detection in non-cultured calvariae, but augmented when calvariae were put in culture for 6 h or even more for 24 h. Furthermore, addition of PTH in the absence or presence of calcitonin led to a strong increase of mRNA levels. Whereas after 6 h Ca^{2+} concentration in the culture medium did not respond to PTH in the absence or presence of calcitonin, after 24 h of culture addition of PTH let to doubling of Ca^{2+} concentration, indicating that bone resorption was occurring. Counter-regulation by calcitonin caused a complete reversal of PTH-induced bone resorption.

Taken together, our experiments suggest that conditioned media from newborn mouse calvariae contain significant amount of MMP-13, MMP-3, MMP-2, MMP-9 and TIMPs. It was shown that MMP-13 is responsible not only for all of the collagenolytic, but also for most of the caseinolytic, gelatinolytic and proteoglycanolytic activities measured in mouse calvariae-conditioned media. Together with the observations of restricted MMP-13 expression during fetal bone development in both rodents [34,35] and humans [36,37], our data suggest that this enzyme also plays an important role in the extracellular matrix remodeling in newborn mice. However, its cellular localization (osteoblasts, osteoclasts) is still the object of debate [34–38]. Furthermore, we demonstrated that in vitro PTH-induced bone resorption is associated with an increase of MMP-13, but not MMP-3, mRNA and protein expression. However, the inhibitory effect of calcitonin on bone resorption is not due to a change in MMP-13 or MMP-3 expression, suggesting a different mode of action for this counter-regulatory hormone.

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