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A Gelatinase A Isoform, GA110, of Human Follicular Fluid Is Degraded by the Bovine Oviductal Fluid Component

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소의 수란관액에 의한 사람 난포액의 Gelatinase A 동위효소인 GA110의 분해

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ABSTRACT : When mammalian oocytes ovulate into the oviduct, associating follicular fluid components are exposed to the oviductal environment, possibly resulting in the mutual interaction between follicular and oviductal fluids. In the present study, we have demonstrated for the first time that components of follicular fluid could be modified by the oviductal fluid. Gelatin zymographic analyses of human follicular fluid (hFF) obtained from IVF patients showed consistently the presence of 110 kDa gelatinase (GA110) in addition to many bands among which 62 kDa gelatinase was predominant. Addition of EDTA or phenanthroline to the gelatinase substrate buffer during gel incubation abolished GA110 band whereas phenylmethylsulfonyl fluoride (PMSF) did not. In contrast, bovine oviductal fluid (bOF) exhibited only 62 kDa gelatinase. Surprisingly, when bOF was added to hFF in 1:1 ratio and then the mixture was incubated for 3 h at 37ºC, GA110 of hFF disappeared. Disappearance of GA110 by bOF was observed even within 30 min after mixing with hFF. Addition of aminophenylmercuric acetate (APMA) to hFF also abolished enzymatic activity of GA110 but increased the activity of 62 kDa gelatinase. However, APMA abolished many other gelatinases as well unlike bOF. Interestingly, treatment of hFF with EDTA for 3 h remarkably increased the enzymatic activity of GA110 but not that of other gelatinases. Addition of phenanthroline, PMSF or soybean trypsin inhibitor (SBTI) did not affect overall gelatinase activities. Again, addition of bOF to the hFF pretreated with any of the above proteinase inhibitors abolished the appearance of GA110. Human serum also showed GA110 of which activity was greatly enhanced by EDTA treatment. Similar to hFF, serum GA110 also disappeared by the addition of bOF. Human granulosa cell homogenate did not reveal any appreciable gelatinase activity except 92 kDa gelatinase. Anti-human gelatinase A antibody reacted with 62 kDa gelatinase of hFF. Based upon these results, it is concluded that bOF could selectively degrade an isoform of gelatinase A present in hFF and human serum.

Key words : Follicular fluid, Oviductal fluid, Granulosa cell, Gelatinase A, Isoform, Zymography.

요 약 : 포유류의 난자가 수란관내로 배란될 때는 난포액 성분도 같이 수란관내로 들어간다. 본 연구에서는 처음으로 난포액의 일부 성분이 수란관액에 의해서 변화하는 것을 관찰하였다. 사람의 난포액을 gelatin zymogram으로 분석한 결과 62kDa gelatinase 이외에 110kDa gelatinase (GA110) 등의 여러 gelatinase 활성이 나타났다. 이 활성들은 EDTA나 phenanthroline에 의해 억제된 반면 PMSF 처리에 의해서는 아무런 변화가 없었다. 소의 수란관액에서는 62kDa gelatinase의 활성만이 주로 관찰되었다. 소의 수란관 액과 사람의 난포액을 1:1로 섞고 이를 37℃에서 3시간 동안 둔 결과 사람의 난포액의 GA110 활성은 사라졌다. 사람의 난포액에 APMA를 첨가한 결과 GA110의 활성은 대부분 감소하고 대신 62kDa gelatinase의 활성은 오히려 증가하였다. 반면에 사람의 난포액에 EDTA를 3시간 동안 처리한 결과 GA110의 활성은 오히려 현저히 증가하였고

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이 때 다른 gelatinases의 활성은 영향을 받지 않았다. PMSF나 SBTI는 난포액내의 gelatinases 활성에 아무런 변화를 일으키지 않았다. EDTA, PMSF 혹은 SBTI 등의 proteinase inhibitor를 미리 처리한 사람의 난포액에 소의 수란관 내액을 섞은 경우에도 GA110의 활성은 여전히 감소하였다. 사람의 혈청에서도 EDTA에 의해 활성이 현저히 증가하는 GA110이 발견되었다. 사람의 난포액과 유사하게 혈청내의 GA110도 소의 수란관액에 의하여 활성이 사라졌다. 그러나 사람의 난포과립세포의 추출물에서는 단지 92kDa gelatinase만 관찰이 되었다. 마지막으로 anti-human gelatinase A 항체를 사용하여 사람의 난포액과 혈청 그리고 난포과립세포의 추출액을 western blotting한 결과 62kDa과 GA110 만이 항원-항체반응을 나타내었다. 이 같은 결과로 미루어 사람의 난포액과 혈청에는 gelatinase A의 독특한 isoform인 GA110이 있으며 특히 난포액내의 GA110은 수란관액 성분에 의해 선택적으로 분해되는 것으로 여겨진다.

INTRODUCTION

The matrix metalloproteinases (MMPs) are a group of structurally-related proteolytic enzymes that degrade extracellular matrix (ECM) and basement membrane (BM) components in a zinc- and calcium-dependent manner (Birkedal-Hansen et al., 1993). The family consists of over 20 enzymes and includes 4 major classes; the collagenases, the gelatinases, the stromelysins, and the membrane-type metalloproteinases (Werb, 1997). Two forms of gelatinases are identified, a 72 kDa gelatinase A and a 92 kDa gelatinase B, referred to as MMP-2 and MMP-9, respectively. Both of the gelatinases have a broad spectrum of substrate specificities including many important components of basement membranes such as type IV collagen, laminin, fibronectin as well as gelatin (Birkedal-Hansen et al., 1993). Of these, 72 kDa gelatinase A is known to be the most widely distributed of all MMPs and has been identified in a variety of cells including cultured human ovarian granulosa cells (Puistola et al., 1995; Stamouli et al., 1996).

Like other MMPs, MMP-2 is synthesized as an inactive precursor and during or after secretion into the extracellular milieu, it is thought to be activated proteolytically. Mast cell proteinases (Suzuki et al., 1995), serine proteinases, such as plasmin and kallikreins (Suzuki et al., 1990; Carmeliet et al., 1997), or other MMPs including the membrane-type MMPs (Sato et al., 1994) have been suggested to be responsible for its activation. The inactive precursor can also be transformed to be active by organomercurials, metal ion, thiol reagents or detergents. Depending on the type of reagents, molecular weight of the resulting polypeptide chain is diverse (Birkedal-Hansen et al., 1993). However, among mammals including human, only a single species of 72～68 kDa polypeptide chain is known as an inactive precursor which turns into 68～62 kDa or 45 kDa upon activation (Nagase et al., 1992; Fridman et al., 1995).

Throughout the reproductive period, mammalian ovarian follicles undergo considerable remodeling of tissues. Many studies have shown that the MMPs are involved in the remodeling process of follicles. Injection of synthetic metalloproteinase inhibitors resulted in the blockade of ovulation in hamster and rat (Ichikawa et al., 1983; Brannstrom et al., 1988). Location and activity of MMP-1 of ovine and rabbit ovaries were concentrated at the site of future rupture in the capillary lumina at the apex of the follicle (Murdoch and McCormick, 1992; Tadakuma et al., 1993). During pro-estrus in rats, maximal expression of collagenase-3 was localized to thecal cells and interstitial tissue of follicles (Balbin et al., 1996). Administration of eCG resulted in the increases of both mRNA expression and metalloproteinase activities of collagenase-3, 72 kDa and 92 kDa gelatinases of the rat ovarian tissues when follicular growth and expansion occurred (Cooke et al., 1999). Thus, MMPs of follicular tissue cells are suggested to play a critical role in follicular development (Hulboy et al., 1997).

Although their physiological role is not clear, MMPs are also found in the follicular fluid of mammals. Human follicular fluid exhibited the type V collagenolytic activity that increased toward ovulation (Puistola et al., 1986). Ovine and porcine follicular fluids, based on the gelatin substrate zymography, showed the presence of many gelatinases (Besnard et al., 1996; Besnard et al., 1997). While these studies have suggested the possible involvement of follicular fluid MMPs in folliculogenesis, the role of these MMPs remains in question until their precise nature is identified.

When mammalian oocytes ovulate, follicular fluid components as well as oocyte-cumulus complex are transported to the oviduct where fertilization and early embryonic development take place. In many laboratory rodents and horses whose ovaries are covered with ovarian bursa, all follicular fluid components

are exposed to the oviductal environment. Even in mammals without bursa, such as human, cows and pigs, highly viscous follicular fluid components bathing oocyte-cumulus complex are also shed from the follicle into the oviduct (Hunter, 1988; Hansen et al., 1991). Therefore, human oviductal environment at the time of fertilization consists of both follicular fluid and oviductal fluid. In this point of view, the products resulting from the mutual interaction between the components of follicular fluid and oviductal fluid as well as unreacted components might affect the process of fertilization. However, most studies have been conducted to focus the role of either fluid components on fertilization or embryonic development, while little is known about the involvement of products of mutual interaction between two body fluids during the events.

The present study was therefore aimed firstly to identify one of the MMPs, gelatinase A, and its isoforms present in hFF and secondly to examine if there is any changes in the biochemical properties of gelatinase A and isoforms upon exposure to oviductal fluid. However, because normal human oviductal fluid was not available, bovine oviductal fluid was used throughout the experiments.

MATERIALS AND METHODS

1. Chemicals

Acrylamide, bisacrylamide, N, N, N, N, -tetramethylethylene-diamine and BCA protein assay kit were purchased from the Bio-Rad (Hercules, CA). Ficoll and gold-labelled goat anti -mouse IgG antibody and IntenSE BL kit were purchased from Amersham International (England). Mouse monoclonal antibody against human MMP-2 was purchased from Calbiochem (San Diego, CA). Other chemicals were purchased from the Sigma Chemical Company (ST. Louis, MO). EDTA was prepared as a stock solution of 50 mmol/l in 3X D.W.. PMSF, phenanthroline and APMA were dissolved into DMSO as 10 mmol/l stock solution, respectively. In preliminary experiments, it was observed that the addition of 10% DMSO to the body fluids during incubation did not affect the gelatinolytic activity. SBTI was dissolved into 3X D.W. to give a conc. of 10 mg/ml as a stock.

2. Preparation of samples

1) Human follicular fluid

Human ovarian follicular fluids and sera were obtained from patients participating in an IVF program at a local hospital (Mirae and Heemang OB/GYN Clinic, Korea). The follicular fluid and oocytes were aspirated transvaginally. Aspirated follicular fluid was centrifuged at 2,000 × g for 30 min. The supernatant was taken and used immediately or kept frozen at −20℃ until use. There was no difference between the gelatinolytic activity of hFF freshly prepared and that of hFF kept frozen. Before use, hFF was centrifuged again to remove any precipitate. When necessary, hFF was mixed with bOF in 1:1 volume ratio and incubated at 37℃ for 3 h.

2) Human ovarian granulosa cell homogenate

The resulting pellet of granulosa cells were overlaid on Ficoll and centrifuged at 400 × g for 30 min. The cells were collected from the interface followed by washing with PBS twice by centrifugation at 300 × g for 10 min. After homogenization on ice in 10 mmol/l sodium phosphate buffer (pH 7.4) containing 1 mmol/l EDTA, 1 mmol/l PMSF and 1 mg/ml SBTI, they were centrifuged again at 4℃, 1,500 × g for 10 min. The supernatant was taken and assayed for the protein content by using BCA assay kit. The homogenate was then mixed with SDS-PAGE sample buffer without mercaptoethanol.

3) Bovine oviductal fluid

Bovine oviductal fluid was prepared as follows. Bovine oviducts were collected from a local abattoir (Incheon, Korea). Reproductive histories of the cows were not available. Oviducts were transported to the laboratory on ice in sterile PBS. After rinsing with PBS several times, they were transferred onto the filter paper and were freed from the adherent lipid using scissors. They were then squeezed out between forceps to liberate inner components into the watch glass. The components were centrifuged at 4℃, 12,000 × g for 60 min. The supernatant was taken as oviductal fluid and was kept at −20℃ until use.

All body fluids were filtered using 0.45 μm membrane filter (Millex-HV, Millipore) before incubation at 37℃. When necessary, EDTA, PMSF, phenanthroline, SBTI or APMA was added to hFF or bOF before incubation. To make a mixture of samples, bOF was added to the same volume of hFF or to the cell homogenate in 1 μl : 30 μg ratio.

3. Gelatin Zymography

SDS-PAGE was used with the addition of 1 mg/ml bovine skin gelatin (type B) to a 8% resolving gel as described previously (Kim et al., 1998). Briefly, samples were dissolved into the SDS sample buffer in the absence of reducing agent without boiling. One microliter equivalent of each body fluid or 30 μg protein of granulosa cell homogenate was loaded onto a single well. For the mixture, 1 μl of hFF or 30 μg protein of cell homogenate was added to 1 μl of bOF and the total mixture was loaded onto a single well. After electrophoresis using Hoefer mini gel kit, gels were washed with 2.5% Triton X-100 in Tris -HCl buffer (pH 8.0), then rinsed with 2X D.W. followed by washing in the 100 ml incubation buffer (5 mmol/l $CaCl_2$, 0.02% NaN_3, 50 mmol/l Tris-HCl, pH 8.0) for 30 min. Then gels were incubated with fresh incubation buffer for 48 h at 37℃. The reacted gels were stained with 0.5% Coomassie brilliant blue G-250 dye in 5% methanol and 7% glacial acetic acid for 30 min and destained with 3X D.W. The clear bands on blue background were regarded as gelatinase bands since gelatinases degrade gelatin present in the acrylamide gel. Wide range molecular markers from Sigma (M-3788) were used as a standard of SDS-PAGE gel.

Where indicated, 5 mmol/l EDTA, 5 mmol/l PMSF, 5 mmol/l phenanthroline or 1 mg/ml SBTI was added to the incubation buffer as a protease inhibitor. Every zymographic results were confirmed by two or more repetitive experiments.

4. Immunoblotting

After nonreducing electrophoresis, the gels were soaked in transfer buffer for 15 min. Transfer buffer was made of 25 mmole/l Tris (pH 8.4), 192 mmole/l glycine and 10% methanol. The proteins were electrotransferred onto PVDF membrane (Immobilon-P, Millipore) for 1 h at 4℃, 200 mA. Before transfer, membranes were hydrated with absolute methanol for 15 sec, 3 X D.W. for 2 min and then with transfer buffer for 5 min. After transfer, membranes were treated with methanol for 10 sec, dried on filter paper and then treated with methanol for 5 min again. To saturate nonspecific binding sites, membranes were treated at 37℃ for 1 h with PT buffer (10 mmole/l sodium phosphate buffer, pH 7.4, 0.05% Tween 20, 10 mmole/l sodium azide) containing 5% BSA. They were then incubated for 1 h with PT buffer containing 1% normal goat serum and 1 μg/ml antibody against human MMP-2. Following washing with PT buffer containing 0.1% BSA three times for 10 min each, membranes were incubated for 1 h with PT buffer containing 1:100 diluted gold-labeled goat anti-mouse IgG antibody. After washing the membranes three times, the signal was revealed by using IntenSE BL kit according to the manufacturers manual. Immunoblotting results were confirmed by three independent experiments.

RESULTS

1. Gelatinases of human follicular fluid

The gelatinases of human follicular fluids from 8 individuals are shown in Fig. 1. There was a little variation in the intensities of each gelatinolytic protein band depending on the individuals. However, most hFFs consistently exhibited gelatinases having MWs of approx. 110 kDa, 92 kDa, 88-84 kDa and 62 kDa from the top of the gel. Of these, 62 kDa gelatinase was the most prominent, giving the brightest band in all 8 hFF samples. A gelatinase of 110 kDa (GA110) was the next in most hFFs. Other gelatinases of 150 kDa and 72 kDa were visible in some samples but not in the rest of samples. Gelatinolytic activities of hFF did not reveal any significant changes when hFF was incubated at 37℃ before zymography. As seen in Fig. 2, the intensity and species of gelatinases of hFF remain constant even after incubation for 24 h.

To determine whether the gelatinolytic bands appearing in gelatin zymogram of hFF might be indeed gelatinases or not,

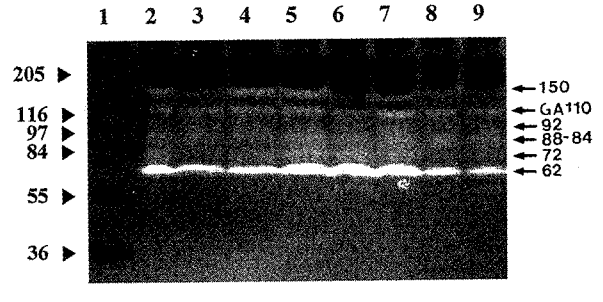

Fig. 1. Gelatinases of human follicular fluid. Lane 1, molecular weight standards with numbers on the left; lanes 2~9, follicular fluids from eight women. Arrowheads on the right indicate typical gelatinases appearing in hFF. From the top of the gel, MWs of the gelatinases are 150 kDa, 110 kDa, 92 kDa, 88~84 kDa, 72 kDa and 62 kDa.

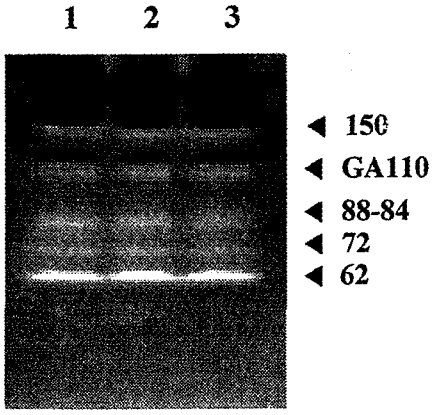

Fig. 2. Gelatinase of hFF after incubation at 37℃ or not. HFF was incubated at 37℃ for 0 h (lane 1), 3 h (lane 2) or 24 h (lane 3) before gelatin-substrate gel electrophoresis. Note that there is no difference among gelatinase activities of hFFs that were incubated or not. Numbers on the right indicate the typical gelatinases of hFF.

various protease inhibitors were added to the gel incubation buffer after electrophoresis (Fig. 3). The addition of 5 mmol/l EDTA, known as an inhibitor of matrix metalloproteinases (MMPs) and Ca^{2+}-dependent proteases, abolished all of the gelatinolytic activities of hFF (Fig. 3A). Similarly, treatment with 5 mmol/l phenanthroline, a specific inhibitor of MMPs, blocked most gelatinase activities except 84~88 kDa gelatinase

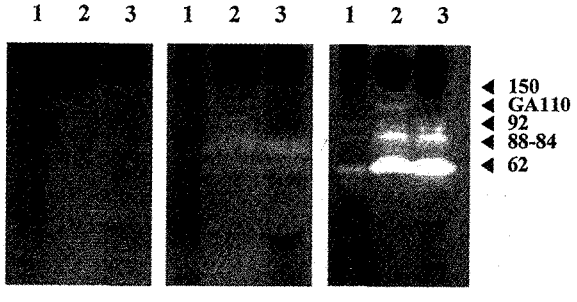

Fig. 3. Effects of proteinase inhibitors on the gelatinase activities of hFF. After gelatin-substrate gel electrophoresis of hFF and bOF, the gels were incubated in the buffer containing 5 mmol/l EDTA (A), phenanthroline (B) or PMSF (C). Note the absence of any gelatinase activity in EDTA-treated gel, while most of gelatinases are active in PMSF-treated gel. Phenanthroline-treated gel shows that only 88~84 kDa bands are active. Lane 1, bOF; lane 2, hFF; lane 3, hFF mixed with bOF followed by incubation for 3 h at 37. Note that bOF alone shows only 62 kDa band which does not appear in EDTA- or phenanthroline-treated gel but appears to be active in PMSF-treated gel.

(Fig. 3B). In contrast, 5 mmol/l PMSF, a serine/threonine protease inhibitor, failed to inhibit the activities of gelatinases (Fig. 3C). The 84~88 kDa protease also remained to be active despite of the PMSF treatment. These results demonstrate that gelatinolytic bands except 84~88 kDa protease appearing in hFF were indeed gelatinases.

2. Effects of bovine oviductal fluid on the gelatinases of human follicular fluid

When hFF was mixed with bOF in 1:1 (vol:vol) ratio and then incubated at 37℃, GA110 gelatinase dramatically disappeared in the subsequent zymogram (Fig. 4A). Separate experiments showed that 150 kDa gelatinase of hFF also disappeared by bOF (data not shown). The change occurred so fast that the disappearance of GA110 was distinct even within 30 min after mixing hFF with bOF. Other gelatinase activities of 92 kDa, 84~88 kDa and 62 kDa did not disappear during bOF treatment. Rather their activities somewhat increased as treatment continued up to 24 h. BOF itself showed little gelatinase activity except the 62 kDa of which enzymatic activity was much weaker compared to that of the 62 kDa of hFF.

Since higher molecular weight gelatinases are likely to be isoforms of a gelatinase, the possibility that GA110 might be an isoform was examined (Fig. 4B). When hFF was treated with 1 mmol/l of APMA, an activator of inactive MMP precursors, and then incubated for 30 min, 3 h or 24 h at 37℃, enzymatic activity of GA110 became weaker as treatment continued and

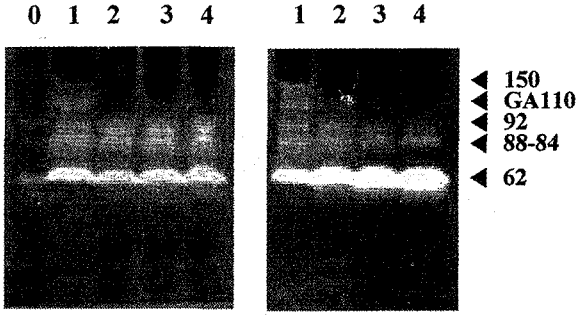

Fig. 4. Effects of bOF or APMA on GA110 of hFF. BOF (A) or 1 mmol/l APMA (B) was added to hFF and the mixture was incubated at 37 for 30 min (lanes 2), 3 h (lanes 3) or 24 h (lanes 4) before gelatin-substrate gel electrophoresis. Untreated hFF (lanes 1) and bOF (lane 0 in A) are shown as controls.

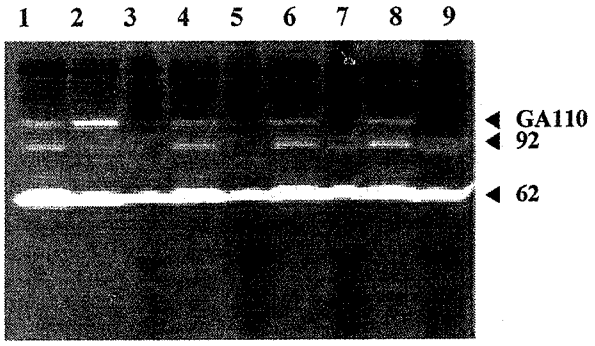

Fig. 5. Effects of bOF on the GA110 of hFF in the presence of proteinase inhibitors. HFF (lane 1) was treated with 5 mmol/l EDTA (lane 2), phenanthroline (lane 4), PMSF (lane 6) or SBTI (lane 8) for 3 h and each treated hFF was mixed with bOF followed by incubation at 37℃ for 3 h (lanes 3, 5, 7 and 9). Note that GA110 always disappeared after incubation with bOF even in the presence of various proteinase inhibitors whereas 92 kDa and 62 kDa were not affected by bOF.

was not discernable after 24 h. Similarly, activities of 150 kDa and 92 kDa gelatinases also became reduced and eventually were invisible after 24 h. In contrast, 62 kDa gelatinase exhibited stronger activity as APMA treatment continued and showed the highest intensity after 24 h. The 84~88 kDa protease did not show any change of its band intensity even after 24 h incubation under the same condition, indicating that it was not a member of gelatinases.

To see if the disappearance of GA110 in hFF by bOF might be mediated by proteolytic reaction, 5 mmol/l EDTA, 5 mmol/l phenanthroline, 5 mmol/l PMSF or 1 mg/ml soybean trypsin inhibitor was added to hFF and then mixed with bOF. After incubation of the mixture at 37℃ for 3 h, the samples were run on gelatin gel and the zymogram was analyzed. As seen in Fig. 5, treatment of hFF with phenanthroline, PMSF or SBTI did not affect the enzymatic activity of most gelatinases including GA110, 92 kDa and 62 kDa (lanes 4, 6 and 8) compared to the untreated hFF (lane 1). However, EDTA treatment reduced the activity of both 92 kDa and 62 kDa gelatinases whereas the activity of GA110 was greatly enhanced (lane 2). Nevertheless, addition of bOF consistently abolished the activity of GA110 whether it was enhanced by EDTA or not, regardless of the presence of an inhibitor (lanes 3, 5, 7 and 9).

3. Identification and origin of GA110 gelatinase

Whether the GA110 and 62 kDa might be indeed gelatinase A isoforms was examined by both zymography and immunoblotting experiment using monoclonal antibody against human MMP-2 which could recognize both inactive and active gelatinase isoforms. Untreated hFF showed gelatinases of GA110, 92 kDa and 62 kDa that are both enzymatically and immunologically active. However, it also showed several isoforms of approximately 300 kDa and 200 kDa doublet that are immunoreactive but enzymatically inactive (lanes 1 in Figures 6A and 6B). The immunoreactive GA110 was barely discernable in untreated hFF, but it was distinguished in hFF treated with EDTA for 3 h (lanes 2 and 3 in Figures 6A and 6B). When this hFF was mixed with bOF and incubated for 3 h, GA110 band disappeared and a little increased intensity of 62 kDa was observed (lanes 4 in Figs. 6A and 6B). In addition, a new immunoreactive 100 kDa doublet appeared (asterisks in lane 4). Several smaller proteins less than 25 kDa were also found to be immunoreactive but they were not enzymatically active. However, these were also seen in bOF alone (lanes 4 and 5 in Fig. 6B). BOF alone showed that only 62 kDa was both enzymatically active and immunoreactive. Some smaller proteins similar to those seen in hFF treated with bOF were found to be immunoreactive though not enzymatically active (lane 5 in Fig. 6B). When hFF pretreated with EDTA was mixed with APMA and incubated for 3 h, both enzymatic activity and immunoreactivity of GA110 were significantly reduced but they did not disappear completely (lanes 3 in Figs. 6A and 6B).

Zymogram of human serum exhibited many gelatinases as in Fig. 6C. Of them, 92 kDa showed the brightest and thick band. Corresponding immunoblot showed that only 62 kDa of them was immunoreactive against anti-human MMP-2 antibody (lanes 1 in Figs. 6C and 6D). When serum was treated with EDTA, both enzymatic activity and immunoreactivity of GA110 increased (lanes 2 in Figs. 6C and 6D). These increased activities of GA110, however, disappeared by bOF. BOF treatment also resulted in the appearance of 100 kDa band(s) in addition to the increased immunoreactivity of 62 kDa (lanes 3 in Figures 6C and 6D).

No gelatinase except 92 kDa gelatinase was observed in the zymogram of granulosa cell homogenate, even though the homogenate was prepared in the presence of EDTA which otherwise could greatly enhance the activity of GA110 as seen

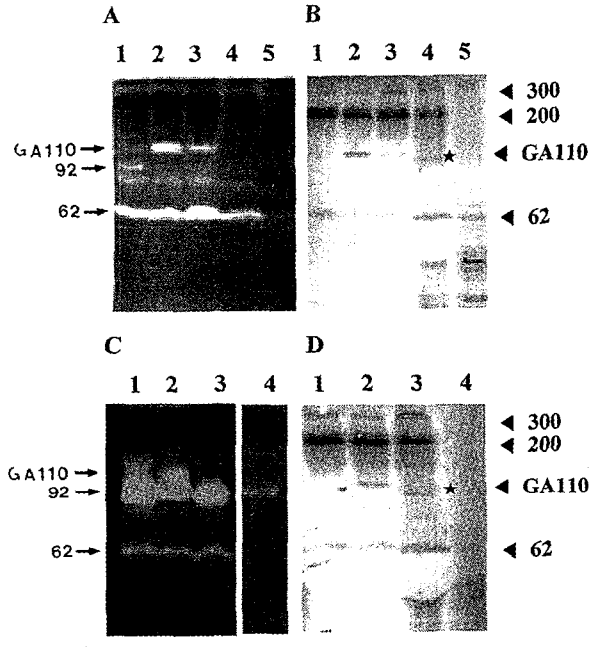

Fig. 6. Zymographic (A,C) and corresponding immunoblotting (B, D) analyses of GA110. In A and B, lane 1, untreated hFF; lane 2, hFF treated with EDTA (hFF+E) for 3 h; lane 3, hFF+E mixed with APMA; lane 4, hFF+E mixed with bOF; lane 5, bOF alone. In C and D, lane 1, untreated human serum; lane 2, human serum treated with EDTA (hS+E) ; lane 3, hS+E mixed with bOF; lane 4, granulosa cell homogenate. Note the disappearance of GA110 and concomitant appearance of 100 kDa bands (asterisks in lanes 4 in B and 3 in D) after mixing hFF+E with bOF (lane 4 in B and lane 3 in D).

in hFF and serum (lane 4 in Fig. 6C). Similarly, no immunoreactive protein against anti-MMP-2 antibody was shown in the immunoblot (lane 4 in Fig. 6D). Both of these results clearly demonstrate that human preovulatory granulosa cell does not synthesize gelatinase A or its isoform, except 92 kDa which is presumed to be a gelatinase B, MMP-9 (Stamouli et al., 1996).

DISCUSSION

Present study demonstrates for the first time that several isoforms of gelatinase A exist in hFF and serum and one isoform, GA110, is proteolytically degraded by the component(s) of bOF.

To date, the only known mammalian gelatinase A isoform is 72 kDa form which is also known as an inactive progelatinase A. Based upon the amino acid sequences of human isoform (Huhtala et al., 1990), the calculated molecular weight mass of the proenzyme is 70,984 Da and the active enzyme proper is 62,067 with a possible variation due to a glycosylation (Reponen et al., 1992). In addition to these two molecules, we have found that other isoforms of gelatinase A are present in human follicular fluid and serum. One of them, GA110, has higher molecular weight than 72 kDa isoform based upon the results from gelatin-substrate zymography and immunoblot analyses. Since gelatinase A and its isoforms are not likely to be synthesized from other than a single known mRNA species (Huhtala et al., 1990), GA110 can not be synthesized de novo. Rather its appearance could be the result of the interaction of progelatinase A with yet unknown other polypeptide(s). The interaction should be a covalent bond as it is not dissociated by SDS.

There are many plasma proteins known to bind MMPs. Some of them bind covalently. Human 2-macroglobulin, pregnancy zone protein, and lipocalin are those which make a complex through covalent bond with MMPs (Sottrup-Jensen and Birkedal -Hansen, 1989; Arbelaez et al., 1997; Grinnell et al., 1998; Monier et al., 2000). When MMPs interact with these molecules, they are known to cleave the bait region of extracellular proteins followed by forming a covalent bond to the fragment produced. It is thus plausible that GA110 might be similarly produced from the covalent binding of progelatinase A to yet unknown protein via bait region cleavage. In the present study, treatment of hFF with APMA resulted in the disappearance of enzymatic activities of 150 kDa, GA110 and 92 kDa gelatinases with the concomitantly increased activity of 62 kDa gelatinase. Since APMA is well known to interact with the precursors of MMPs resulting in the autolytic cleavage of enzyme itself to generate a processed form (Birkedal-Hansen et al., 1993), changes of the enzymatic activities of these gelatinases by APMA indicate that 150 kDa, GA110 and 92 kDa were proteolytically processed to a 62 kDa form. However, among these, GA110 showed the strongest enzymatic activity and was the only immunoreactive isoform being recognized by the anti-human MMP-2 antibody, indicating that it was indeed an isoform of gelatinase A.

The isoforms of gelatinase A present in hFF do not appear to be synthesized by ovarian granulosa cells but is probably originated from serum. The presence of GA110 was also identified in normal serum and both of its enzymatic and immunological activities were greatly increased by the treatment of serum

with EDTA, which was also observed in hFF treated with EDTA. In contrast, GA110 and 62 kDa gelatinase A were hardly discernable in zymogram and immunoblot of granulosa cell homogenate even though the homogenate was prepared in the presence of EDTA. The finding is consistent with the previous reports that gelatinase A was not detected in freshly isolated human granulosa cells (Puistola et al., 1995; Stamouli et al., 1996). Based upon these observations, it is believed that the gelatinase A isoforms present in hFF are originated from serum. However, circulating serum isoforms do not appear to be simply accumulated within ovarian follicles. Our results showed that there are many differences between the gelatinase profiles of hFF and serum. For example, hFF showed 62 kDa gelatinase A as the most prominent while serum exhibited very intense 92 kDa gelatinase as a predominant. Thus it is rather obvious that gelatinase A isoforms found in hFF might be a result of the selective serum transudation by follicle cells.

Interestingly, we have observed that bOF also induced proteolytic modification of gelatinase A isoforms. Similar to APMA, bOF quickly abolished the activity of 150 kDa and GA110 within 30 min after its addition to hFF. GA110 present in serum was also disappeared by bOF. However, the action mechanism of bOF was not the same as that of APMA. Addition of bOF to hFF failed to reduce the activity of 92 kDa even after 24 h incubation whereas treatment with APMA resulted in the disappearance of 92 kDa as well as 150 kDa and GA110. BOF addition did not significantly alter the activity of 62 kDa, while APMA treatment induced the increase of 62 kDa activity. BOF addition to hFF or serum pretreated with EDTA generated new 100 kDa doublet proteins that were immunoreactive against anti-MMP-2 antibody. However, APMA did not induce any appearance of new immunoreactive proteins. These observations lead to suggest that bOF could proteolytically degrade the isoforms of gelatinase A present in hFF and serum.

The proteolytic activity of bOF appears to be specific to gelatinase A isoforms. Human serum consistently showed very intense band of 92 kDa gelatinase, which was presumed to be a progelatinase B (Besnard et al., 1997) and was not recognized by the anti-gelatinase A antibody in this study. Addition of bOF to serum, however, did not alter the activity of 92 kDa. Similarly, bOF did not affect the activity of 92 kDa present in hFF either. I.e., the proteolytic activity of bOF was not effective on the gelatinase B isoform.

Little information is available regarding to the proteinases of bOF. Recent experiments has shown that both oviductal secretion and oviductal tissue extract of superovulated females in hamster species showed proteolytic activity some of which was ascribed to a plasminogen activator (Jimenez Diaz et al., 2000). However, this type of oviductal enzyme, of which activity was inhibited by SBTI or PMSF, is not responsible for the degradation of GA110 of hFF and serum, since the degradation of GA110 by bOF was not inhibited by EDTA, phenanthroline, PMSF or SBTI.

Compared to a number of gelatinases and isoforms in hFF and serum, bOF exhibited only a single 62 kDa gelatinase of which enzymatic activity was much weaker than those of hFF and serum. As revealed by the zymographic and immunoblotting analyses, it was identified as active 62 kDa gelatinase A. Any other enzymatically active or immunoreactive gelatinases was not found in bOF. Previous investigators also reported that bovine oviductal tissue did not exhibit any gelatinase activities except presumptive MMP-1 of which activity disappeared near to the time of ovulation (Einspanier et al., 1999). These observations imply that bovine oviductal cells, if they do, synthesize and secrete only a trace amount of gelatinase A.

Ovine and porcine follicular fluids have also been shown to exhibit MMP-2 and -9 (Besnard et al., 1996; 1997). In the same zymograms, however, isoforms similar to 150 kDa and GA110 observed in hFF were not detected. In the present study, gels after SDS-PAGE were incubated for 48 h in the buffer to increase gelatinolytic activity, whereas gels of the previous studies were incubated for 24 h in the same buffer. This difference of incubation time might account for the absence of isoforms in follicular fluids of porcine and ovine in the previous studies.

We have consistently observed that EDTA treatment of hFF and serum greatly increased the band intensity of GA110 in the subsequent zymogram. Since EDTA is well known to inhibit the enzymatic activity of MMPs by chelating the divalent ions needed, the increased band intensity of GA110 by EDTA is not due to an increased enzymatic activity but to an increase of amount of GA110 molecules. Our immunoblotting experiments showed that both hFF and serum exhibited the presence of 300 kDa and 200 kDa doublet proteins that were strongly reacted

with the antibody. Whether these molecules can serve as a storage form of gelatinase A remains to be answered.

The physiological function relevant to the proteolytic degradation of gelatinase A isoforms present in hFF by bOF is not known. Following ovulation, the oviductal fluid could be mixed with follicular fluid of which components may be beneficial (Anderson et al., 1994; Kulin et al., 1994; Eriksen et al., 1997; Huyser et al., 1997) or harmful (Gearon et al., 1994; Yao et al., 1998) for the gametes to undergo fertilization and development in the oviductal environment. Therefore it is reasonable to suggest that oviductal components might possess mechanisms to selectively activate or inactivate specific follicular fluid components. The bOF components inducing the degradation of gelatinase A isoforms might be one of them involved.

While our results demonstrate that follicular fluid of human could interact with oviductal fluid of bovine *in vitro* resulting in the degradation of particular hFF components, it is not known whether the similar reaction could happen *in vivo* apart from the species difference between human and bovine. Further studies on the nature of bOF components might give an insight to understand the role of mutual interaction between follicular fluid and oviductal fluid in fertilization and/or embryonic development.

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