Increased Expression of Urokinase Type Plasminogen Activator(u-PA), Plasminogen Activator Inhibitor-1(PAI-1), and Collagenases in Caco-2 Cells Infected by *Salmonella typhimurium*

Salmonella penetrates the basement membrane of intestinal epithelial cells into deeper tissues, in which process extracellular matrix proteases should be required. Hypothesizing that the proteases might be provided by host cells, we investigated the changes of expression of urokinase type plasminogen activator(u-PA), plasminogen activator inhibitor-1(PAI-1), and collagenases in epithelial cells(Caco-2) infected with *Salmonella typhimurium*. The change of mRNA levels, amount of the enzyme secretion and functional activity were analyzed by Northern blot, ELISA, and Zymography. The mRNA level of u-PA was elevated by *Salmonella* infection itself without any exogenous transcription regulators. u-PA was actively secreted into the medium and was enzymatically active. The synthesis and secretion of PAI-1 was increased over time from 2hrs post infection(pi) to 8hrs pi. Zymographic assay revealed that the secretion of collagenases(type IV, type V and interstitial collagenase) were also increased. Taken together, *S. typhimurium* infection might induce accumulation of pericellular proteolytic activity and consequently degrade the extracellular matrix surrounding the infected cells. These in turn might enable *Salmonella* to invade into deeper tissues. ***(JKMS 1997; 12: 23~31)***

Key Words : *Salmonella typhimurium, u-PA, PAI-1, collagenase, Caco-2 cell*

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Received : August 8, 1996
Accepted : November 7, 1996

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* This work was supported by the Fund for Basic Medical Science of the Korean Medical Association(1994) and the Basic Medical Research Fund from the Ministry of Education, Korea(1995) to Yoon-Hoh Kook

INTRODUCTION

Salmonella causes acute gastroenteritis or enteric fever. Unlike *Shigella* and enteroinvasive *Escherichia coli*, which invade the colonic epithelial cells producing rather superficial and localized infection (1, 2), *Salmonella* invades intestinal epithelial cells to reach deeper tissues and produces systemic infection (3, 4). Since the electron microscope studies could show the sequential events leading to penetration of intestinal epithelial cells by *Salmonella typhimurium* (4), many investigators have revealed how *Salmonella* attaches (5~10), and enters (3, 11, 12). The genes related to invasion have also been investigated (8, 11, 13~20). The adherence of *Salmonella* to cultured mammalian cells is accompanied by the activation of the epidermal growth factor receptor (EGFR) (8). It activates the signal transduction pathway leading to the membrane ruffling (21) and the ruffles induced are subverted by *Salmonella* so that it enters epithelial cells (12). However, it has not been well understood how *Salmonella* disseminates and causes septicemia. For *Salmonella* to gain access to blood stream or lymphatic system it has to reach lamina propria. Between epithelial cells and lamina propria is a continuous thin mat of specialized extracellular matrix of basal lamina (22) which has been a selective barrier (2). The basal lamina is mainly composed of type IV collagen, proteoglycans and laminin tightly cross-linked with each other (23, 24) and it is considered to be a solid sheet pierced by multiple small pores with average diameters of 9-10nm as suggested by the model derived from permeation physiology (2). For the *Salmonella,* an average size of 1 μm, to cross this barrier the basal lamina would have to be disrupted. On the transmission electron microscope finding, the basal lamina at the bottom of the M cell attacked by *S. typhi* was disappeared (25), being suggestive of disruption of basal lamina.

Several extracellular proteolytic enzymes including metalloproteinases and serine proteases, which have been shown to degrade extracellular matrix such as collagen, laminin and fibronectin have been known to be responsible for the degradation of the basement membrane (26,

27). Urokinase type plasminogen activator (u-PA), one of serine protease, acts as the specific trigger in a proteolytic cascade. It converts plasminogen to plasmin, a neutral protease of broad specificity, which in turn degrades extracellular proteins either directly or through activation of latent matrix metalloproteinases (28~31). Ultrastructural pathologic changes such as loss of polarity, disruption of tight junctions in intestinal epithelial cells attacked by enteroinvasive *Salmonella* are also suggestive of involvement of extracellular proteolytic enzymes in the *Salmonella* invasion process (32).

Several bacteria are known to have plasminogen activators. *Yersinia pestis*, the causative agent of plague, has a plasminogen activator called 'Pla protease' which enables *Y. pestis* to reach liver and spleen from subcutaneous tissue (33~36). Highly invasive strains of *Escherichia coli* which invade through epithelial cell barriers to proliferate in the underlying mucosa, eventually establishing a systemic infection, have been known to possess a protease which activates plasminogen to plasmin (37). However, *Salmonella* has not been reported to have any extracellular proteases of its own. As extracellular proteases must be involved in the pathogenesis of *Salmonella* infection, it is likely that the enzymes are induced from host cells interacting with *Salmonella*. We, therefore, investigated the changes of expression of u-PA, plasminogen activator inhibitor-1 (PAI-1), and collagenases in epithelial cells (Caco-2) infected with *Salmonella typhimurium* by Northern blot, ELISA, zymographic assay and fibrin-autography.

MATERIALS AND METHODS

Bacterial strains and culture :

Salmonella typhimurium strain LT2(ATCC 19585) was obtained from American Type Culture Collection(ATCC, Rockvile, MD) and *Escherichia coli* DH5α(F^-, endA1, hsdR17(r_k^-, m_k^+), supE44, thi-1, λ^-, recA1, gyrA96, relA1, ϕ80dlacZΔM15) was purchased from Bethesda Research Laboratories(BRL, Gaithersburg, MD, USA). Bacteria were grown anaerobically (38) at 37℃ in Luria Bertani (LB) broth in an anaerobic jar(BBL, Cockeysville, MD, USA) with GasPak Anaerobic Systems(BBL, Cockeysville, MD, USA). Exponentially growing cultures were washed twice in phosphate buffered saline (10mM, pH7.4, PBS) and suspended in Eagle's minimal essential medium with Earle's salts and nonessential amino acids (GIBCO/ BRL, Gaithersburg, MD, USA, EMEM) prior to infection. Inactivation of bacteria was done by incubating harvested bacteria in 3% buffered formalin for 18 hrs. Inactivated bacteria were washed four times in PBS and suspended in EMEM prior to infection.

Culture and inoculation of Caco-2 cells with bacteria :

The human colon carcinoma cell line Caco-2 was obtained from ATCC (HTB 37) and used between passage 18 and 30. Cells were grown in Eagle's minimal essential medium with 20% fetal bovine serum (GIBCO/ BRL, Gaithersburg, MD, USA) with penicillin and streptomycin (Cat. No. P 0906, Sigma, St. Louis MO, USA) in 5% CO_2 atmosphere.

Monolayers were prepared by seeding 9×10^5 cells into 60mm-diameter plastic tissue culture dishes (Cat. No. 3002, Becton Dickinson and Company, Lincoln Park, New Jersey, USA) and incubating for 6 days with a change of media every 3 days. Before bacterial addition, monolayers were rinsed 3 times with EMEM without antibiotics. Monolayers were inoculated with bacteria by adding 250 μl (5×10^8 cfu) of freshly grown or formalin inactivated bacteria in EMEM. One ml of fresh EMEM was added to dishes and then incubated at 37℃ for 2 hrs in 5% CO_2 atmosphere to allow bacterial invasion to occur (the time was determined by invasion assay, data not shown). After this incubation period, the culture supernatant was harvested, centrifuged at 4℃, 10,000×g for 10min and then filtered. The filtered supernatant was used as conditioned media of 2 hr post inoculation (pi) and stored at -20℃. The plates were washed three times with EMEM and then further incubated for additional 6 hrs with the addition of 2 ml of EMEM containing 100 μg/ml of gentamicin to kill extracellular bacteria (39) and 1 ml of conditioned media prepared at 2 hr pi. At 4, 6, 8 hrs after inoculation, the conditioned media were prepared respectively as described above. After the supernatant was removed, each of the cells was harvested at 2, 4, 6, 8 hrs pi and washed three times with PBS. Cell extracts were prepared by extracting the cells with 0.1% Triton X-100 in 0.1 M Tris, pH 8.1 and they were stored at -20℃. Protein quantitation of cell lysates was performed with Bio-Rad protein assay kit according to the manufacturer's instruction (Cat No. 500-0006, Bio-Rad Laboratories, Hercules, CA, USA).

Quantitation of u-PA and PAI-1 :

These were performed by using ELISA kit (Cat. No. 894, and 822/1S, American Diagnostica Inc., Greenwich, CT, USA) according to the manufacturer's instruction. Briefly, 100 μl of samples (conditioned media or cell extracts) were incubated in micro-test-strip-wells precoated with a murine anti-human u-PA or PAI-1 monoclonal antibody as the capture antibody. A second, biotinylated antibody recognized the bound u-PA or PAI-1 molecules. Streptavidin conjugated horseradish peroxidase (HRP) and a perborate/3,3',5,5'-tetramethylbenzidine substrate were used as detection complex. Human standard curve

was included in every assay. Statistical analysis was done by using PC-SAS version 6.04.

Northern blot analysis :

Messenger RNAs were extracted from bacterial infected cells at 1, 2 and 4 hrs pi by Quick Prep Micro mRNA Purification Kit (Cat. No. 27-9255-01, Pharmacia Biotech, Milwaukee, Wisconsin, USA) and electrophoresed on 1% denaturing formaldehyde-agarose gels (3 μg RNA/lane). The RNAs were transferred onto nitrocellulose membrane (Cat. No. 439-386 Schleicher & Schuell, Dassel, Germany). The membrane was hybridized overnight at 42℃ in 50% formamide, 25 mM KPO_4, pH 7.4, 5× SSC, 5×Denharts solution, 50 μg/ml salmon sperm DNA, 10% dextran sulfate, and 1×10^6 cpm/ml probe. u-PA cDNA of 0.6 kb obtained by *Eco*RI digestion of plasmid pUK0321 was labeled by random priming with [^{32}P]dCTP to a specific activity of $3\text{-}7\times10^8$ cpm./μg DNA. Following hybridization the membrane was washed to a final stringency of 0.2×SSC and 0.1% sodium dodecyl sulfate (SDS) at 65℃. The washed membrane was used to expose Kodak XAR-5 film with an intensifier at -70℃ for 48 hrs. After stripping, the membranes were rehybridized with murine GAPDH cDNA. Hybridization and washing conditions were as described above. Quantitation of bands on autoradiograms was performed by using a Bio-Profil Image Analyzer (Vilber Lourmat, Cambridge, UK) with accompanying software.

SDS-PAGE and Fibrin Autography :

Samples were electrophoresed in the presence of SDS under nonreducing condition in 10% polyacrylamide gels using the buffer system of Laemmli (40). Fibrin autography of u-PAs was performed as described (41). Briefly, fibrin-agar indicator gel was prepared by mixing agarose [3 parts of NuSieve GTG agarose (FMC Bioproducts, cat No. 50082, Rockland, ME, USA) and 1 part of agarose (Sigma, cat No. A-9539, St. Louis MO, USA); final concentration 1%] with prewarmed (45℃) PBS containing plasminogen (American diagnostica, Product #400B, Greenwich, CT, USA ; final concentration, 25 μg/ml) and thrombin (Sigma, cat No. T9000, St. Louis, MO, USA ; from bovine plasma, 500 u/ml). Fibrinogen (Calbiochem Corporation, cat No. 341573, La Jolla, CA, USA; bovine, purified; final concentration, 2.4 mg/ml) in PBS was added and the solution was mixed and poured onto a prewarmed (45℃) glass plate. After SDS-PAGE, the gel was soaked in 50 ml of 2.5% Triton X-100 (Sigma, cat No. X-100, St. Louis, MO, USA ; two changes, 45 min each) and layered over an fibrin-agarose indicator gel. Fibrin autograms were allowed to develop at 37℃ in a moist chamber, and photographs were taken under dark- ground illumination.

Zymographic assay of collagenases :

Collagenases were identified by zymography (42). Briefly, conditioned media harvested at 2, 4, 6, and 8 hrs pi were electrophoresed on 10% polyacrylamide gels copolymerized with 1 mg/ml of porcine gelatin (Cat No. G6144, Sigma, St. Louis, MO, USA). The gels were washed in 2.5% Triton X-100 for 30 min, incubated for 40 hrs at 37℃ in buffer containing 50 mM Tris-HCl, pH 7.7, 5 mM $CaCl_2$, and 0.02% NaN_3, stained for 10 min in Coomasie blue, and destained to identify the zone of lysis.

RESULTS

Increased secretion of u-PA and PAI-1 in Caco-2 cells infected with *Salmonella typhimurium* :

As it has been reported that *Salmonella* was more invasive when it is grown in low-oxygen condition and in logarithmic phase, *S. typhimurium* LT2 and *E. coli* DH5α were cultured anaerobically and harvested when they were in logarithmic phase. Unpolarized monolayers of Caco-2 cells prepared as described in Materials and Methods were infected with *S. typhimurium* and *E. coli* DH5α. The integrity of infected monolayer cells was intact until 8hrs post infection (pi), which was confirmed by inverted microscope. After 2 hrs of incubation, non-internalized bacteria were killed by the addition of gentamicin, which did not readily kill intracellular bacteria. The amount of u-PA and PAI-1 in conditioned media from cells infected with *S. typhimurium*, *E. coli* DH5α and uninfected cells (control) respectively, was determined by sandwich ELISA at 2, 4, 6, and 8 hrs pi. The amount of secreted u-PA and PAI-1 significantly increased ($p<0.05$) over time from 0.247 ± 0.110 at 2 hrs pi to 1.953 ± 0.204 at 8 hrs pi and from 0.143 ± 0.055 at 2 hrs pi to 4.707 ± 0.125 at 8 hrs pi, respectively in *S. typhimurium*-infected cells. Repeated Measures Analysis of Variances revealed that the level of u-PA and PAI-1 was significantly higher ($p<0.05$) in *S. typhimurium*-infected cells than in cells infected with noninvasive bacteria, i.e., *E. coli* DH5α or control after controlling time effect (Fig. 1). This showed that *S. typhimurium* infection significantly stimulated the secretion of u-PA and PAI-1 in Caco-2 cells.

The amount of u-PA in conditioned media was determined from cells inoculated with formalin-inactivated bacteria. The time course of the concentration of u-PA showed no remarkable increase from 2 hrs pi to 8 hrs pi and the level of u-PA was not significantly higher in *S. typhimurium* inoculated cells (Fig. 2). This result sug-

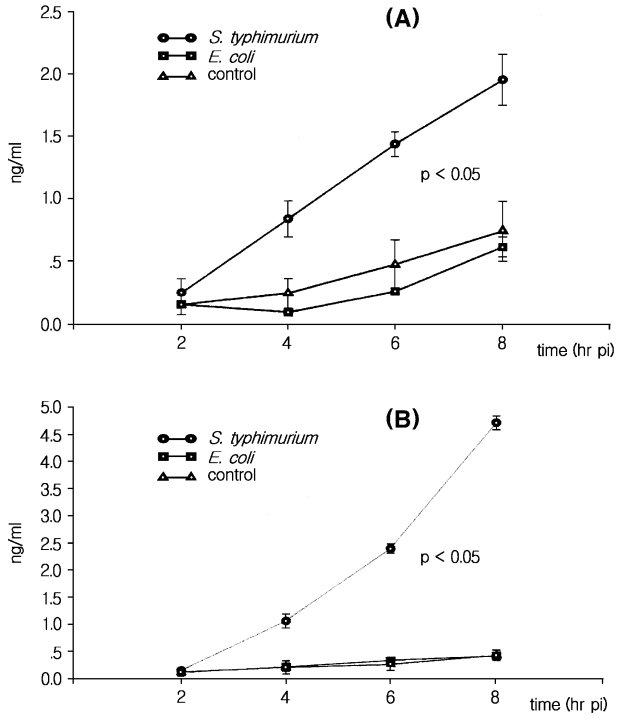

Fig. 1. The time course of concentration of u-PA and PAI-1 in the conditioned media. Cultures of Caco-2 cells were infected with bacteria as described in Materials and Methods. The conditioned media were harvested at 2, 4, 6, and 8 hrs pi and the concentration of u-PA and PAI-1 was determined by ELISA. The values are means ±standard errors of those obtained from the three independent experiments. $P<0.05$ was determined by Repeated Measures Analysis of Variances. (A); u-PA, (B); PAI-1.

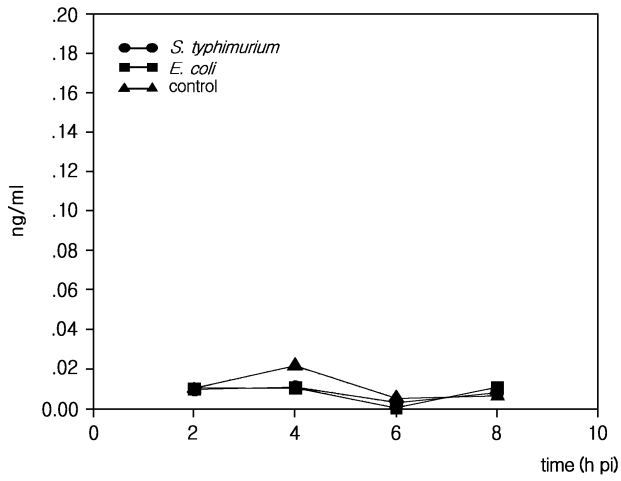

Fig. 2. The time course of concentration of u-PA in the conditioned media from cells inoculated with formalin-treated bacteria. Cultures of Caco-2 cells were inoculated with formalin-treated bacteria as described in Materials and Methods. The conditioned media were harvested at 2, 4, 6, and 8 hrs pi and the concentration of u-PA was determined by ELISA. The values are means of those obtained from the two independent experiments.

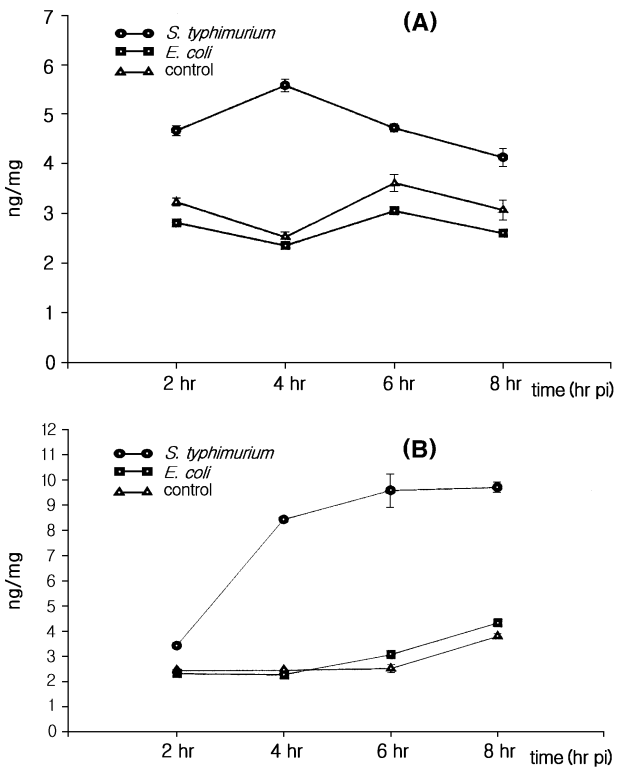

Fig. 3. Time course of the concentration of u-PA and PAI-1 in cell extracts. Cultures of Caco-2 cells were infected with bacteria as described in Materials and Methods. The cells were extracted with 0.01% Triton X-100 in 0.1 M Tris-HCl at 2, 4, 6, and 8 hrs pi and the concentration of u-PA and PAI-1 was determined by ELISA. The values are averages of those obtained from the three independent experiments. (A); u-PA, (B); PAI-1.

gested that the stimulation of secretion of u-PA by *S. typhimurium* required its viability.

Increased synthesis of u-PA and PAI-1 :

Cell extracts were prepared by extracting the cells harvested at 2, 4, 6, and 8 hrs pi with 0.1% Triton X-100 in 0.1 M Tris-HCl, pH 8.1 and the levels of u-PA and PAI-1 were assayed. The amount of u-PA and PAI-1 determined by ELISA was significantly higher in *S. typhimurium*-infected cell extracts than in control or *E. coli*-infected cell extracts (Fig. 3). This suggested that *S. typhimurium* infection induced the synthesis of u-PA and PAI-1 molecules. In extracts of *S. typhimurium*-infected cells, the amount of u-PA increased between 2 hrs and 4 hrs pi, peaked at 4 hrs pi and then decreased to 8 hrs pi. In extracts of *E. coli* infected and control cells, however, the amount of u-PA decreased between 2 hrs and 4 hrs pi, peaked at 6 hrs pi and decreased to 8 hrs pi (Fig. 3A). The peak level of PAI-1 was observed at 8 hrs pi and the most rapid increase in the amount occurred between 2 hrs and 4 hrs

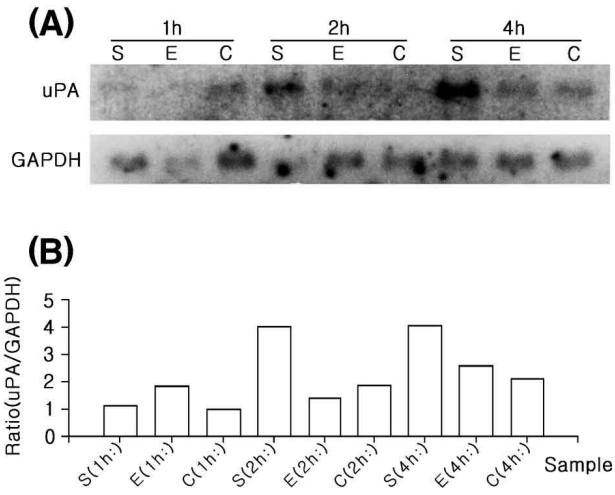

Fig. 4. Northern blot analysis. (A) Autoradiogram; Messenger RNA was extracted at 1, 2 and 4 hrs pi. Three micrograms of mRNA per lane was size fractionated and blotted onto nitrocellulose membrane. The membrane was probed with 0.6 kb u-PA cDNA, then washed free of probe and rehybridized with a murine glyceraldehyde-3-phosphate dehydrogenase(GAPDH) cDNA as a control. (B) The relative intensities of u-PA mRNA by densitometric scanning of autoradiograms. The ratio(u-PA/GAPDH) value is relative to that of C(1 hr) that is assigned the value 1.0. S, sample from *Salmonella typhimurium*-infected cells; E, sample from *Escherichia coli*-infected cells; C, control, sample from uninfected cells.

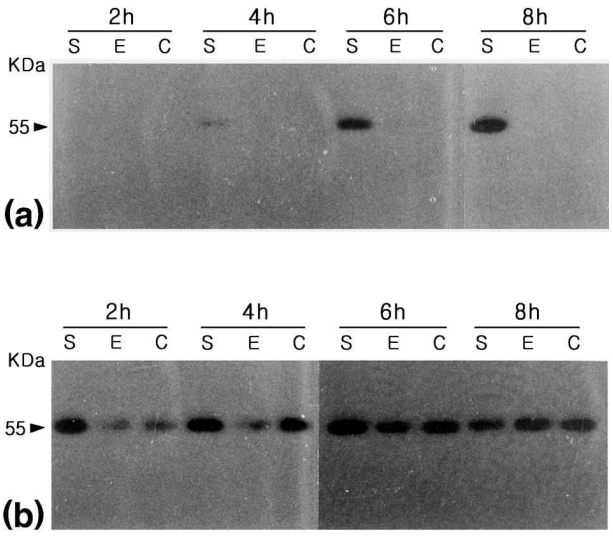

Fig. 5. u-PA activity in conditioned media and cell extracts analyzed by fibrin autography. Cultures of Caco-2 cells were infected with bacteria as described in Materials and Methods. The conditioned media and cell extracts were prepared at 2, 4, 6, and 8 hrs pi and analyzed by SDS-PAGE and zymography. Zymograms were allowed to develop at 37℃ and pictures were taken after 15 hrs. S, sample from *Salmonella typhimurium*-infected cells; E, sample from *Escherichia coli*-infected cells; C, control, sample from uninfected cells. (a); conditioned media, (b); cell extracts

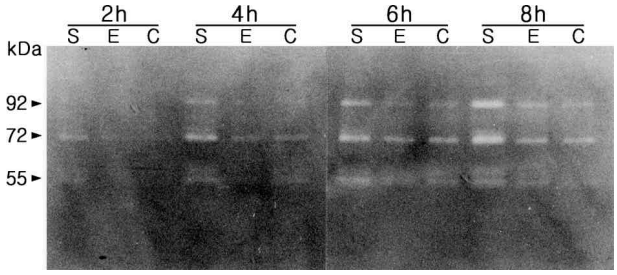

Fig. 6. Zymographic assay of collagenases secreted in conditioned media. Cultures of Caco-2 cells were infected with bacteria as described in Materials and Methods. The conditioned media harvested at 2, 4, 6, and 8 hrs pi were mixed with sample buffer and electrophoresed on a 10% polyacrylamide gel copolymerized with 1 mg/ml of gelatin and developed as described in Materials and Methods. S, sample from *Salmonella typhimurium*-infected cells; E, sample from *Escherichia coli*-infected cells; C, control, sample from uninfected cells.

(Fig. 3B). The samples for mRNA analysis of u-PA, therefore, were prepared at 1, 2 and 4 hrs after bacteria inoculation.

Increased messenger RNA level of u-PA :

The mRNA level for u-PA was determined in cells infected with *S. typhimurium*, *E. coli* DH5α and control cells . Messenger RNAs from the cultures were prepared at 1, 2 and 4 hrs pi and separated by denaturing formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose membrane. The membrane was hybridized with [^{32}P]dCTP-labeled cDNA probes. u-PA mRNA was detected in all of *S. typhimurium*-infected cells, *E. coli*- infected cells and uninfected cells of 1, 2 and 4 hrs pi. But the level of transcripts was much higher in *S. typhimurium*-infected cells than *E. coli*-infected cells or uninfected cells. Quantitation of bands on autoradiograms revealed that mRNA level was highest at 2 hrs pi (Fig. 4B). These results showed that *S. typhimurium* infection stimulated synthesis of mRNA of u-PA and this was paralleled by the increased secretion or synthesis of u-PA.

Enzymatically active u-PA synthesized and secreted :

Conditioned media and cell extracts prepared at 2, 4, 6, 8 hrs pi respectively were subjected to SDS-PAGE, and the gels were layered over a fibrin-agarose indicator gel. Fibrin autograms were developed. No PA activity was observed with the conditioned media from control, *E. coli*- and *S. typhimurium*-infected cells at 2 hrs pi. From 4 hrs pi, one major form of PA activity which migrated to the 55kDa appeared with the conditioned media of *S. typhimurium*-infected cells only. The size and clarity of the lytic zone increased over time to 8 hrs pi

(Fig. 5A). The fibrin autogram of cell extracts also showed one major form of PA activity of 55kDa from 2 hrs pi to 8 hrs pi (Fig. 5B). The size and clarity of lytic zone in the fibrin- autography correlated well with the amount of u-PA determined by ELISA, showing that the u-PA synthesized and secreted was enzymatically active.

Increased secretion of collagenases :

Conditioned media obtained at 2, 4, 6, and 8 hrs pi were analyzed by electrophoresis in a polyacrylamide gel copolymerized with 1 mg/ml of gelatin, followed by zymography. Three major bands of lysis were observed from 2 hrs pi to 8 hrs pi in control, *E. coli*- and *S. typhimurium*-infected cells. The size and clarity of lytic zones increased with increasing time of infection (Fig. 6). The two slower moving bands, calculated as 92kDa and 72kDa in size, represent Type V collagenase (MMP-9) and Type IV collagenase (MMP-2), respectively. The fastest moving band, calculated as 55kDa in size, seen as doublet, represents interstitial collagenase (MMP-1). The lysis zone of three bands with conditioned media of *S. typhimurium*-infected cells was stronger and clearer than those generated by the conditioned media of control and *E. coli*-infected cells. This meant *S. typhimurium* infection also stimulated secretion of collagenases.

DISCUSSION

In this report, we showed that *S. typhimurium* significantly stimulated the synthesis and secretion of u-PA, which has been known to play a central role in extracellular proteolysis by generating plasmin from plasminogen in Caco-2 cells. Plasminogen has been known to be present in plasma and extracellular fluids at a 1-2 mM concentration, in the range of the Km of the activation reaction (43). The secretion of collagenases were also increased. Collagenases, one of matrix metalloproteinase family, are responsible for degradation of the extracellular matrix components such as collagen and proteoglycans in normal embryogenesis and remodeling and in many disease processes. Thus u-PA and collagenases might exert degradation of extracellular matrix (ECM) and basement membrane (BM) cooperatively. PAI-1 has been known to inhibit PA-catalyzed proteolysis. However, recent study has revealed that co- expression of u-PA and PAI-1 is necessary for optimum invasiveness of cultured lung cancer cells (44). In our experiment, the secretion and synthesis of PAI-1 was increased by *S. typhimurium* infection (Fig. 1B, 3B). This might lead the overall balance towards enhanced proteolysis.

Increased synthesis and secretion of u-PA was considered to be active processes requiring viability of both bacteria and host cells. When the bacteria were treated with 3% buffered formalin (Fig. 2) or Caco-2 cells were irradiated with ultraviolet light (300 nm) (data not shown), we could not observe any increased synthesis and secretion of u-PA. The secretion of u-PA was increased linearly over time to 8 hrs pi (Fig. 1). Northern analysis showed the increased synthesis and secretion of u-PA was attributed to the increased expression of mRNA. Messenger RNA level was increased over time to 4 hrs pi (Fig. 4). The functional u-PA in cell extracts followed a similar course, with an approximately 2-hour lag of mRNA level (Fig. 4). This result was consistent with other's investigation in which transcriptional mechanisms have been shown to play a determining role in regulation of expression of u-PA (45).

The mechanism of induced production of extracellular proteases and their related factors by *Salmonella* could be speculated through activation of epidermal growth factor receptor (EGFR). The invasion of *Salmonella* to epithelial cells has been accompanied by autophosphorylation of EGFR (8, 9). This activates the signal transduction pathway leading to the phosphorylation and activation of the mitogen activated protein kinase (MAP kinase), phospholipase A2 (PLA2) and Ca^{2+} channel (21). MAP kinases (also called extracellular signal regulated kinases) are turned on by a wide range of extracellular proliferation and differentiation-inducing signals, some of which activate receptor tyrosine kinase including EGFR, while others activate G-protein-linked receptors. This suggests that activation of EGFR by *Salmonella* and subsequent signal pathway may be same as that activated by EGF. The biosynthesis of extracellular proteases has been shown to be under the control of hormones and growth factors. It has been shown EGF can induce u-PA (46) and collagenases (47). Therefore *Salmonella* may induce u-PA and collagenases through activation of EGFR. The same nuclear factor (PEA3) has been reported to participate in regulation of u-PA and collagenases expression (45). Simultaneous increase in expression of u-PA and collagenase by *S. typhimurium* infection could support the notion that the stimulation by *Salmonella* might be through activation of EGFR.

Zymographic assay revealed that secreted u-PA was enzymatically active. A polarity of secreted enzyme has been another factor which may influence the physiological role of secreted u-PA. Epithelial cells carry out vectorial transport functions of secretion and/or absorption and constitute a boundary between distinct extracellular domains: the apical compartment, usually in continuity with the external milieu, and the basolateral compartment which faces the subepithelial connective tissue and includes the basement membrane and other extracellular matrix constituents. This, together with the

presence of tight junctions around the apex of epithelial cells that restrict the intercellular diffusion of ions and macromolecules, implies that extracellular proteases released by epithelial cells might have different targets depending on whether the enzymes are secreted apically or basolaterally, and their intracellular sorting would result in spatially restricted proteolysis. As we used unpolarized culture system, we could not know whether u-PA would be secreted apically or basolaterally. However, it has been shown that the polarity of secretion is dependent on the cell type. u-PA has been reported to be secreted basolaterally in polarized cultured Caco-2 cells (48). Anyhow, it might be speculated that basolaterally accumulated u-PA would trigger the reaction to degrade basal lamina and apically accumulated u-PA would participate in degradation of tight junctions.

In our experiment, Caco-2 cells were used as host cells. Caco-2 cells, although isolated from an adult human colon, express several characteristic markers of normal small intestinal villus cells, such as microvillar hydrolases (49) and are also thought to be closely analogous to enterocytes of the fetal colon. When a tissue culture system is used as a model by which the invasion process of *Salmonella* infection is studied, the target cell of *Salmonella* infection has been assumed to be enterocytes (11). However, studies using animal models have revealed that the primary site of infection is Peyer's patches of terminal ileum and in which the systemic infection after oral challenge is established (10, 25, 50). The target cells of the pathogenic *Salmonella* species are known to be M cells of the Peyer's patches (25, 32). M cells are specialized epithelial cells that are found exclusively in lymphoid FAE (follicle-associated epithelium) (51, 52). These cells form tight junctions with adjacent enterocytes, possess microvilli, and line up the basement membrane (53). Recent studies (25, 32) have provided indirect evidences suggesting that u-PA or collagenases may be involved in degradation of ECM when M cells are infected by enteroinvasive *Salmonella* species in animal model systems. The transmission electron micrograph has revealed that the basal lamina at the bottom of the M cell attacked by *S. typhi* was not visible and a number of bacteria appeared near the basal lamina of epithelial lining (25). Another EM study using ligated ileal loop model has revealed that enterocytes adjacent to *S. typhimurium*-infected M cells are depolarized and detached from basement membrane and the tight junctions are disrupted (32). These are thought to be closely related to the activation of extracellular proteases. Because the basal lamina not only function as barriers that block the passage of cells and macromolecules but also determine cell polarity, the disruption of basal lamina may lead to the loss of polarity. Plasmin degrades glycoproteins of basement membrane that are involved in cell-cell and/or cell-matrix adhesion and this may lead to the disruption of tight junctions and sloughing off the enterocytes from basal lamina.

There are a number of chemical and physical barriers preventing disease causing bacteria from invasion into host cells. In the gastrointestinal tract, the mucous membrane and tight junctions are regarded as the first line of defense. The basement membrane, functioning as a selective barrier and providing a scaffolding on which the integrity of cells are maintained and along which regenerating cells can migrate, seems also to be important as a defense line. The disruption of these barriers might be critical in establishing a pathogenicity of enteroinvasive bacteria. In this study, we suggested that extracellular proteases of host cells might be utilized by *Salmonella* in destruction of these physical barriers. Conclusively, *S. typhimurium* stimulated increased synthesis and secretion of enzymatically active u-PA and collagenases as well as increased secretion of PAI-1. These might result in accumulation of proteolytic activity and consequently the possible destruction of ECM surrounding infected cells which might enable *Salmonella* to invade into deeper tissues.

Acknowledgment

We thank Dr. L. Ossowski for critical reading of the manuscript and discussion.

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