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Activation of Human Progelatinase A/Promatrix Metalloproteinase 2 by *Escherichia coli*-Derived Serine Proteinase

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Treatment of human uterine cervical fibroblasts with commercial lipopolysaccharide (LPS) preparations from different serotypes of *Escherichia coli* effectively augmented the processing of mammalian progelatinase A/promatrix metalloproteinase (proMMP)-2 to a 62-kDa form of MMP-2. When purified proMMP-2 was incubated with LPS preparations, the proenzyme was similarly processed into the 62-kDa active MMP-2 in a time- and dose-dependent manner. By contrast, progelatinase B/proMMP-9 and prostromelysin 1/proMMP-3 were not activated. A serine proteinase inhibitor, phenylmethylsulfonyl fluoride, completely interfered with this LPS-mediated activation of proMMP-2. This is novel evidence that *E. coli* serine proteinase is a specific activator of proMMP-2. Thus, it is very likely that *E. coli* infection plays a crucial role in the degradation of connective tissues via the activation of proMMP-2, and the resultant active MMP-2 participates in the dysfunction of connective tissues such as in the preterm rupture of fetal membranes.

Matrix metalloproteinases (MMPs) are a family of potent enzymes that participate in the physiological and pathological breakdown of extracellular matrix components such as collagens, elastin, laminin, fibronectin and proteoglycans (for review, Refs. 1, 2). Most MMPs are secreted from a variety of cells as inactive zymogens (proMMPs) and then activated by several proteinases such as plasmin, plasma kallikrein, trypsin, neutrophil elastase and active MMPs in a stepwise manner (1, 2). Among these proMMPs, progelatinase A/proMMP-2 is resistant to activation by the above proteinases (1, 2). Recent studies revealed that proMMP-2 is activated by plasma-membrane-bound metalloproteinases called membrane type-MMPs (MT-MMPs) (3–7). The expression of most proMMPs is strictly regulated by several stimuli such as cytokines, growth factors and hormones, but the production of proMMP-2 is not regulated by these stimuli. This is the case except for transforming growth factor (TGF)-β and extracellular matrix metalloproteinase inducer (EMMPRIN) (1). Thus, the activation is thought to be a key step for the control of enzymatic activity of MMP-2.

Intrauterine bacterial infection is recognized to be closely related to chorioamnionitis, premature rupture of fetal membranes and preterm birth (8–12). The uterine cervix and fetal membranes are typical connective tissues consisting of collagen types I, III, and V and proteoglycans (13). A significant decrease in the density of collagen types I, III, and V is observed in the zone of altered morphologic structure at term (13) and those collagens function to maintain the tensile strength of the tissues. Type V collagenolytic activity is thought to be responsible for the maintenance of the strength of the fetal membranes (14). In this point of view, the fact that some bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* produce a number of proteinases including collagenolytic enzyme is of much interest (12). Thus, these enzymes degrade the extracellular matrix components and thereby result in dysfunction of connective tissues in the reproductive tract. Moreover, bacterial proteinases also activate some zymogens of MMPs including proMMP-1/procollageanse 1 and proMMP-9/progelatinase B, but not proMMP-2 (15–17).

Abbreviations used: MMP, matrix metalloproteinase; MT-MMP, membrane type-MMP; LPS, lipopolysaccharide; Con A, concanavalin A; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; FBS, fetal bovine serum; LAH, lactalbumin hydrolysate; PMSF, phenylmethylsulfonyl fluoride.

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However, very little is known about the activation of proMMP-2 by bacterial infection.

In this study we report novel evidence that serine proteinase from *E. coli* effectively activates proMMP-2 but no other proMMPs including proMMP-3 and proMMP-9. Our study suggests that intrauterine infection is likely to result in a reduction of tensile strength of reproductive tract and to accelerate the preterm rupture of fetal membranes and/or preterm birth.

MATERIALS AND METHODS

Materials. *E. coli* lipopolysaccharide (LPS) (serotypes; O26:B6, O55:B5, O111:B4 and O127:B8), protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), lactalbumin hydrolysate (LAH) and alkaline phosphatase conjugated donkey anti-(sheep IgG)IgG were from Sigma. Minimum essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) were from Life Technologies, Inc. Concanavalin A (Con A) was from Seikagaku Kogyo Co. Gelatin was from DIFCO. Fetal bovine serum (FBS) was from Biowhitakker. Human purified proMMP-3 and sheep anti-(human MMP-3)antiserum were kindly provided by Prof. Hideaki Nagase, University of Kansas Medical Center. Other reagents used were of analytical reagent grade.

Cultures and treatment of human and rabbit fibroblasts. Human uterine cervical fibroblasts were prepared and maintained in MEM containing 10% (v/v) FBS as described previously (18). In all experiments, cells up to the 6th passage were used. Rabbit dermal fibroblasts were prepared by tissue explant culture from the skins of Japanese white rabbits. Fibroblasts grown out of tissue explants in DMEM/10% (v/v) were detached with 0.1% trypsin/0.02% (w/v) EDTA and subcultured in the same medium. In all experiments, cells up to the 8th passage were used. Treatment of respective cell species was carried out as follows; confluent cells plated in 24-multiwell plate were once washed with PBS(−) and then treated with LPS in 1.0 ml of the respective culture medium/0.2% (w/v) LAH for 24 h. The conditioned culture media were harvested and stored at −20°C until use.

Purification of proMMP-2 from the culture medium of human uterine cervical fibroblasts. Human proMMP-2 was purified from the culture medium of confluent human uterine cervical cells as described previously (19). Briefly, the harvested culture medium was applied to a column of gelatin-Sepharose 4B equilibrated with 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/0.02% (w/v) NaN_3. ProMMP-2 was eluted from the column with 5% (v/v) dimethyl sulfoxide in the same buffer. The proMMP-2 preparation was free from an active form of MMP-2.

Assay for gelatinolytic activity. Gelatinolytic activity was detected by gelatin zymography using a 10% (w/v) acrylamide slab gel containing 0.9 mg/ml of gelatin under nonreducing conditions as described previously (19). The gelatinolytic activity was also examined by monitoring degradation of heat-denatured type I collagen (gelatin). An aliquot (50 μl) of enzyme preparation and 50 μg of gelatin were incubated for 1 h at 37°C in a total volume of 75 μl 50 mM Tris–HCl (pH 7.5)/0.15 M NaCl/10 mM $CaCl_2$/0.02% (w/v) NaN_3. Then the reaction mixture was applied to SDS–PAGE using 7.5% (w/v) acrylamide under reducing conditions (20), and the digestion of gelatin was monitored by staining proteins with Coomassie brilliant blue R-250.

Western blot analysis. The sample was mixed with 1/5 volume of 20% (w/v) trichloroacetic acid. The resultant precipitates were dissolved in reducing SDS–PAGE sample buffer (20), and the portion was first subjected to SDS–PAGE (20) using 10% (w/v) acrylamide slab gel under reducing conditions, and then proteins in the gel were electro-transferred onto a nitrocellulose membrane. The membrane was reacted with sheep anti-(human MMP-3)antiserum which was then complexed with alkaline phosphatase-conjugated donkey anti-(sheep IgG)IgG. Immunoreactive proMMP-3 was visualized indirectly using 5-bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium as described previously (21).

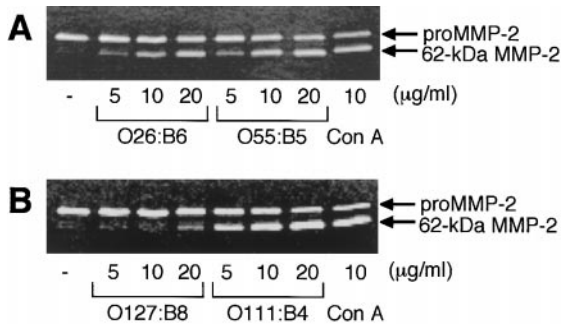

FIG. 1. Effect of LPS on the activation of progelatinase A/proMMP-2 in cultured human uterine cervical fibroblasts. Confluent cells at the 5th passage in 24-multiwell plate were treated with LPS derived from *E. coli* (serotypes; O26:B6, O55:B5, O111:B4 and O127:B8) or Con A (10 μg/ml) in 1.0 ml of MEM/p0.2% (w/v) LAH. After 24 h culture media were harvested and a portion (10 μl) was subjected to gelatin zymography as described under Materials and Methods.

RESULTS

LPS preparations from E. coli induced the activation of proMMP-2 in human uterine cervical fibroblasts. Since LPS is well known to exert many biological activities on many cell species, we first investigated whether LPS induces the activation of proMMP-2 and/or production of specific proMMP-2 activator, MT1-MMP, in human uterine cervical fibroblasts. When confluent human uterine cervical cells were treated with LPS from several serotypes of *E. coli*, the processing of 62-kDa MMP-2 was accelerated along with the decreasing proMMP-2 in a dose dependent manner (5–20 μg/ml) as shown in Fig. 1. The activation was observed in all four preparations of LPS examined and other commercial LPS from *Pseudomonas aeruginosa and Salmonella typhimurium* also processed 62-kDa MMP-2 (data not shown). This newly processed 62-kDa MMP-2 is very likely to be an active MMP-2 since its relative molecular mass was identical to that of active MMP-2 produced by human uterine cervical cells treated with Con A which is well characterized to induce a proMMP-2 activator of MT1-MMP in many cell species (19). Surprisingly, neither immunoreactivity nor enzymatic activity of MT1-MMP were detected in the 62-kDa MMP-2 by LPS preparations even when the fibroblasts were cotreated with LPS and polymyxin B which is a typical inhibitor of LPS (22) (data not shown). These results suggest that the activation of proMMP-2 in LPS-treated cells is not due to the action of LPS itself.

Then we examined whether LPS itself processed proMMP-2 to 62-kDa form of MMP-2. When purified

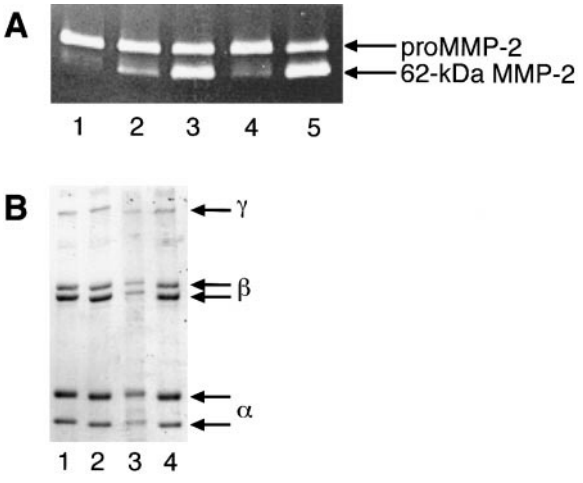

FIG. 2. LPS directly activated purified proMMP-2. (A) Effect of LPS preparations on the purified proMMP-2. Purified human proMMP-2 (500 ng) was incubated with 10 μg of LPS derived from each serotypes of *E. coli* for 24 h at 37°C in a total 0.5 ml of MEM/0.2% (w/v) LAH/0.02% (w/v) NaN_3. After the incubation a portion (10 μl) of the incubation mixture was subjected to gelatin zymography as described under Materials and Methods. Lane 1, untreated conditioned medium and lanes 2–5, conditioned medium incubated with LPS of stereotypes O26:B6, O55:B5, O127:B8 and O111:B4, respectively. (B) 62-kDa MMP-2 digested heat-denatured type I-collagen (gelatin). MMP-2 with 62-kDa produced by LPS preparation as in A and gelatin were incubated at 37°C for 1 h and then the reaction mixture was applied to SDS–PAGE as described in the text. Lane 1, undigested gelatin; lane 2, gelatin treated with proMMP-2; lane 3, gelatin treated with the 62-kDa MMP-2 and lane 4, gelatin treated with the 62-kDa MMP-2 in the presence of 20 mM EDTA. α, β and γ correspond to α, β- and γ-chains of heat denatured type I-collagen (gelatin).

proMMP-2 was incubated with LPS preparations, all four serotypes of LPS augmented the appearance of 62-kDa MMP-2. Both 055:B5 and 0111:B4 were effective among them (Fig. 2A). The processing of 62-kDa form by 055:B5 LPS was found to be time-dependent and fairly stable for at least 24 h (data not shown). These results strongly suggest that the LPS-mediated processing of 62-kDa MMP-2 is due to an activator contained in LPS preparations used. In addition, the appearance of 62-kDa form of MMP-2 paralleled an increase in gelatinolytic activity against heat-denatured type I collagen (gelatin), and EDTA effectively interfered with this gelatinolytic activity as shown in Fig. 2B. This further supports the finding that the 62-kDa form of MMP-2 is an active MMP-2 and the activator in LPS-preparations directly activates proMMP-2.

Characterization of proMMP-2 activator in LPS preparations. Next, we characterized the activator in the LPS preparations. When LPS preparation was preincubated with commercial *E. coli* proteinase inhibitor (Sigma) of which composition was EDTA, 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, bestatin and trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64), the processing of active MMP-2 was completely inhibited (data not shown). Pretreatment of LPS preparations with a serine proteinase inhibitor, PMSF, also interfered with the activation of proMMP-2 (Fig. 3A). Furthermore, the boiling of LPS preparations for 30 min also destroyed the activity of the activator (Fig. 3B). These results suggest that proMMP-2 activator in LPS preparations is predominantly due to serine proteinase(s).

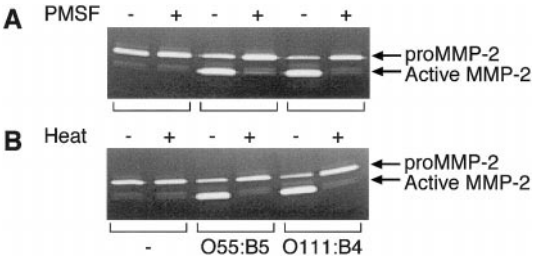

FIG. 3. Effect of serine proteinase inhibitor and heat on the LPS-mediated activation of proMMP-2. LPS (20 μg, *E. coli* O55:B5 and O111:B4) in 0.5 ml of MEM/0.2% (w/v) LAH/0.02% (w/v) NaN_3 was preincubated with a final concentration of 1 mM PMSF at 37°C for 30 min (A) or was stood in a boiling water bath for 30 min (B), and then the LPS was further incubated with 1 μg of human proMMP-2 at 37°C for 24 h in a total 1.0 ml of MEM/0.2% (w/v) LAH/0.02% (w/v) NaN_3. The activation of proMMP-2 was monitored by gelatin-zymography as described in the legend to Fig. 1.

E. coli serine proteinase selectively activated proMMP-2. We further examined whether proteinase from *E. coli* activates other proMMPs. Both LPS preparations from *E. coli* 055:B5 and 0111:B4 rapidly processed rabbit proMMP-2 to 62 kDa active form, indicating that *E. coli* proteinase is able to activate proMMP-2 from different origins. By contrast, *E. coli* proteinase could not activate proMMP-9/progelatinase B (Fig. 4). Human proMMP-3/prostromelysin 1 was rapidly degraded into small species without the appearance of an active form (Fig. 5). Therefore, it is likely that *E. coli* serine proteinase selectively activates proMMP-2.

DISCUSSION

Although MMP-2 is known to rapidly hydrolyze gelatins and types I, IV, and V collagens, other connective

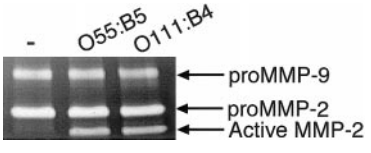

FIG. 4. Effect of LPS on the activation of progelatinase B/proMMP-9. Conditioned medium of rabbit dermal fibroblasts which contained proMMPs-2 and -9 was incubated with a final 20 μg/ml of LPS from *E. coli* (stereotypes O55:B5 and O111:B4, respectively) at 37°C for 24 h, and then changes in molecular mass of proMMPs-9 and -2 was monitored by gelatin zymography as described in the legend to Fig. 1.

FIG. 5. Effect of proteinase in LPS on the activation of prostromelysin-1/proMMP-3. Human purified proMMP-3 (1 μg) was incubated with 20 and 60 μg of LPS from *E. coli* 055:B5 for 24 h at 37°C in a total 2.0 ml of MEM/0.5% (w/v) LAH/0.02% (w/v) NaN_3. After the incubation a portion (1.4 ml) of the incubation mixture was concentrated with a final 4.0% (w/v) trichloroacetic acid and the resultant precipitate was subjected to Western blot analysis for MMP-3 as described under Materials and Methods. Lane 1, untreated proMMP-3, and lanes 2 and 3, proMMP-3 treated with LPS (10 and 30 μg/ml, respectively).

tissue components including aggrecan, elastin, fibronectin and laminin are also degraded (2). Thus, MMP-2 plays central roles in connective tissue degradation and re-modeling. ProMMP-2 is constitutively produced by many cell species including connective tissue cells and tumor cells in culture. Therefore, the activation of proMMP-2 is a major rate-limiting step in regulating its activity. It is generally known that neither serine proteinases nor active MMPs activate mammalian proMMP-2, and MT-MMPs are characterized as a specific proMMP-2 activator (2, 19). When connective tissue cells are treated with Con A, MT1-MMP was transcriptionally induced and localized on the cell surface and thereby 72-kDa proMMP-2 was effectively processed to 62-kDa active MMP-2 along with the appearance of a 64-kDa intermediate MMP-2 (19). During the course of our investigation of MT1-MMP inducers in human uterine cervical fibroblasts, we observed that commercial LPS-preparations form 4 serotypes of *E. coli*, *P. aeruginosa* and *S. typhimurium* effectively produced fully active 62-kDa MMP-2 without induction of any MT1-MMP in fibroblasts. Furthermore, the serine proteinase derived from *E. coli* was found to activate proMMP-2 into its active form.

Some bacterial proteinases such as thermolysin-family metalloproteinase are known to activate proMMP-1, proMMP-8/neutrophil collagenase and proMMP-9 (15–17). However activation of proMMP-2 by bacterial proteinases is not understood well. In this respect, *E. coli* is known to produce a number of proteinases including serine proteinases (23, 24), and we demonstrated that the serine proteinase as well as mammalian MT1-MMP functions as an activator of mammalian proMMP-2. In addition, the serine proteinases from four serotypes of *E. coli* was similarly able to activate mammalian proMMP-2, suggesting that serine proteinase from most serotypes of *E. coli*. is likely to ubiquitously activate proMMP-2. It is of interest that *E. coli* proteinase selectively activates proMMP-2 but not proMMP-9 and proMMP-3 since mammalian serine proteinases such as plasmin and trypsin have a potential to activate both proMMP-3 and proMMP-9, but not proMMP-2. At present, we can not explain in detail the above discrepancy between the mammalian serine proteinases and the *E. coli* one. Further studies are necessary to clarify the exact action mechanisms of *E. coli* proteinase during proMMP-2 activation.

It is reported that *E. coli* is frequently found in amniotic fluid and intrauterine tissues in cases of intraamniotic infections and closely participates in chorioamnionitis, premature rupture of fetal membrane and preterm birth (25 and for review, Ref. 26). Furthermore, it is also suggested that MMP-2 in human amniotic fluid and/or fetal membrane plays a critical role in premature rupture of fetal membrane (27, 28). These studies and our present works emphasize that *E. coli* infection is very likely to play a crucial role in the destruction of connective tissues via the activation of proMMP-2 and that the processed active MMP-2 as well as *E. coli* serine proteinase, at least in part, causes the dysfunction of connective tissues.

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