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PDI-like Enzyme in Human Follicular Fluid Converts 72 kDa Gelatinase into GA110

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사람 난포액에 존재하는 72 kDa Gelatinase로부터 GA110을 만드는 PDI-like Enzyme

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ABSTRACT : Previously, we discovered a new MMP-2 isoform, GA110, of which appearance in human follicular fluid(FF) and serum was increased by EDTA. The present study was conducted to investigate how GA110 can appear by EDTA. To examine possible involvement of protein disulfide isomerase(PDI), an enzyme responsible for the dimerization of protein via disulfide formation, effect of PDI inhibitor on the appearance of GA110 by EDTA was investigated. When PDI inhibitor added to FF before EDTA treatment, the gelatinolytic activity of GA110 was abolished in a concentration dependent manner. By contrast, the activity of 72 kDa gelatinase increased. However, the PDI inhibitor added to FF after EDTA treatment, the gelatinolytic activity of GA110 was unaffected. To find out the nature of the enzyme which converts 72 kDa gelatinase into GA110, chromatographic separation method of FF proteins was done. Using hydroxyapatite column, fractions rich in 72 kDa gelatinase were isolated and pooled. By using this pool as substrate for the 72 kDa converting enzyme, protein fractions containing the converting activity were obtained from chromatographic separation of FF onto glutathione sepharose fast flow column. When immunoblotting was performed on this enzymatically active protein fractions against polyclonal anti-PDI antibody, distinct immunoreactivity was observed, although appeared in smaller molecular weight region. Based on these observations, it is suggested that the appearance of GA110 in FF by EDTA treatment could be due to an activation of PDI-like enzyme, which dimerizes 72 kDa gelatinase into GA110 via the formation of disulfide bond between molecules.

Key words : GA110, 72 kDa gelatinase, PDI-like enzyme, Human follicular fluid.

요 약 : 최근 사람의 난포액에 존재하는 gelatinase 중 EDTA 처리에 의해 활성이 크게 증가하는 GA110을 발견하였으며, 본 연구에서는 이러한 GA110이 만들어지는 기작을 알아보고자 하였다. 먼저, protein disulfide isomerase(PDI)가 관여하는지 알아보기 위해 PDI 저해제를 처리하여 GA110의 활성을 조사하였다. 난포액에 EDTA를 처리하기 전에 저해제를 첨가하여 반응시키면 저해제의 농도가 증가할수록 GA110의 활성이 감소하였으나 반면 72 kDa gelatinase의 활성은 증가하였다. 그러나 EDTA를 처리한 후 저해제를 첨가하여 반응시키면 GA110의 활성에 영향을 주지 못하였다. 다음으로 72 kDa gelatinase로부터 GA110이 만들어지는 과정에 관여하는 효소를 분리하기 위하여 chromatography 방법을 이용하였으며 이렇게 분리한 효소와 기질을 반응시켜 GA110이 만들어지는 것을 확인하였다. 또한 PDI 항체를 이용하여 immunoblotting을 수행한 결과 난포액 내에 PDI보다 분자량은 약간 작지만 항체와 반응하는 단백질이 존재하는 것으로 나타났다. 이러한 결과로 보아 EDTA 처리로 인해 난포액에서 나타나는 GA110은 난포액 내에 존재하는 PDI와 유사한 효소의 활성화로 인해 72 kDa gelatinase 서로간에 이황화 결합이 형성되어 결국 72 kDa gelatinase dimer인 GA110이 만들어지는 것으로 추측된다.

INTRODUCTION

Extracellular matrix(ECM) components can induce the differentiation of cells by directly acting on the specific cells or are indirectly involved in the determination of all kind of cell fates by providing an appropriate environment(Werb, 1997).

Mammalian follicles are surrounded by outer theca cells along basement membrane inside of which layers of mural granulosa cells are located. As follicles grow, fluid-filled cavity appears

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within follicles and oocytes with the surrounding cumulus granulosa cells are floating in the follicular fluid. Upon LH surge, follicular oocytes undergo maturation to get ready to be fertilized and are ovulated from the follicles into the oviduct. Remaining follicles after ovulation luteinize to produce progesterone for some time but later they degenerate. Most follicles failed to grow or to ovulate also degenerate eventually(Rodgers et al., 2003). To allow this programed remodeling to occur, specific mechanism must operates. Of the molecules comprising the follicle structure, many are ECM components and found in every place within follicles. Basement membrane of mammalian follicles consists of various ECM molecules such as fibronectin, type IV collagen, laminin, heparan sulfate proteoglycan(Rodgers & Irving-Rodgers, 2002; Akkoyunlu et al., 2003), perlecan, nidogen I(Rodgers et al., 2003), versican(Russell et al., 2003). Mural granulosa cell layers within follicles also contain ECM components such as cartilage link protein(Sun et al., 2002), thromospondin-1 and -2(Petrik et al., 2002). In contrast, cumulus granulosa cell layers consist of hyaluronan and versican(Rodgers et al., 2003; Russell et al., 2003). Therefore, rearrangement of these diverse components needs various enzymes to act on specific targets during remodeling.

Gelatinases are a member of matrix metalloproteinases(MMPs) responsible for the degradation of extracellular matrix and basement membrane components during remodeling of the most tissues. Two forms of gelatinases identified are 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 such as type IV collagen, laminin, fibronectin as well as gelatin(Birkedal-Hansen et al,. 1993). Of these, the 72 kDa gelatinase A is the most widely distributed of all MMPs and has been identified in a variety of tissues, including mammalian ovaries(Ny et al., 2002). Previously, it was found that follicular fluid and sera of human and bovine species contain a new isoform of MMP-2, GA110, and gelatin zymogram showed that the gelatinolytic activity of the isoform was greatly increased by the treatment of EDTA(Kim et al., 2003). Since EDTA is generally known as an inhibitor of MMPs and since the activity increase of GA110 by EDTA was due to increased amount of GA110 protein, it is important to clarify the mechanism how EDTA addition to the FF could result in the appearance of GA110. However, as revealed by the reducing gel electrophoresis, it was observed that GA110 molecule was constructed via disulfide bond between molecules. Thus a possibility can be raised that an enzyme might catalyze oxidation of intermolecular sulfhydryl residue of MMP-2 resulting in the dimerization.

Protein disulfide isomerase(PDI), an enzyme found in the endoplasmic reticulum of higher and lower eukaryotes, catalyses the formation, reduction, and isomerization of disulfide bonds during the folding pathway of secretory proteins(Freedman et al., 1994). PDI is a well characterized 57 kDa oxidoreductase(Edman et al., 1985) and its redox function is based on the presence of two active sites in a Cys-Gly-His-Cys(CXXC) motif. When the cysteins of CXXC are oxidized, PDI interacts with two cysteinyl thiol groups of a neighboring peptide to form a disulfide bond. When CXXC bears two cysteinyl thiols, it cleaves neighboring disulfide bonds. PDI has been found to be secreted by various cells including hepatocytes(Terada et al., 1995), pancreatic exocrine cells(Yoshimori et al., 1990), endothelial cells (Hotchkiss et al., 1998), and activated platelet(Chen et al., 1992). Secretion of over expressed ERp72(Dorner et al., 1990) and ERp57(Hirano et al., 1995) from cultured cells has also been observed. While the biological importance of these secreted proteins remains in most cases obscure, a function of PDI secreted by thyrocytes into the lumen of the thyroid follicles has been identified(Delom et al., 2001). It has been shown that the enzyme is involved, together with the BiP protein(the immunoglobulin heavy chain-binding protein) in the control of thyroglobulin folding and multimerization, probably by reducing the intermolecular disulfide bridge and thus limiting the extent of multimers formation.

The present study investigated the nature of the enzyme converting 72 kDa MMP-2 into GA110 by the treatment of FF with EDTA.

MATERIALS AND METHODS

1. Preparation of human follicular fluid(FF)

FF was obtained from patients participating in an IVF program at a local hospital. The use of human material for research was based on informed consent and was approved by the Local Ethics Committee. Follicles were aspirated through the vaginal wall and follicular fluid devoid of oocytes was spun at 2,000 × g for 30 min. The supernatant was taken as FF and used

immediately or kept at −20℃ until use. The pellet of granulosa cell was washed several times and also kept frozen at −20℃ until use. There was no difference between the gelatinolytic activity of fresh FF and that of frozen FF. Before using, FF was spun and millipore-filtered to remove any precipitate. Where indicated, FF was treated with 5 mM ethylenediaminetetraacetic acid(EDTA) and then incubated for 3 h at 37℃. Human serum was donated from healthy women.

2. Treatment of PDI inhibitor

DTNB(6,6'-dinitro-3,3'-dithiodibenzoic acid) or PAO(phenylarsine oxide) was added to FF as PDI inhibitor. Firstly, FF was pretreated with PDI inhibitor at 37℃ for 3 h and treated with 5 mM EDTA at 37℃ for another 3 h. Secondly, FF was pretreated with EDTA at 37℃ for 3 h and treated with PDI inhibitor at 37℃ for another 3 h. The inhibitor was prepared as a stock solution of 100 mM in DMSO and final inhibitor concentration was prepared as 0.1, 1, or 5 mM.

3. Gelatin zymography

Gelatin substrate SDS-PAGE was used by the addition of 1 mg/ml bovine skin gelatin(type B) to 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. Unless specified elsewhere, 30 μg protein in 0.75 μl of FF was loaded onto a single well. After gelatin SDS electrophoresis, gels were soaked with 2.5% Triton X-100 in 50 mM Tris-HCl buffer(pH 8.0) for 30 min and washed in the 100 ml of incubation buffer(5 mM $CaCl_2$, 0.02% NaN_3, 50 mM Tris-HCl, pH 8.0). Then gels were incubated in fresh incubation buffer overnight at 37℃ for the development of gelatinolytic activity. The reacted gels were stained with Coomassie brilliant blue R-250 dye(acetic acid : isopropyl alcohol : water = 1 : 5 : 4) and the clear bands on blue background were regarded as gelatinase bands since gelatinases degrade gelatin in the acrylamide gel including gelatin.

4. Isolation of 72 kDa MMP-2 as an enzyme substrate

To isolate 72 kDa gelatinase, FF was applied onto a hydroxyapatite column(Bio-rad, Hercules, USA). After washing the loaded column with the 10 ml of starting buffer(10 mM sodium phosphate, pH 7.8), each 8 ml fraction was eluted with the two different concentration of NaCl, 0.3 M(eluate I) and 1 M(eluate II), respectively. After zymographic analysis of 5 μl each fraction, those showing the strong 72 kDa gelatinase activity were selected, pooled and used as an enzyme substrate after desalting with the starting buffer.

5. Isolation of the enzyme which converts 72 kDa MMP-2 to GA110

Ten ml of FF was loaded onto the 5 ml glutathione sepharose fast flow(Amersham International, England) at 4℃. After washing with the starting buffer(10 mM sodium phosphate, pH 7.8), the bound proteins were eluted with the same buffer, but containing 0.2 M NaCl and 10 mM reduced glutathione(GSH). These later eluted fractions were individually tested for the existence of the enzymatic activity. Protein fractions collected by the glutathione sepharose column chromatography were concentrated using the Centricon(Millipore, USA) and Microcon (Millipore, USA). After mixing two solutions, the mixture was incubated overnight at 37℃ and the reaction product was investigated using the gelatin zymography.

6. Immunoblotting

After nonreducing electrophoresis, the gels were soaked in a transfer buffer made of 25 mM Tris(pH 8.4), 192 mM glycine, and 10% methanol. Proteins on the gel were electrotransferred onto PVDF membrane(Immobilon-P, Millipore, USA) for 1 h at 4℃, 200 mA. Before transfer, membranes were hydrated with absolute methanol, distilled water, and then with transfer buffer. To saturate nonspecific binding sites, membranes after transfer were incubated at 37℃ for 1 h in a reaction buffer(10 mM sodium phosphate buffer, pH 7.4, 0.05% Tween 20, 10 mM sodium azide) containing 5% BSA. They were then incubated for 1 h in the buffer containing 1% normal goat serum and 1 μg/ml rabbit polyclonal antibody against bovine PDI. Following washing several times, membranes were incubated for 1 h in the buffer containing 1:100 diluted gold-labeled goat anti-goat IgG antibody(Amersham International, England). After reaction, the signal was visualized by using IntenSE BL kit(Amersham International, England) according to the manufacturer's manual. As a control, purified bovine PDI was used.

7. Reagents

Reagents used throughout this experiment were obtained from Sigma(St.Louis, USA) unless otherwise mentioned.

RESULTS

1. Gelatinases in human follicular fluid(FF)

Typical gelatinases found in FF are shown in Fig. 1. When FFs obtained from five women were zymographically examined after treatment with EDTA for 3 h at 37℃, all of them consistently exhibited the appearance of an intense 110 kDa gelatinase(GA110) band and two weak bands of 98 kDa and 72 kDa gelatinases. Two bands of 88 and 84 kDa are not gelatinases(Kim et al., 2001).

2. Effects of PDI inhibitor on the gelatinolytic activity of GA110

To examine the properties of the enzyme responsible for the conversion of 72 kDa gelatinase into GA110, DTNB and PAO were used as PDI inhibitors. When DTNB was added before EDTA treatment, the gelatinolytic activity of GA110 was abolished as the concentration of the inhibitor was increased such that significant gelatinolytic activity of GA110 was observed at 0.1 mM concentration of the inhibitor whereas complete absence of the activity was found at 1 or 5 mM concentration. Concomitantly with the reduction of GA110 activity, gelatinolytic activity of 72 kDa gelatinase was increased. By contrast, when the same PDI inhibitor was added after EDTA treatment, the gelatinolytic activity of GA110 was not affected(Fig. 2A). Another PDI inhibitor PAO also showed similar results as revealed by the disappearance of GA110 activity at 5 mM concentration of the inhibitor(Fig. 2B). However, the inhibitory activity of PAO was not as effective as DTNB as seen by the incomplete absence of GA110 at 1 mM concentration. Again the addition PAO after EDTA treatment did not affect the GA110 activity.

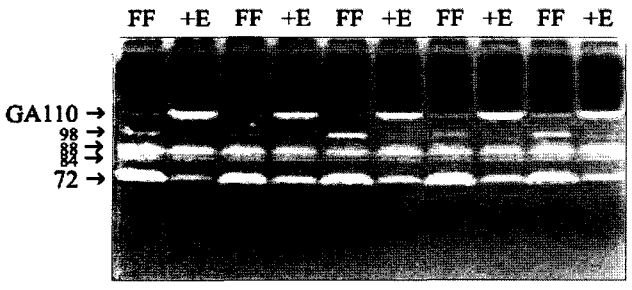

Fig. 1. Gelatin zymogram of GA110 in human follicular fluid(FF). FF indicates an untreated follicular fluid, and +E indicates an FF treated with EDTA fors 3 h at 37℃. FFs were collected from five women and the results show that every FF produced intense GA110 activity after treatment with EDTA.

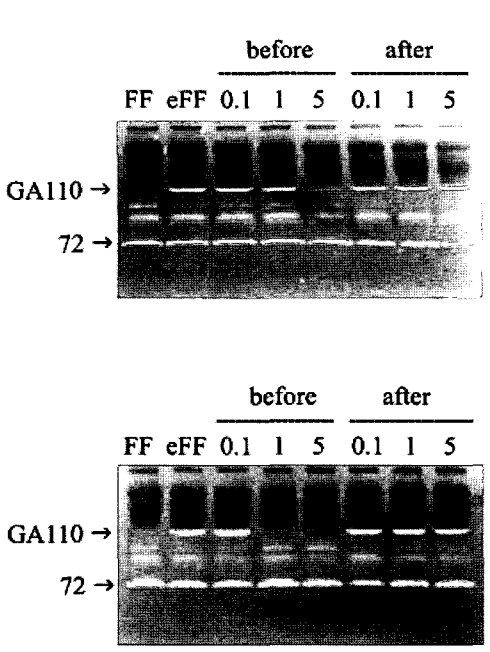

Fig. 2. Effects of PDI inhibitors DTNB(A) and PAO(B) on the gelatinolytic activity of GA110 in follicular fluid. *before* indicates the FF pretreated with 0.1 mM, 1 mM, and 5 mM PDI inhibitor for 3 h and added 5 mM EDTA for 3 h. *after* indicates the FF pretreated with 5 mM EDTA for 3 h and processed with 0.1, 1, or 5 mM PDI inhibitor for 3 h.

3. Isolation of 72 kDa gelatinase as the enzyme substrate

After loading fresh FF onto hydroxyapatite column, elution was done in a three-step gradient consisting of 0, 0.3, and 1.0 M of NaCl concentration(Fig. 3A). When fractions of the three groups were examined zymographically for the presence of gelatinases, fractions eluted with by 0 M NaCl exhibited the presence of an intense gelatinolytic activity corresponding to 98 kDa gelatinase, thought to be MMP-9 molecule(Besnard et al., 1997). By contrast, fractions eluted with 1.0 M NaCl showed most 72 kDa activity. Although some of 72 kDa activity also appeared in 0.3 M NaCl fractions, these were discarded because proteinases other than 72 kDa gelatinase were also retained(Fig. 3B).

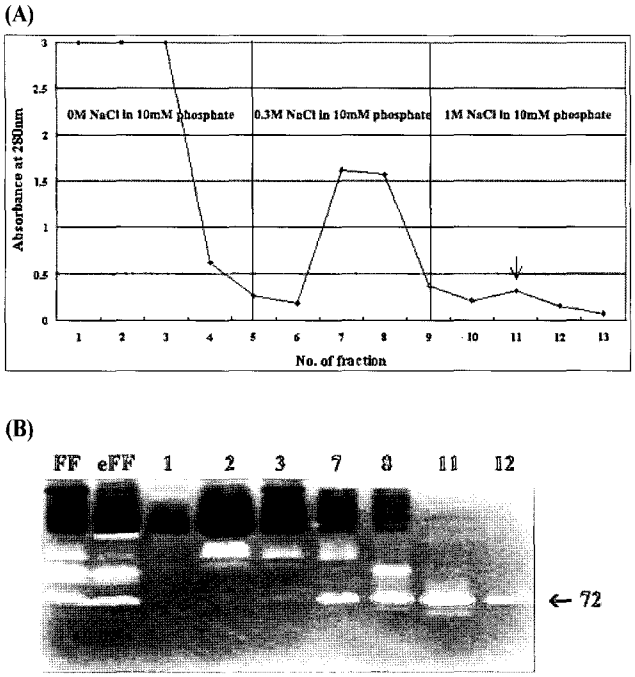

Fig. 3. Separation of 72 kDa MMP-2 by hydoxyapatite column. A, eluiton profile of FF by hydoxyapatite column; B, corresponding gelatinase activity of FF fractionates in A. An arrow in A indicates the fraction which retained most of the 72 kDa gelatinase.

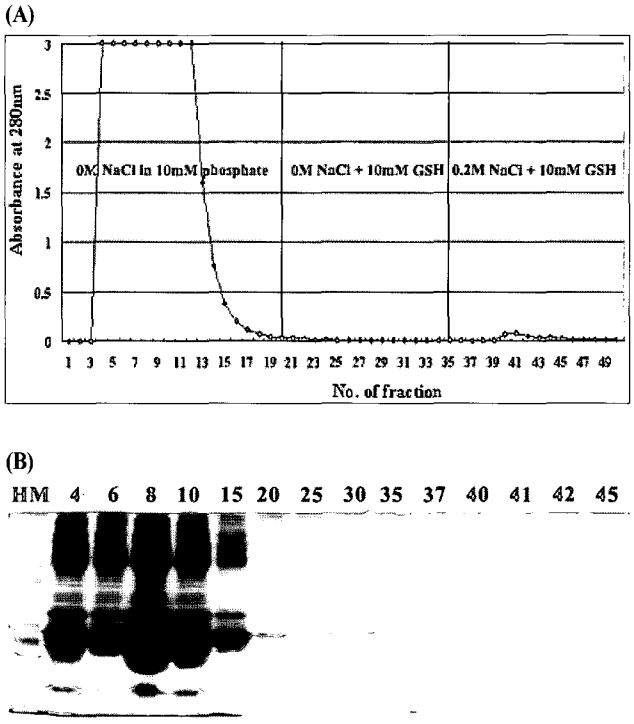

Fig. 4. Separation of PDI-like enzyme in human follicular fluid by glutathione sepharose fast flow chromatography. A, eluiton profile of FF pretreated with EDTA by glutathione sepharose column; B, silver stained gel of separated FF by glutathione sepharose column.

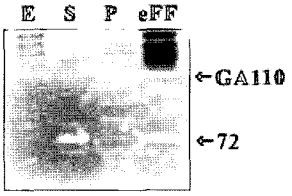

Fig. 5. Enzymatic activity of FF component separated by glutathione sepharose column with crude 72 kDa gelatinase. Partially purified FF components as described in Fig. 4 were allowed to react with the crude 72 kDa gelatinase substrate as prepared in Fig. 3. E, the fractionate eluted with 10 mM GSH and 0.2 M NaCl; S, the 72 kDa substrate as prepared as in Fig. 3; P, the mixture of E and S kept overnight at 37℃; eFF, FF treated with EDTA for 3 h at 37℃. Note the discrete band corresponding to GA110 in lane P.

4. Preparation of an enzyme which converts 72 kDa gelatinase into GA110

After treating 10 ml of FF with EDTA, isolation of an enzyme converting 72 kDa gelatinase into GA110 was performed using glutathione sepharose column. When elution was done three steps consisting of 0 mM GSH, 10 mM GSH, and 10 mM GSH with 0.2 M NaCl, most of FF proteins did not adsorb onto the glutathione sepharose beads shown by absorption spectrum(Fig. 4A). Silver stained electrophoresed gel also revealed that little protein was present in fractions of the latter two groups(Fig. 4B). However, when the concentrated solution of fractions eluted lastly was mixed with the 72 kDa gelatinase substrate prepared as described in the above and kept overnight at 37℃, a discrete band corresponding to GA110 appeared in the mixture as seen in Fig. 5.

5. Immunoblot analysis of the enzyme against anti-bovine PDI antibody

Finally to see if the above enzymatically active solution might contain PDI, immunoblot analysis was performed using rabbit polyclonal anti-bovine PDI antibody. As seen in Fig. 6A, FF exhibited only one weak immunoreactivity corresponding to 53 kDa one. Similarly the enzyme solution active in converting the 72 kDa gelatinase into GA110 showed only weak band with 53 kDa size. By contrast, human granulosa cell extract showed one intense immunoreactive band corresponding to 57 kDa PDI and

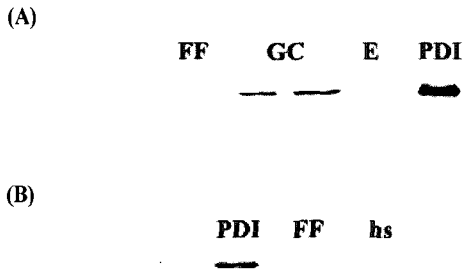

Fig. 6. Immunoblot analysis using anti-bovine PDI antibody. In(A), immunoreactivity of FF, human ovarian granulosa cell(GC), enzyme solution used in Fig. 5(E), and purified bovine PDI(PDI) is shown. Note that an intense 57 kDa protein(upper band) appears in GC and PDI and a weak 53 kDa protein(lower band) appears in FF, GC, and E. In(B), only 57 kDa protein appears in PDI and only 53 kDa protein appears in both FF and human serum(hs).

a weak activity with 53 kDa one. When human serum was examined similarly, again only 53 kDa was immunoreactive as in FF.

DISCUSSION

Previously, it was observed that a new MMP-2 isoform, GA110, was present in FF and its appearance was greatly increased by EDTA(Kim et al., 2003). Moreover, GA110 was found to consist of homodimer of 72 kDa gelatinase via disulfide bond between molecules. It has been reported that thrombospondin, an adhesive protein released from the activated platelets, forms SDS-stable complex with itself or other proteins by disulfide bond accelerated by EDTA(Speziale & Detwiler, 1990). EDTA has been suggested to chelate Ca^{++} that inhibits intramolecular thiol-disulfide exchange in thrombospondin (Huang et al., 1997). Further studies are needed to see if induction of GA110 in FF by EDTA could be due to a similar mechanism.

PDI catalyzes formation, reduction and isomerization of disulfide bonds in proteins and is believed to play an important role in folding of disulfide-bonded proteins in the cell. Consequently, to examine the possible involvement of PDI in the mechanism by which GA110 is induced by EDTA in FF, effect of PDI inhibitor on the appearance of GA110 was investigated. When PDI inhibitor was pretreated and then EDTA was added to FF, the GA110 activity was inhibited in a concentration-dependant manner. DTNB at 1 mM and PAO at 5 mM were enough to abolish most GA110 activity. On the other hand, when the EDTA was added to FF before PDI inhibitor treatment, gelatinase activity of GA110 was not abolished. These observations suggest that PDI or PDI-like enzyme could be activated by EDTA, thus enabling the conversion of 72 kDa gelatinase into GA110.

In addition, DTNB seems to be a better inhibitor than PAO, because it acts on different mechanism. The membrane-impermeable reagent DTNB forms mixed disulfides with thiol groups, PAO, and its derivative As^{+3} with the vicinal thiols of the CXXC motif of proteins such as PDI(Gallina et al., 2002). Therefore, the PDI or the PDI-like enzyme is thought to be related to converting 72 kDa to GA110.

Although PDI is known to exist in the lumen of the rough endoplasmic reticulum(ER), PDI and PDI family proteins have been also found outside of the cell(Turano et al., 2002). Platelets secrete PDI, some of which localize to the plasma membrane and remains active(Essex et al., 1995). Although platelets have no identified ER, internal PDI is diffusely localized in the cytosol and at the cell surface. Platelets activated by thrombin or the calcium ionophore A 23187 secrete PDI into the surrounding media(Chen et al., 1992; Chen et al., 1995). The PDI secreted by the activated platelets is apparently released from its internal cytosolic localization by vesiculation. PDI secreted by platelets is active in *in vitro* assays, suggesting that it may have a significant physiological role in protein-thiol mediated events in the region of the wound. PDI catalyzes disulfide isomerization reaction on thrombospondin I and its 120 kDa fragment in the calcium binding region of the molecule, which may affect their interaction with thrombin(Hotchkiss et al., 1996; Huang et al., 1997).

To investigate whether the enzyme producing GA110 in FF might be a PDI or related one, glutathione sepharose fast flow column chromatography was performed to isolate the enzyme with the found properties. Glutathione sepharose fast flow is designed for purification of glutathione S-transferase(GST) fusion protein and glutathione binding proteins. Most proteins are eliminated with void fractions, and only a small part of the proteins were adsorbed to the glutathione sepharose beads. These fractions were concentrated and were used as the enzyme source. Meanwhile, to isolate 72 kDa gelatinase for a substrate of the enzyme, hydroxyapatite column was used. This fraction eluted

with high concentration of salt was too high in salt concentration, thus desalting was needed to use it as the substrate. The reaction product of the enzyme and the substrate was observed using the zymography method. Zymogram of the product revealed the clear zone in the similar place to 110 kDa, while 72 kDa band has disappeared. To verify the nature of the enzyme, immunoblotting was performed with polyclonal rabbit antibody against bovine PDI. The enzyme fraction resulted in the appearance of a weak immunoreactive band corresponding to 53 kDa protein of which size is smaller than 57 kDa of the known PDI molecular weight(Edman et al., 1985). Whether this smaller protein might be a variant of PDI appearing only in follicular fluid, immunoblotting was done on human granulosa cell homogenate along with the FF. The homogenate was found to contain both 57 kDa PDI and unknown 53 kDa protein which was also found in FF and the enzyme isolated. Human serum similarly exhibited the presence of 53 kDa protein only as in FF. Based on these results, several explanations can be made. Firstly, assuming that both forms of PDI might be synthesized from the same species of mRNA, PDI destined to function within a cell remains cytosol as 57 kDa form without further modification after being synthesized, whereas PDI destined to be secreted from the cell undergoes further processing to become a 53 kDa form before release. Another possibility is that 57 kDa and 53 kDa PDI could be synthesized from the different species of mRNA but share epitopes with each other. Again in this case, 57 kDa might be a cytosolic form while 53 kDa might be an extracellular form. Previous reports have shown that in addition to a major 55 kDa PDI, a novel 53 kDa polypeptide of PDI was found in chick embryos. Interestingly, this 53 kDa was not identical to the chick 55 kDa PDI but was able to react with the anti-bovine PDI antibody, and it shares epitopes with the bovine 57 kDa PDI(Bassuk & Berg, 1991).

Taken together, these results suggest that the enzyme converting 72 kDa gelatinase into GA110 in FF treated with EDTA possesses PDI-like activity and could be a member of PDI family. Further studies are needed to unveil the nature of this enzyme.

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