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Modulation of Neutral Matrix Metalloproteinases of Involuting Rat Mammary Gland by Different Cations and Glycosaminoglycans

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Abstract The synthesis and regulation of the matrix metalloproteinases (MMPs) are important factors contributing to the involution of mammary gland. In order to understand the role of these MMPs in involution and in remodeling of the mammary gland, the different MMPs (130K, 68K, and 60K gelatinases) were partially purified by gel filtration and affinity chromatography over gelatin Sepharose and subjected to kinetic analysis. Comparative analysis of the different gelatinases showed that the 130K that appears at the early involutary phase and the constitutive 68K enzyme are more specific for Col IV of the basement membrane, while the inducible 60K that appeared at the later phase of involution degraded Col I more efficiently. These neutral proteinases required Ca^{2+}/Zn^{2+} for their activity and the analysis of cation dependence revealed that Ca^{2+} at 10 mM concentration and above completely inhibited the enzyme. The 60K was active at very low concentration of Zn^{2+} (5 μM); but at higher concentration of Zn^{2+} (2 mM), where the 68K and 130K were active, the 60K gelatinase was inhibited, indicating a difference in the cation dependence of these enzymes. Chondroitin sulfate A and chondroitin sulfate C caused inhibition of the 130K, 68K, and 60K, while hyaluronic acid and heparin did not show any effect, suggesting that the chondroitin sulfate proteoglycan that decorates collagen in the ECM can modulate the activity of the collagenases in vivo. These results suggest that the 130K gelatinase expressed during the early phase of involution degraded Col IV of the basement membrane, making the 60K gelatinase formed at a later stage of involution more accessible to its preferred substrate (Col I of the underlying stroma), highlighting the role of these MMPs in mammary gland involution. J. Cell. Biochem. 73:218–226, 1999. © 1999 Wiley-Liss, Inc.

Key words: gelatinase; mammary gland; involution; inhibition by glycosaminoglycans; modulation by cations; substrate specificity

The interactions of cells with the extracellular matrix (ECM) molecules have important regulatory and structural consequence for cells. Studies have demonstrated the role of ECM in embryonic development, growth regulation, and the control of cellular phenotypes in specialised tissues [Ekblom et al., 1986; Bissell and Aggeler, 1987; Hay, 1991, 1993; Carey et al., 1986; Lin and Bissell, 1993]. Remodeling of the ECM occurs during wound healing [Galit and Clark, 1994] and in tissues such as mammary gland [Blum et al., 1987; Streuli et al., 1991], which undergoes proliferation, differentiation, and involution in adult life. The variation in topographical distribution of various ECM components and their degradation in the mammary gland during development probably play a key role in modulating cell functions [Li et al., 1987]. The changes in the structure of ECM during involution, which is accompanied by remodeling of the mammary gland, includes the discontinuation of the basement membrane around the secretory alveoli and a localized degradation of the ECM macromolecules [Warburton et al., 1982; Martinez-Hendez et al., 1976]. In vitro studies [Li et al., 1987; Streuli and Bissell, 1990] also suggested that basement membrane remodeling might be central to the regulation of mammary epithelial cell growth and function during mammary gland development. Reconstitution of the basement membrane is brought about by the degradation of the ECM macromolecules (e.g., collagen, fibronectin, and laminin) [Bernfield et al., 1984]. A group of metal-dependent endoproteinases that act at neutral pH termed as matrix metalloproteinases

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(MMPs) function in a coordinated fashion, bringing about the degradation of the ECM macromolecules [Birkedal-Hansen et al., 1993; Mignatti et al., 1996]. These enzymes are subject to transcriptional regulation as well as regulation by tissue inhibitor of metalloproteinases (TIMP) [Martisian, 1990] and hormones [Feng et al., 1995]. An increase in the production of MMPs during involution of mammary gland has been demonstrated [Dickson and Warburton, 1992; Strange et al., 1992]. Myoepithelial cells in mammary gland synthesize and secrete certain MMPs during involution [Andersson et al., 1994] and correlation between the expression of TIMP, MMP activity, and the loss of differentiated function has been demonstrated [Talhouk et al., 1992a]. Studies on mammary gland on different days of involution showed the presence of three gelatinases of different relative molecular weights [Talhouk et al., 1991; Ambili et al., 1997] (130K, 68K, and 60K). These gelatinases are regulated throughout development and are lowest during lactation. In order to understand the role of these MMPs in involution and in remodeling of the mammary gland, these enzymes were purified and characterized. The results reported in this paper indicate that 130K and 68K are more specific for Col IV of the basement membrane, while the inducible 60K is more active against Col I and are subject to inhibition by chondroitin sulfates, suggesting that the MMPs are also regulated by proteoglycans, a major component of the ECM.

MATERIALS AND METHODS

Materials

Heparin, hyaluronic acid, chondroitin sulfate A and chondroitin sulfate C were obtained from Sigma Chemical Co. (St. Louis, MO) and gelatin from Serva (Heidelberg, Germany). Col I from calf skin was kindly provided by J. Rauterberg (Munster, Germany) and Col IV was kindly provided by R.C. Hughes (NIMR, London). Sepharose 4B-CL and Sepharose 4B were from Pharmacia (Uppsala, Sweden). ^{125}[I]-NaI was a product of BARC (Mumbai, India).

Methods

Processing of mammary tissue. The gelatinases were purified and characterized from the 2nd (48 h after weaning) and 6th (144 h after weaning) involuting day mammary tissues from female Sprague-Dawley rats. Tissues were excised and extracted with phosphate buffer (1:4 w/v [0.1 M pH 7.5]). The supernatant after precipitation with 70% ammonium sulfate was dialyzed against phosphate buffer.

Fractionation of 130K and 68K gelatinase. The dialyzed supernatant after ammonium sulfate precipitation was concentrated and applied over a Sepharose 4B-CL column (1.5 cm $\times$ 35 cm), and the active fractions corresponding to 130K and 68K were pooled. The activity of the enzyme was checked at each step by zymography.

The fractions corresponding to 68K activity obtained from the Sepharose 4B-CL column were pooled and subjected to gelatin Sepharose affinity chromatography and eluted with, 1 M NaCl. Purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970], followed by silver staining [Ambili et al., 1997].

The 60K gelatinase was purified from the 6th involuting day mammary tissue by gelatin Sepharose affinity chromatography [Ambili et al., 1997]. Protein was determined either by measuring the absorbance at 280 nm or by the method of Lowry et al. [1951].

Zymography. Collagenase activity was assayed by preparing zymograms [Ambili et al., 1997]. Zymogram gels consisted of 10% polyacrylamide impregnated with gelatin (2 mg/ml). After electrophoresis, the gels were washed twice for 30 min each in 2.5% Triton-X-100 solution at room temperature, then incubated for 24 h in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3 pH 7.5) at 37°C. The gels were stained with Coomassie blue R250 for 1 h and destained in water. Gelatinase activity was seen as clear bands, indicating the degradation of gelatin.

Estimation of sugar. The glycoprotein nature of the enzyme was studied by analyzing the protein-bound carbohydrate by the phenol sulfuric acid method. D-Glucose served as the standard [Dubois et al., 1956].

Binding to Con A-Sepharose. The glycoprotein nature of these enzymes was also examined by studying the binding of the enzymes to ConA-Sepharose in acetate buffer (0.1 M pH 6.0) containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The bound material was eluted with increasing concentration of methyl-α-D-mannoside in the same buffer [Balasubramanian et al., 1975].

Substrate digestion. Gelatin, Col I, and Col IV were radiolabeled with 200 µCi ^{125}[I]-NaI, using chloramine-T [Roth, 1975]. The radiolabeled substrates (spec. act. 52,500 cpm/mg, protein) were incubated with the enzyme in phosphate buffer (0.1 M pH 7.5) in a total volume of 260 µl at 37°C for the required time interval. The enzyme action was stopped by the addition of an equal volume of 10% trichloroacetic acid (TCA). The soluble radioactivity was measured in a LKB-mini gamma counter. Different concentration of different substrates were used and the V_{max} and K_m of each enzyme for each substrate were calculated from double reciprocal plot.

Cation dependence. The cation dependence of the enzyme was studied using radiolabeled substrate as well as by zymography. The enzymes (25–30 µg/100 µl) in phosphate buffer (0.1 M pH 7.5) were preincubated with the cations (2 mM each of Ca^{2+}, Zn^{2+}, Mg^{2+}, and EDTA) and used for assay. Parallel controls without cations were taken. Cation dependence was also tested by incubating the zymograms in the substrate buffer containing 2 mM each of the cations.

Inhibition by glycosaminoglycans. The purified enzymes (25–30 µg) were preincubated with (6–8 µg) each of heparin, hyaluronic acid, chondroitin sulfate A, and chondroitin sulfate C in phosphate buffer (0.1 M pH 7.5). The samples were assayed using radiolabeled substrates, as well as by zymography. Parallel controls were taken without glycosaminoglycans. In order to assay the enzyme on substrate impregnated gels, the zymograms were prepared by copolymerizing gelatin (2 mg/ml), acrylamide (10%), and the enzyme (with or without GAG) in a multiwell plate. After polymerization, the gels were subjected to the same process as in zymographic analysis.

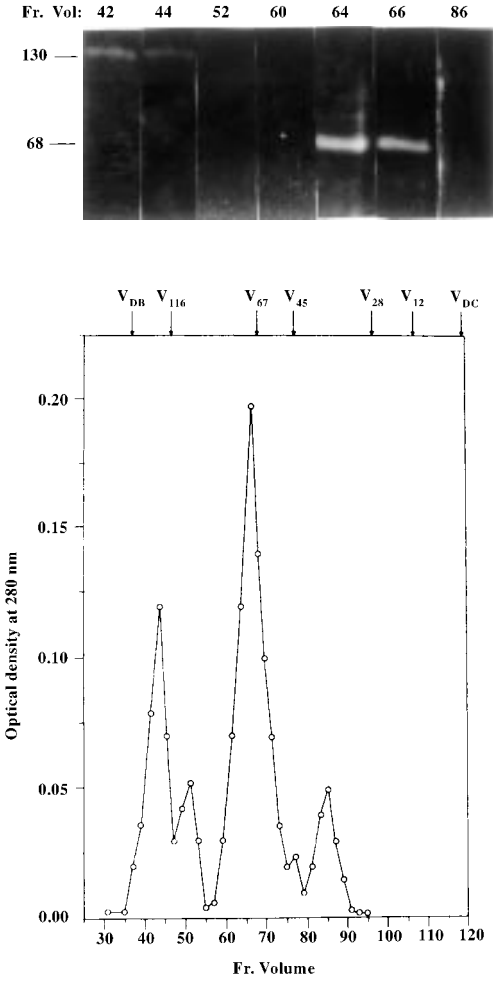

Fig. 1. Fractionation of 130K and 68K gelatinase by gel filtration. Mammary tissue from involuting gland (48 h after weaning) was extracted with phosphate buffer (0.1 M, pH 7.5). Proteins precipitated by 70% $(NH_4)_2SO_4$ were removed and the supernatant was dialysed, concentrated and subjected to gel filtration over sepharose 4B-CL (3 × 35 cm) in phosphate buffer (0.1 M, pH 7.5); 2-ml fractions were collected **(bottom)** V_{DB} and V_{Dc} represent the elution volumes of dextran blue and potassium dichromate, respectively. Zymographic analysis using 100 µl from each fraction was carried out **(top)**. Fractions corresponding to 130K and 68K were pooled.

RESULTS

Fractionation of 130K and 68K Gelatinase

In order to examine the characteristics of the different gelatinases, the 130K, 68K, and 60K gelatinases were isolated. The 2nd involuting day mammary tissue was processed and subjected to gel filtration as described under Methods (Fig. 1). Fractionation by gel filtration over Sepharose 4B-CL yielded about 462-fold purification of 130K gelatinase, while the affinity chromatography over gelatin-sepharose gave about 240–250-fold purification for 68K and 60K gelatinases. Analysis of the purified enzyme by SDS-PAGE showed that they were not contaminated with any other protein (Fig. 2); parallel zymographic assays showed that the gelatin degrading activity corresponded to 130K, 68K, and 60K protein.

The glycoprotein nature of the three gelatinases from mammary gland was studied by measuring the carbohydrate associated with the purified enzyme. All three enzymes contained covalently bound carbohydrate. The 130K gelatinase contained about 13% carbohydrate,

while 68K contained only about 6%; the 60K had about 59 M equivalent of glucose, while it was only about 20 M equivalent in the case of 68K. The glycoprotein nature was further checked by the ability of the enzyme to bind to Con A. The 60K gelatinase bound to ConA-Sepharose and could be eluted with 1 M methyl α-D-mannoside.

Substrate Specificity

The substrate specificity of the three MMPs was studied using radiolabeled gelatin, Col I, and Col IV, as described under Materials and Methods. All three enzymes degraded gelatin to a greater extent than native collagens under similar conditions. There was a progressive increase in the amount of substrate degraded over time, and the maximum degradation of the substrates occurred in about 4 h. Substrate specificity was further examined by studying the effect of concentration of different substrates on these enzymes. There was an increase in the velocity of the reaction with increased concentration of each substrate, reaching a maximum velocity at high concentrations. V_{max} was the highest for 130K, 68K, and 60K for gelatin, but for other substrates, a significant difference in V_{max} and K_m was seen (Table I). The V_{max} for 130K and 68K gelatinase was higher for Col IV than for Col I ($P \leq 0.05$); in the case of 60K, the V_{max} was higher for Col I than for Col IV ($P \leq 0.05$). All three enzymes showed the lowest K_m value for gelatin. The K_m value of 130K and 68K for Col IV was lower than that for Col I, while the 60K has a much lower K_m value for Col I than for Col IV ($P < 0.01$). These results indicate that although denatured collagen (gelatin) is efficiently hydrolyzed by all three enzymes, among the native collagen, Col IV was the preferred substrate for 130K and 68K, while Col I was the preferred substrate for 60K.

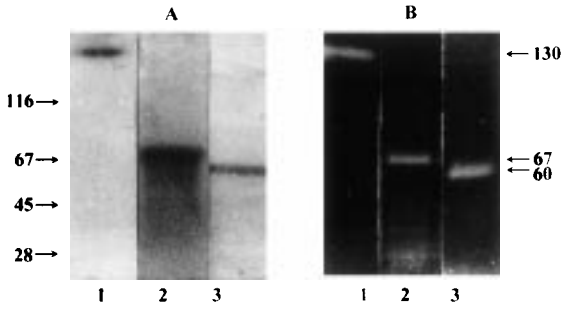

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of the purified gelatinases. The active fraction containing the 68K protein after gel filtration was further purified by affinity chromatography over gelatin Sepharose (0.5 × 5 cm) in phosphate buffer (0.1 M, pH 7.5). The bound protein was eluted by 1 M NaCl and tested by electrophoresis over 10% SDS-PAGE and located by silver staining **(A)** and Zymogram **(B).** 130K **(lane 1),** 68K **(lane 2)** gelatinase. **Lane 3,** purified 60K gelatinase.

pH Dependence

Zymographic analysis at different pH values (pH 4–9) showed that all three gelatinases have maximum activity at neutral pH. This was further examined by measuring the TCA soluble activity, using iodinated gelatin at different pH values. Maximum activity was found at pH 7.5 in all three cases (Fig. 3).

Cation Dependence

In order to study the metal ion dependence, the enzymes were pretreated with 2 mM each of Ca^{2+}, Zn^{2+}, Mg^{2+}, and EDTA and assayed by following the degradation of radiolabeled gelatin. All three enzymes degraded gelatin in the presence of Ca^{2+} ions (Fig. 4). As the concentration of Ca^{2+} ions increased, there was a loss in activity, causing complete inhibition of the activ-

TABLE I. Kinetic Constants of 130K, 68K, and 60K Gelatinases for Different Substrates[a]

	V_{max} (substrate degraded μg/mg protein)			K_m (mg × 10^{-5} × ml^{-1})		
Enzyme	Gelatin	Col I	Col IV	Gelatin	Col I	Col IV
130K	0.21 ± 0.023*	0.10 ± 0.021	0.14 ± 0.020**	3.9 ± 1.2‡	18 ± 2.3	6 ± 1.6***
68K	0.19 ± 0.021*	0.09 ± 0.019	0.13 ± 0.018**	4.2 ± 0.91‡	42 ± 1.8	6 ± 1.0***
60K	0.23 ± 0.020*	0.15 ± 0.021	0.11 ± 0.020**	4.5 ± 0.013	8 ± 1.4§	25 ± 2.0***,§

[a]Values given are the average of four to five experiments.
*$P < 0.01$ when gelatin was compared with Col I and Col IV.
**$P < 0.05$ when Col IV was compared with Col I.
***$P < 0.005$ when Col IV was compared with Col I.
‡$P < 0.005$ when gelatin was compared with Col I and Col IV.
§$P < 0.005$ when 60K was compared with 130K and 68K.

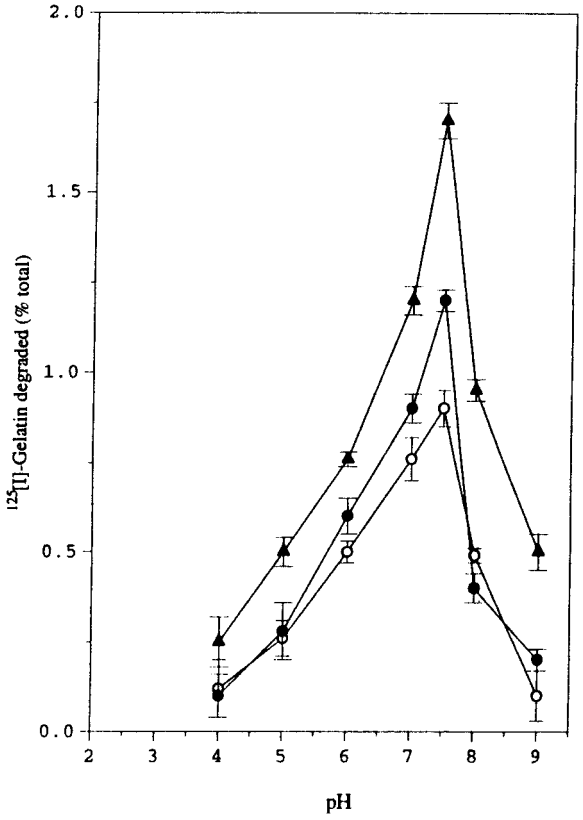

Fig. 3. pH dependence. Purified 130K (○), 68K (●), and 60K (▲) gelatinases were incubated with radioiodinated gelatin in phosphate buffer (0.1 M) at varying pH values (pH 4–9) at 37°C for 2 h. The trichloroacetic acid (TCA)-soluble radioactivity was measured and expressed as a percentage of the total. The values are the average of four assays ±SD.

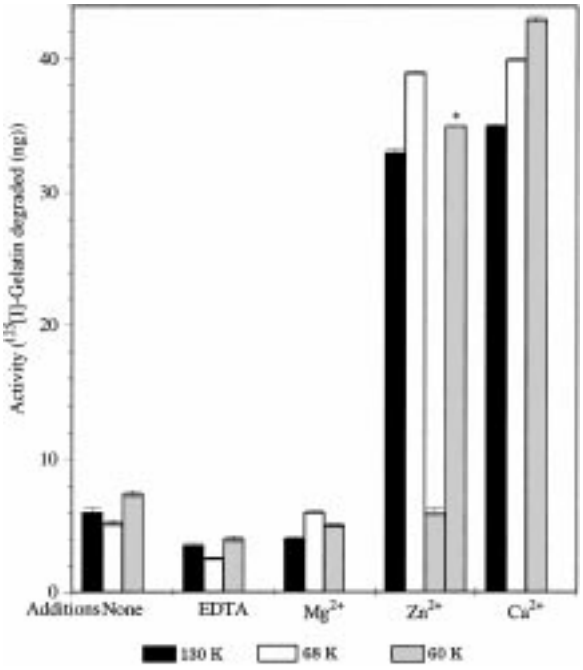

Fig. 4. The 130K, 68K, and 60K (30 µg each) gelatinases were pretreated with 2 mM each of Ca^{2+}, Zn^{2+}, Mg^{2+}, and EDTA for 20 min. Parallel controls were taken without ions. These enzymes were then incubated with radioiodinated gelatin (0.13 mg/ml) in a total volume of 200 µl for 2 h and the trichloroacetic acid (TCA)-soluble activity analyzed and expressed as the amount of gelatin degraded in 2 h. The values given are the average of four assays. ±SD (Zn^{2+}, 5 µM*).

ity of 130K and 68K at a concentration of 10 mM. 130K and 68K showed gelatin degrading capacity in the presence of 2 mM Zn^{2+} as well, while at this concentration, Zn^{2+} caused inhibition of 60K gelatinase, but when the concentration of Zn^{2+} was reduced to 5 µM, the 60K enzyme was active.

Zymographic analysis carried out by incubating the gels in buffer containing the corresponding ions (2 mM) also indicated that 130K and 68K enzyme degraded gelatin in the presence of both Ca^{2+} and Zn^{2+}. Significant activity was shown by 60K gelatinase in the presence of 2 mM Ca^{2+}. However, 2 mM Zn^{2+} inhibited the activity of the enzyme significantly. At a low concentration of Zn^{2+} (5 µM), zymography also showed activity for 60K gelatinase, but at higher concentration, Zn^{2+} was inhibitory.

Inhibition of Gelatinases by Glycosaminoglycans

Proteoglycans are a major group of macromolecules present in the ECM. The possibility of proteoglycans influencing MMP activity was examined in vitro by measuring the activity of the different gelatinases of mammary gland after pretreatment with different glycosaminocycans (heparin, hyaluronic acid, chondroitin sulfate A, and chondroitin sulfate C).

The addition of hyaluronic acid or heparin did not affect the ability of 130K, 68K, and 60K gelatinases to degrade gelatin, Col I, or Col IV both in radiolabeled substrate degradation assay (Fig. 5A) and in substrate-impregnated zymographic assay in multiwell plates (Fig. 5B), but both chondroitin sulfate A and chondroitin sulfate C inhibited 130K, 68K, and 60K activity. The ability of the enzymes to degrade gelatin, Col I, and Col IV was reduced by about 40–60% by 6–8 µg of chondroitin sulfate A and chondroitin sulfate C.

DISCUSSION

The production and regulation of MMPs in mammary gland during involution have important roles in the remodeling of the ECM [Sakakura, 1991; Talhouk et al., 1992b]. The

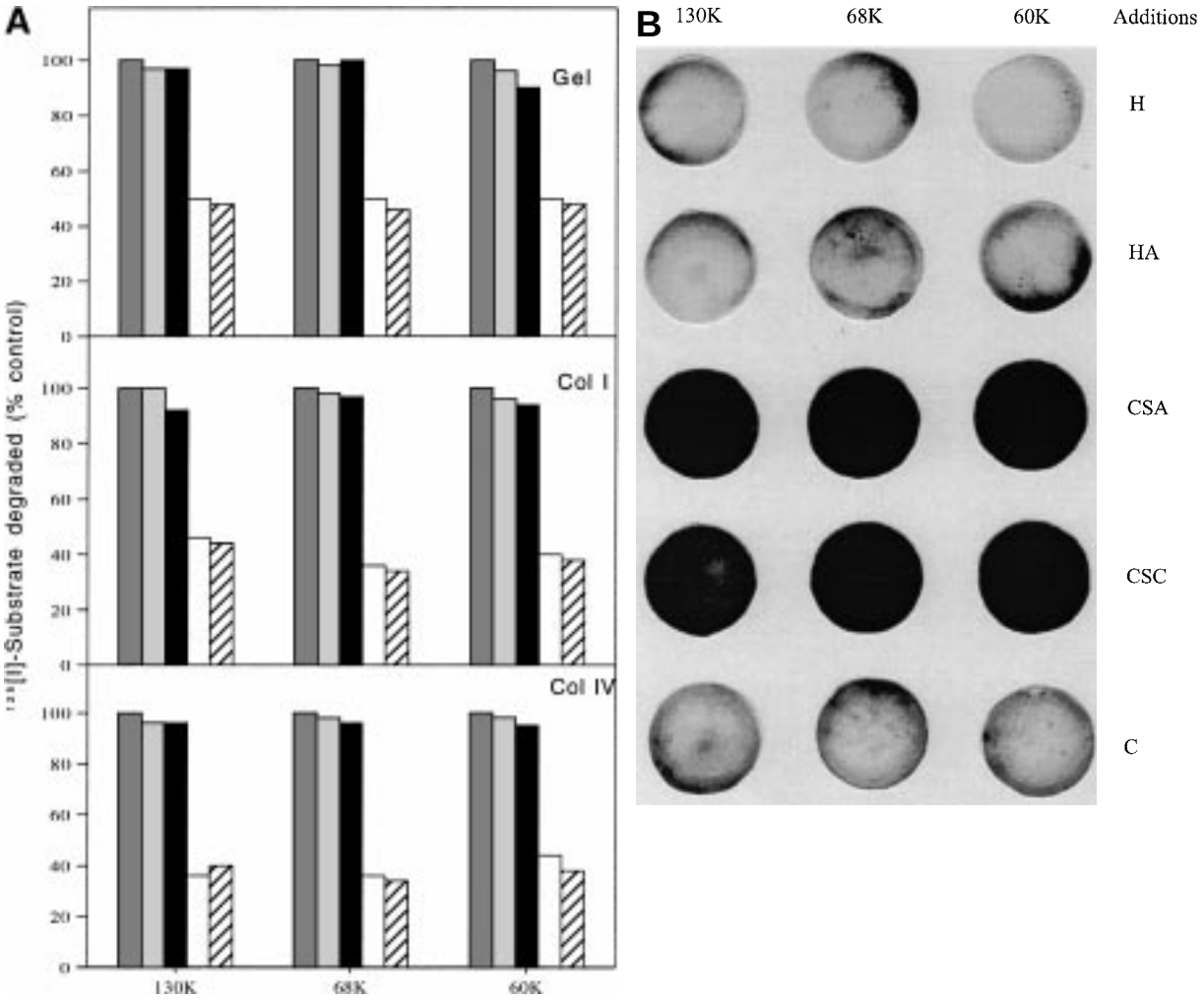

Fig. 5. Effect of glycosaminoglycans on 130K, 68K, and 60K activity. **A:** Radioiodinated gelatin, Col I, and Col IV (0.1 mg/ml) were incubated for 3 h at 37°C with 130K, 68K, and 60K gelatinases (30 µg each) after pretreatment for 30 min with heparin (7 µg), hyaluronic acid (6.0 µg), chondroitin sulfate A (7 µg), and chondroitin sulfate C (7.2 µg) in a total volume of 250 µl. Parallel controls were taken without GAG. The trichloroacetic acid (TCA)-soluble radioactivity was measured. The enzymatic activity was expressed as a percentage of control. The values given are the average of four assays. **B:** 130K, 68K, and 60K gelatinases were pretreated with heparin (H), hyaluronic acid (HA), chondroitin sulfate A (CSA), and chondroitin sulfate C (CSC), as in A. The enzymes were then copolymerized with gelatin (2 mg/ml)-impregnated polyacrylamide in multiwell plates. After polymerization, the gels were incubated in substrate buffer, stained with Coomassie Blue, and destained with distilled water.

results presented above indicate certain important characteristics of these enzymes which provide some insight into their action in mammary gland involution. The 68K gelatinase seems to be a constitutive enzyme present throughout all phases of development of mammary gland, whereas the 130K, which appears at the earlier phase of involution, and the 60K, which appears at the later phase of involution, are inducible. The three enzymes that are present during different phases of involution exhibited maximum activity at neutral pH, and degradation of the substrate by these enzymes took place only in presence of divalent cations particularly Ca^{2+} and Zn^{2+}, indicating that these are neutral matrix metalloproteinases.

Significant gelatin degrading activity was exhibited by 130K, 68K, and 60K in the presence of 2–5 mM Ca^{2+} but as the concentration of Ca^{2+} increased, there was considerable loss in enzymatic activity. 130K and 68K are active in the presence of 2 mM Zn^{2+} as well. But the 60K gelatinase required only a very low concentration of Zn^{2+} (5 µM) for its activity and was inhibited at 1–2 mM Zn^{2+} concentration. These results indicate a difference in cation depen-

dence, particularly that of Zn^{2+}, among the different MMPs of mammary gland. The difference in the requirement of the cations, particularly zinc, may also contribute to the differential modulation of the activity of the three enzymes in the mammary gland.

Myoepithelial cells have been reported to be the source of gelatinase A and gelatinase B in mammary gland [Andersson et al., 1994]. We have found that mammary epithelial cells in culture produce 130K, 68K, and 60K enzymes under appropriate conditions. These enzymes can act on basement membrane components and the underlying stroma. Having Col IV as the preferred substrate for 130K gelatinase highlights the importance of the enzyme in remodeling the basement membrane in the mammary gland. Degradation of the basement membrane by 130K gelatinase during the early phase of involution makes the 60K gelatinase that appears during the later phase of involution more accessible to the stroma and degrades Col I, which seems to be the preferred substrate for 60K. The two interstitial collagenases MMP-1 and MMP-8 [Goldberg et al., 1986] have been reported to cleave collagen molecule to 3/4- and 1/4-length fragments. This denatured collagen is then acted on by MMP-2 and MMP-9 [Collier et al., 1988]. These metalloproteinases are found both in normal as well as in malignant cells [Carey et al., 1986; Collier et al., 1988]. Further, MMP-2 is also capable of cleaving Col IV [Birkedal-Hansen et al., 1993]. Other important members of the MMP family include a 92K gelatinase B, which is produced by monocytes and macrophages, and a 72K gelatinase A of the fibroblast, which degrades Col IV, Col V, fibronectin, entactin, and insoluble elastin [Murphy et al., 1991; Wilhelm et al., 1989]. The 60K gelatinase reported in this paper is a Ca^{2+}-dependent enzyme that has a preferential ability to degrade Col I. 130K and 68K gelatinase prefers Col IV as its substrate. Inhibition by iodoacetate and zinc dependence suggests that, like other MMPs, histidine may be one of the active site residues in 130K gelatinase as well. But a higher molecular size of the active enzyme indicates the possibility of a different domain structure [Mignatti and Rifkin, 1993] with additional domain inserts. The constitutive 68K gelatinase appears to belong to the gelatinase A type. This is indicated by the cation dependence, substrate specificity, and expression pattern, and it appears to be the same as the 72K [Talhouk et al., 1991] and characterized as gelatinase A, using riboprobes [Lund et al., 1996].

Talhouk et al. [1992] suggested that 60K gelatinase is an activated form of 68K/72K gelatinase. Our results indicate that the three enzymes are different. This was further proved by immunodiffusion and Western blot analysis using antibody against 60K gelatinase. 68K and 130K gelatinases did not cross-react with antibodies against 60K gelatinase. We have also found that the 60K gelatinase is subject to regulation by hormones, particularly β-estradiol, which promotes mammary gland involution, whereas the 68K and 130K were not affected by β-estradiol treatment [Ambili et al., 1998].

One of the suggested mechanisms for the regulation of MMPs in vivo is through TIMP [Talhouk et al., 1992a]. As a constitutive enzyme, 68K gelatinase is supposed to take an active part in degrading the collagen, even during the lactating phase. It was suggested that the regulation of this enzyme is through TIMP [Talhouk et al., 1992a]. The expression of TIMP was highest during lactation and early involution, where MMP activity was suppressed. The 130K enzyme is high during the early involutary phase, when TIMP expression is also high. In vitro studies [Talhouk et al., 1991] with added TIMP showed that the 130K gelatinase is not inhibited by TIMP, indicating that 130K is not regulated by TIMP. 60K appears during the later phase of involution, when TIMP is downregulated. The observation that glycosaminoglycans, particularly chondroitin sulfate A and chondroitin sulfate C, inhibited the 130K, 68K, and 60K gelatinases suggests the possibility of another regulatory mechanism for the control of the activity of collagenases in vivo through proteoglycans. In the ECM, proteoglycans are present in association with other components, particularly collagen. Decorin is a major chondroitin sulfate proteoglycan that is bound extensively to collagen [Fleischmajer et al., 1991]. Glycosaminoglycans have been shown to affect the production of MMPs under certain conditions. Heparin caused an increase in the MMP activity in fibroblast [Tyagi et al., 1997] and mesangial cells [Kitamura et al., 1994], whereas it suppressed the interleukin-1β (IL-1β)-mediated expression of stromelysin, gelatinase B, and collagenase in primate arterial smooth muscle cells [Kenagy et al., 1994]. The

cellular effects of proteoglycans and glycosaminoglycans, particularly heparin, have been suggested to occur at the level of transcription of the MMPs. But our results indicate that the proteoglycan can also regulate the activity of MMPs at the extracellular sites, where substrate degradation and matrix remodeling occur. Of the different GAGs, heparin has the maximum negative charge and hyaluronic acid the minimum. Heparin possesses the maximum capacity to bind to cations and hyaluronic acid the minimum. Since HA and H did not inhibit MMP activity, it is unlikely that removal of cations by GAG is the reason for the inhibitory effect of GAG. This also suggests that the inhibition caused by chondroitin sulfate is specific and may not be due to their polyanionic nature alone. Although the precise mechanism of inhibition of MMPs by chondroitin sulfates is not clearly understood, it is possible that the ability of the chondroitin sulfate proteoglycans to bind to collagen may exert a masking effect on the cleavage sites of the substrate, making it unavailable for enzyme digestion. Investigations from our laboratory showed qualitative and quantitative changes in the GAGs as the mammary gland undergoes developmental changes [S. Philip and P.R. Sudhakaran, unpublished data]. The amount of chondroitin sulfate was reduced during involution of mammary gland. It may therefore facilitate the collagen degradation by MMP during involution.

The differential expression of these matrix-degrading metalloproteinases during mammary gland ontogeny [Talhouk et al., 1991] regulation of their activity by TIMP [Talhouk et al., 1992a] and β-estradiol [Ambili et al., 1998] and inhibition by proteoglycans, particularly chondroitin sulfate A and chondroitin sulfate C highlight the importance of these enzymes in matrix remodeling of the mammary gland.

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REFERENCES

Ambili M, Radhakrishna Pillai M, Sudhakaran PR. 1997. Characteristics of a 60K gelatinase involved in rat mammary gland involution. Ind J Biochem Biophys 34:347–353.

Ambili M, Jayasree K, Sudhakaran PR. 1998. 60K gelatinase produced during mammary gland involution is regulated by β-oestradiol. Biochim Biophys Acta 1403:219–231.

Andersson LM, Dundas SR, O'Hare MJ, Gusterson BA, Warbrutton MJ. 1994. Synthesis of gelatinases by rat mammary epithelial and myoepithelial cell lines. Exp Cell Res 212:389–392.

Balasubramanian KA, Abraham D, Bachhawat BK. 1975. Partial purification of testicular hyaluronidase using concanavalin A-sepharose affinity chromatography. Ind J Biochem Biophys 12:204–206.

Bernfield M, Banerjee SD, Koda JE, Rapraeger AC. 1984. Remodeling of the basement membrane as a mechanism of morphogenetic tissue interaction. In: Trelstad R, editor. The role of extracellular matrix in development. New York: Alan R Liss. p 545–572.

Bissell MJ, Aggeler J. 1987. Dynamic reciprocity: how do extracellular matrix and hormone direct gene expression? In: Cabot M, editor. Mechanisms of signal transduction by hormones and growth factors. New York: Alan R Liss. p 251–262.

Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. 1993. Matrix metalloproteinases: A review. Crit Rev Oral Biol Med 4:197–250.

Blum JL, Zeigler ME, Wicha MS. 1987. Regulation of rat mammary gene expression by extracellular matrix components. Exp Cell Res 173:322–340.

Carey DJ, Todd MS, Rafferty CM. 1986. Schwann cell myelination: induction by exogenous basement membrane-like extracellular matrix. J Cell Biol 102:2254–2263.

Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He C, Bauer EA, Goldberg GI. 1988. H-ras oncogene transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J Biol Chem 263:6579–6587.

Dickson SR, Warburton MJ. 1992. Enhanced synthesis of gelatinase and stromelysin by myoepithelial cells during involution of the rat mammary gland. J Histochem Cytochem 40:697–703.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356.

Ekblom P, Vestweber D, Kemler R. 1986. Cell-matrix interactions and cell adhesion during development. Annu Rev Cell Biol 2:27–47.

Feng Z, Marti A, Jehn B, Altermatt HJ, Chicaizi G, Jaggi R. 1995. Glucocorticoid and progesterone inhibit involution and programmed cell death in mouse mammary gland. J Cell Biol 131:1095–1103.

Fleischmajer R, Fischer LW, McDonald ED, Jacobs L, Perlish JC, Jermine JD. 1991. A decorin interacts with fibrillar collagen of embryo and adult human skin. J Struct Biol 106:89–91.

Galit L, Clark RAF. 1994. Wound repair in the context of extracellular matrix. Curr Opin Cell Biol 6:717–725.

Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ. 1986. Human fibroblast collagenase: Complete primary structure and homology to an oncogene transformation induced rat protein. J Biol Chem 261: 6600–6605.

Hay ED. 1991. Collagen and other matrix glycoproteins in embryogenesis. In: Hay ED, editor. Cell biology of extracellular matrix. New York: Plenum. p 419–462.

Hay ED. 1993. Extracellular matrix alters epithelial differentiation. Curr Opin Cell Biol 5:1029–1035.

Kenagy RD, Nikkari ST, Welgus HG, Clowes AW. 1994. Heparin inhibits the induction of three matrix metalloproteinases (stromelysin, 92K gelatinase and collagenase) in primate arterial smooth muscle cells. J Clin Invest 93: 1987–1993.

Kitamura M, Maruyama N, Mitarai T, Nagasawa R, Yokoo T, Sakai O. 1994. Heparin selectively inhibits gene expression of matrix metalloproteinase transin in cultured mesangial cells. Biochem Biophy Res Commun 203:1333–1338.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage-T4. Nature 227:680–685.

Li ML, Aggeler J, Farson DA, Hatier C, Hassell J, Bissell MJ. 1987. Influence of a reconstituted basement membrane and its components on casein expression and secretion in mouse mammary epithelial cells. Proc Natl Acad Sci USA 84:136–140.

Lin CQ, Bissell MJ. 1993. Multifaceted regulation of cell differentiation by extracellular matrix. FASEB J 7:737–743.

Lowry OH, Rosebrough NJ, Farr A, Randall RJ. 1951. Protein measurement with folin-phenol reagent. J Biol Chem 193:265–275.

Lund LR, Romer J, Thomasset N, Solberg H, Pyke C, Bissell MJ, Dano K, Werb Z. 1996. Two distinct phases of apoptosis in mammary gland involution: Proteinase independent and dependent pathways. Development 122:181–193.

Martinez-Hendez A, Fink LM, Pierce GB. 1976. Removal of basement membrane in involuting breast. Lab Invest 34:455–462.

Martisian LM. 1990. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 6:121–125.

Mignatti P, Rifkin DB. 1993. Biology and biochemistry of proteinases in tumor invasion. Physiol Rev 73:161–195.

Mignatti P, Rifkin DB, Welgus HG, Parks WC. 1996. Proteinases and tissue remodeling. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum. p 427–473.

Murphy G, Cockett MI, Ward RV, Docherty AJP. 1991. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan: A quantitative comparison of the activities of a 95K and 75K gelatinase, stromelysin-1 and -2 and punctuated metalloproteinases (PUMP). Biochem J 277:277–279.

Roth J. 1975. Methods for assessing immunologic and biologic properties of iodinated peptide hormones. Methods Enzymol 37:223–233.

Sakakura T. 1991. New aspects of stroma-parenchyma relations in mammary gland differentiation. Int Rev Cytol 125:165–202.

Strange R, Li F, Saurer S, Burkhardt A, Friis RR. 1992. Apoptotic cell death and tissue remodeling during mouse mammary gland involution. Development 115:49–58.

Streuli CH, Bissell MJ. 1990. Expression of extracellular matrix components is regulated by substratum. J Cell Biol 110:1405–1415.

Streuli CH, Bailey N, Bissell MJ. 1991. Control of mammary epithelial differentiation: Basement membrane induces tissue specific gene expression in the absence of cell-cell interaction and morphological polarity. J Cell Biol 115:1383–1395.

Talhouk RS, Chin JR, Unemori EN, Werb Z, Bissell MJ. 1991. Proteinases of the mammary gland: Developmental regulation in vivo and vectorial secretion in culture. Development 112:439–449.

Talhouk RS, Bissell MJ, Werb Z. 1992a. Coordinated expression of extracellular matrix degrading proteinases and their inhibitors regulates mammary epithelial function during involution. J Cell Biol 118:1271–1282.

Talhouk RS, Werb Z, Bissell MJ. 1992b. Functional interplay between extracellular matrix and extracellular matrix degrading proteinases in mammary gland: A coordinate system for regulating mammary epithelial function. In: Fleming TP, editor. Epithelial organisation and development. London: Chapman & Hall. p 329–347.

Tyagi SC, Kumar S, Katwa L. 1997. Differential regulation of extracellular matrix metalloproteinase and tissue inhibitor by heparin and cholesterol in fibroblast cells. J Mol Cell Cardiol 29:391–404.

Warburton MJ, Mitchell D, Ormerod EJ, Rudland P. 1982. Distribution of myoepithelial cells and basement membrane proteins in resting, pregnant and involuting rat mammary gland. J Histochem Cytochem 30:667–676.

Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI. 1989. SV-40 transformed lung fibroblast secrete a 92K type IV collagenase which is identical to that secreted by normal human macrophage. J Biol Chem 264:17213–17221.