Shiga Toxins Induce Apoptosis in Pulmonary Epithelium–Derived Cells

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The effect of Shiga toxins (Stxs) produced by Escherichia coli on human lung epithelial cells was investigated. Specific antibodies for Stxs positively stained lung tissue from a patient who died of hemolytic uremic syndrome associated with Stx-producing E. coli (STEC) infection, indicating the deposition of Stxs in the lung. Binding experiments with normal lung tissue revealed apparent Stx binding to both vascular endothelium and to portions of the pulmonary epithelium. CD77-positive lung carcinoma cell lines, which are derived from lung epithelium, showed binding to Stx and a high susceptibility to Stxs, as determined by MTT assay. Consistent with our previous reports on renal tubular epithelium, apoptosis is involved in the Stx-mediated cytotoxicity of these lines. These data indicate that lung epithelium is another target for Stxs, and Stx-mediated injury to lung epithelial cells is thought to play an important role in the pathogenesis of pulmonary involvement associated with STEC infection.

The incidence of infections due to Shiga toxin (Stx)–producing Escherichia coli (STEC) as a cause of hemorrhagic colitis has been increasing worldwide. The seriousness of this colitis is emphasized by its association with fatal complications, namely hemolytic uremic syndrome (HUS) [1]. HUS is defined by a triad of clinical symptoms consisting of hemolytic anemia, thrombocytopenia, and acute renal failure [2]. However, precise analysis of recent major outbreaks of STEC infections has revealed the existence of extrarenal events, such as neurologic, cardiovascular, pancreatic, and pulmonary involvement [3–6].

Pulmonary involvement is rather uncommon in HUS but important because of its potentially life-threatening feature [3, 4, 6]. For example, sudden unexpected pulmonary edema and pulmonary hemorrhage have been described as the cause of death of HUS patients [3, 4]. Brandt et al. [5] reported that 3 of 37 children with postdiarrheal HUS following the ingestion of hamburger contaminated by STEC O157:H7 developed adult respiratory distress syndrome. Siegler et al. [6] presented the case of a 9-year-old girl with HUS-associated acute respiratory failure due to lung edema. Most recently, cold symptoms were reported as an initial sign of illness among 5.2% (550 people) of the cases in a massive outbreak of STEC O157:H7 infection in Sakai city, Osaka, Japan [7]. Martin et al. [8] reported that 6 (37.5%) of 16 patients who had atypical HUS presented with a respiratory prodrome [8]. They also pointed out that 22 (19%) of 117 patients presented respiratory symptoms during the course of HUS [8]. In addition, although clinically apparent lung involvement is uncommon, thrombotic angiopathy of the pulmonary microvasculature is frequently seen on autopsy [4, 9–11].

The mechanisms leading to pulmonary involvement in HUS have not been clarified. However, on the basis of histopathologic findings [4, 9–11], pulmonary edema or hemorrhage (or both) are thought to be due to thrombotic angiopathy of the pulmonary capillaries, with subsequent anoxic damage and increased capillary permeability [6]. Since experimental observations have indicated that cultured endothelial cells express globotriaosylceramide (Gb3)/CD77, a functional receptor for Stx [12–14], and are susceptible to the cytotoxicity associated with Stx [15–20], it has been postulated that Stx directly injures endothelial cells in vivo and that the resulting microangiopathy plays a central role in the pathogenesis of HUS [15, 21]. It has been argued consistently that widespread Stx-mediated endothelial injury of pulmonary capillaries contributes to the development of lung involvement in HUS [6]. Recent studies, however, have shown that Stx directly affects several different cell types, including B lymphocytes [22], macrophages/monocytes [23, 24], renal tubular epithelial cells [25–28], and mesangial cells [29, 30]. These reports indicate that endothelial cell damage may not be the sole cause of HUS and that further investigation is required to understand this disease.

In an attempt to clarify the pathogenesis of pulmonary involvement associated with Stx infection–mediated HUS, we examined the effect of Stx on pulmonary tissues and lung epi-
thelium-derived cell lines. Herein, we discuss the possibility that the lung epithelial cells are a target for Stxs.

Materials and Methods

**Lung tissues, cell lines, Stxs, and antibodies.** Lung tissues were obtained from 2 autopsied patients. One patient, a 21-month-old girl, died following HUS associated with O157:H7 STEC infection. Deposition of Stxs in her kidney tissue was identified as described elsewhere [31]. The other patient, an 80-day-old girl, died of a congenital anomaly; she had not had HUS or STEC infection. The lung tissue of the latter patient showed no abnormality on histopathologic examination.

Lung carcinoma cell lines, including A-549 (RCB0098 [32]), PC-14 (RCB0446 [33]), and RERF-LC-AI (RCB0444 [34]), were obtained from Riken Cell Bank (Koyadai, Tsukuba Science City, Japan). Stxs and antiserum for Stxs were prepared as described elsewhere [35, 36]. The monoclonal antibodies (MAbs) used in this study were CD34 (QBEnd10; Coulter/Immunotec, Westbrook, MA); Apo2.7, which is an antibody against 7A6 antigen, an early stage marker of the apoptotic process [37] (Coulter); CD77 (38.13 [Coulter] and 1A4; provided by Otsuka Assay Laboratories, Karawauchi-cho, Tokushima, Japan); anti-Stx1 (13C4, ATCC CRL 1794 [38]); and anti-Stx2 (11E10, ATCC CRL 1907 [39]). Anticytokeratin MAbs were purchased from DAKO (Glostrup, Denmark) and ICN Biochemicals (Costa Mesa, CA). Fluorescein-conjugated and enzyme-conjugated secondary antibodies were purchased from Jackson Laboratory (West Grove, PA).

**Immunohistochemical analysis.** The frozen lung sections were prepared by cryostat (Tissue-Tek II; Miles Scientific, Naperville, IL) and fixed in 100% acetone at 4°C for 15 min. For cell lines, suspended cells, which were produced by treating with a nondenzytimatic cell dissociation solution (Sigma, St. Louis), were immobilized onto glass slides with Cytospin 2 (Shandon, Pittsburgh) and fixed in a manner similar to that for the frozen sections. Immunoperoxidase staining was done as described elsewhere [40]. To detect binding of Stxs, we incubated the tissues and cells with 100 ng/mL of Stx1 or Stx2 for 1 h at room temperature prior to staining with specific antibody. Two-color immunostaining was done with a combination of horseradish peroxidase-conjugated mouse anticytokeratin MAb (DAKO) and rabbit anti-Stx2 antiserum followed by alkaline phosphatase-conjugated donkey anti-rabbit immunoglobulin (Jackson). TrueBlue (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and FastBlue (DAKO) were used as the substrates for horseradish peroxidase and alkaline phosphatase, respectively.

**Immunofluorescence study.** Suspended cells prepared in a manner similar to that outlined above were stained with MAbs and analyzed by flow cytometry (EPICS-XL; Coulter) as described elsewhere [41]. To detect Stx binding, we incubated 10⁶ cells in suspension with and without 100 ng/mL of Stx1 or Stx2 for 1 h at 4°C and then washed the cells intensively before staining. To detect 7A6 antigen, which is located in the cytoplasm, we fixed and permeabilized the cells prior to staining with phycoerythrin-Cy5-conjugated APO2.7 antibody, as described elsewhere [37].

**MTT assay.** To assess growth and viability, we plated A549, PC14, and RERF-LC-AI cells onto 96-well plates (Corning, Corn- ing, NY), with and without Stxs, at a concentration of 10⁵ cells in 100 μL of complete medium per well. MTT assays were done as described elsewhere [42].

**DNA fragmentation assay.** Cells were assayed for DNA ladder formation, by gel electrophoresis. Cells were either treated or not treated with Stxs, and then DNA was extracted and separated in 1.5% agarose gel by electrophoresis and examined under UV light, as described elsewhere [43]. DNA fragmentation was also investigated by the TUNEL method, by use of an in situ apoptosis detection kit (TACS 2 TdT; Trevigen, Gaithersburg, MD). Experiments were done according to the manufacturer’s protocol. To evaluate relative DNA contents, we stained cells with propidium iodide (Sigma) and then analyzed them by flow cytometry, as described elsewhere [43].

Results

**Detection of Stxs in the lung of a patient with STEC infection.** We examined the kidney of a child who died of STEC-associated HUS and found deposits of Stx1 and Stx2 [31]. In an attempt to identify the distribution of Stxs in human tissues during STEC infection, we tested other tissues obtained from the same patient and observed clear positive staining in the lung by immunohistochemistry with anti-Stxs antibodies (figure 1A). However, no staining was observed when control mouse immunoglobulin was used (figure 1B). Because the specificity of the anti-Stxs antibodies used in this study has been confirmed previously [27, 31, 38, 39], the results show the specific detection of Stxs in the lung, indicating that Stx produced in the intestine reaches the lung via the blood stream and binds to lung tissue during STEC infection. Although we tried to identify the cell type to which Stxs were bound in the lung, it was difficult because of massive hemorrhage and edema (figure 1A).

**Stxs bind to lung tissue from non-HUS patient.** In an attempt to test whether Stx can bind to normal lung tissue, we examined the lung of a patient without HUS. As shown in figure 2A, the lung tissue showed clear positive staining with anti-Stxs antibodies following incubation with Stxs, whereas no staining was observed when control mouse immunoglobulin was used (figure 2A). In addition, anti-Stxs antibodies did not stain lung tissue that was not preincubated with Stxs (figure 2A), indicating that anti-Stxs antibodies specifically detected Stxs bound to lung tissue. When the lung tissue was stained with anti-CD77 antibody, a staining pattern similar to that of Stx binding was obtained (figure 2B).

Since endothelial cells of small vessels have been suggested as a candidate in vivo target for Stxs by a number of experimental studies [15–20], it was easily predicted that Stxs would bind to those endothelial cells in the lung. To investigate the Stxs-binding site in the lung, we stained the tissue with other antibodies that can identify the components of lung, including anti-CD34 antibody (a marker for vascular endothelium [44]) and anticytokeratin antibody (a marker for pulmonary epi-
Figure 1. Identification of Shiga toxin (Stx) 1 in lung of patient with hemolytic uremic syndrome (HUS). A. Hematoxylin-eosin (HE) staining revealed massive edema and hemorrhage. B. Fresh-frozen section of lung tissue from HUS patient (described in detail by Uchida et al. [31]) was stained with anti-Stx1 monoclonal antibody (MAb) 13C4 (Anti-Stx1) or anti-Stx2 MAb 11E10 (Anti-Stx2). No staining was observed when isotype-matched control mouse immunoglobulin (Ms-Ig) was used or in the absence of primary antibody (W/O 1st Ab) on same tissue preparation. Arrows (bottom right panel) indicate brown staining in typical positive cells. Original magnifications are indicated on figure.
Figure 2. Analysis of Shiga toxin (Stx)-binding and antigen expression of normal lung tissue. A, Fresh-frozen sections from normal lung tissue from autopsied patient without hemolytic uremic syndrome were incubated with Stx1 (Anti-Stx1 [With Stx1]; lower left panel) or with Stx2 (Anti-Stx2 [With Stx2]; lower right panel). After being intensively washed, lung sections were immunohistochemically stained with anti-Stx1 monoclonal antibody (MAb) 13C4 (Anti-Stx1 [With Stx1]; lower left panel) or anti-Stx2 MAb 11E10 (Anti-Stx2 [With Stx2]; lower right panel), which revealed bound Stxs. No staining was observed when isotype-matched control mouse immunoglobulin (Ms-Ig) was used or in the absence of primary antibody in protocol (W/O 1st Ab) on same tissue without preincubation with Stxs (W/O Stxs; upper left panels). Although preincubated with Stxs, mouse immunoglobulin failed to stain same tissue (Ms-Ig [With Stx1] and Ms-Ig [With Stx2]; upper middle panels). In addition, anti-Stx MAbs did not stain same tissue when not preincubated with Stxs (Anti-Stx1 [W/O Stx1] and Anti-Stx2 [W/O Stx2]; upper right panels). B. Tissue preparation used in A was also stained immunohistochemically with specific MAbs as indicated. C. Same tissue preparation was treated with Stx2 as described in A and stained immunohistochemically with anti-cytokeratin antibody and anti-Stx2 antibody, which visualized blue and red, respectively. Arrows indicate double-positive cells, which stained purple. Original magnifications are indicated on figure.
CD77 expression and Stxs binding on lung carcinoma cell lines. To extend further the results obtained in tissue, we tested three cell lines derived from different histology of lung carcinomas as a model of cultured lung epithelium. First, we examined the expression of Gb3/CD77 and found a different profile in each line. Alveolar cell type adenocarcinoma cell line A-549 [32], which expresses a low level of cytokeratin in immunohistochemical assay (figure 3A), showed no expression of CD77 by flow cytometry (figure 3B). In contrast, squamous cell carcinoma cell line RERF-LC-AI [34], which moderately expresses cytokeratin (figure 3A), showed prominent expression of CD77 (figure 3B). On the other hand, undifferentiated adenocarcinoma line PC-14 [33], which strongly expresses cyto-

Figure 3. Analysis of antigen expression and Shiga toxin (Stx) binding of lung cancer cell lines. A. Three cell lines established from different histology of lung cancers were cytocentrifuged and immunohistochemically stained with control mouse immunoglobulin (Mouse Ig) or anti-cytokeratin monoclonal antibody (MAb; Cytokeratin). B. Lung cancer cell lines in suspension were stained with anti-CD77 MAb 1A4 and analyzed by flow cytometry; resulting histogram (dark lines) was superimposed on histogram of negative control (light lines). After cell lines were incubated with Stx2, binding of Stx2 to cell surface was detected by staining with anti-Stx2 MAb 11E10. Data obtained were presented in manner similar to that for anti-CD77. Similar experiments with a combination of Stx1 and anti-Stx1 antibody revealed identical results (data not shown). FITC, fluorescein isothiocyanate.
keratin (figure 3A), showed moderate expression of CD77 (figure 3B). The binding ability of Stx to these cell lines was examined by use of flow cytometry, which revealed that Stx bound to 2 lines expressing CD77: PC-14 and RERF-LC-AI, but not A-549, bound Stx, and the binding of Stx was higher in RERF-LC-AI than in PC-14 (figure 3B).

Cytotoxic effect of Stxs on lung carcinoma cells. Next, we examined whether Stxs affected the growth and survival of lung carcinoma cells. When incubated with Stx1 or Stx2, the number of viable cells of the CD77-expressing cell lines (RERF-LC-AI and PC-14), as assessed by MTT assay, decreased markedly in a dose-dependent manner (figure 4). The cytotoxic effect of Stxs correlated with the expression levels of CD77. Thus, RERF-LC-AI, which expresses a high level of CD77, is more sensitive for Stx-mediated cytotoxicity than is PC-14, which intermediate expresses CD77. In contrast, Stxs did not affect the growth of A-549 cells, which do not express CD77, indicating that Stxs specifically injure cells expressing CD77.

Detection of apoptosis in the Stxs-induced cell death of RERF-LC-AI cells. Since it has been reported that apoptosis is involved in the mechanism of Stx-mediated cytotoxicity of a variety of cells [22, 25–27, 46–50], we investigated the possibility that the cytotoxic effect of Stxs on RERF-LC-AI cells also involves apoptotic mechanisms. First, we tested whether cleavage of nuclear DNA, a major feature of apoptosis, occurs during Stx-induced death of RERF-LC-AI cells. As shown in figure 5A, DNA prepared from RERF-LC-AI cells pretreated with Stx2 exhibited an oligonucleosomal ladder fragmentation pattern on agarose-gel electrophoresis. The DNA fragmentation in RERF-LC-AI cells treated with Stx2 was also confirmed by in situ detection with the TUNEL method (figure 5B). Moreover, Stx2-induced cleavage of the nuclear DNA in RERF-LC-AI cells was further confirmed by detection of subploid cells, with propidium iodide staining (figure 5C).

7A6, a 38-kDa protein detected by APO2.7 antibody, was identified as an early-stage marker of the apoptotic process in various cells [37]. As shown in figure 6, APO2.7 stained RERF-LC-AI cells treated with Stx2. No remarkable staining was observed when APO2.7 was reacted with nontreated RERF-LC-AI cells. These findings showed that apoptosis had occurred in RERF-LC-AI cells following treatment with Stxs.

Discussion

On the basis of histopathologic findings and in vitro experiments, it has been generally believed that vascular endothelial cells are a prime target for Stx-mediated cytotoxicity, and the damage caused to them by Stxs is the major event that triggers Stx-mediated HUS [15–21, 51]. However, recent reports have shown that Stxs induce cell death in various other cells, such as intestinal epithelium, Burkitt’s lymphoma cells, Vero and MDCK renal–derived cells, ACHN renal adenocarcinoma cells, and renal epithelial cells [22, 25–28, 46–50]. Thus, it is now clear that Stxs can injure a variety of cell species and that vascular endothelial cells are not the unique targets of Stxs.

The data presented herein clearly demonstrate the presence in lung tissue of Gb3/CD77-expressing cells, which can bind to Stxs both in vitro and in vivo. Precise immunohistochemical observation suggested that not only the vascular endothelial cells but also portions of pulmonary epithelium contain Gb3/CD77-expressing cells. The evidence that some of the human lung carcinoma cell lines express Gb3/CD77 and can bind Stxs further confirms the above notion. In addition, the Gb3/CD77-expressing lung carcinoma cells are highly sensitive to Stx-mediated cytotoxicity. Collectively, our data indicate that some portions of the lung epithelium expressing a receptor for Stxs are susceptible to Stxs and can be directly damaged during STEC infection. Thus, in lung, both vascular endothelium and...
lung epithelium are the major targets for Stxs, and the damage mediated by Stxs should contribute to the pathogenesis of pulmonary involvement associated with Stx-induced HUS.

Although it is true that certain patients with Stx-induced HUS suffer pulmonary compromise [3–6, 7, 8], it is rather striking that most patients do not experience severe pulmonary consequences in the absence of pulmonary overload. A possible interpretation may be the presence of counteracting mechanisms that lessen the effect of Stxs on human pulmonary cells. Mitra et al. [52] reported that plasma from patients with sporadic HUS or a related disorder, thrombotic thrombocytopenic purpura, can injure vascular endothelium of renal, cerebral, or dermal but not pulmonary origin in the culture system. They also described that a deficiency of prostacyclin production was observed in these susceptible cells but not in the resistant pulmonary endothelium, suggesting a correlation of prostacyclin production with the counteracting mechanisms against certain toxic substances that induce sporadic HUS or thrombotic thrombocytopenic purpura. Since previous studies have reported various abnormalities in prostacyclin synthesis and metabolism, such as the defective renal synthesis of prostacyclin in postdiarrheal HUS [53–55], the correlation between prostacyclin and the counteracting mechanisms that affect tissue susceptibility to Stxs should be of interest for future study.

Our data also clearly show that the mechanism of apoptosis is involved in the Stx-mediated cell damage to lung carcinoma cell lines. Fragmentation of genomic DNA of Stx-treated lung carcinoma cell lines clearly indicates the presence of nucleosomal DNA breakdown, a phenomenon typical of apoptosis. The expression of an apoptosis marker, 7A6 [37], provides further evidence that apoptosis has occurred. It is a widely accepted idea that the cytotoxic effect of Stx is mediated by RNA N-glycohydrolase activity of the A-toxic subunit, which cleaves a specific adenine residue on the 28S ribosomal subunit, resulting in inhibition of protein synthesis [56–59]. However, in recent studies, including our own, apoptosis was found to be involved...
Figure 6. Induction of apoptotic antigen 7A6. After incubation with 100 pg/mL Shiga toxin (Stx) 2 for 2 or 4 days, RERF-LC-AI cells were fixed and stained with APO2.7 monoclonal antibody or isotype-matched control immunoglobulin and then were analyzed by flow cytometry. To identify positive cells more clearly, fluorescence intensity was displayed as 2-dimensional histogram against side light scatter. x axis, fluorescence intensity. % of positive cells are presented. Similar experiments with Stx1 revealed identical results (data not shown).

in the mechanism of Stx-mediated cytotoxicity [22, 25–27, 46–50].

Protein-synthesis inhibition and apoptosis are not always contradictory, and in some instances inhibition of protein synthesis has caused apoptosis or has operated as an apoptosis cofactor [60–62]. In fact, a complete form of holotoxin is required to induce apoptosis in Vero cells [51], suggesting that inhibition of protein synthesis mediated by Stx is linked to apoptosis in this case. Our previous observation that Stx-mediated apoptosis in ACHN renal carcinoma cells can be enhanced with a protein-synthesis inhibitor [26] supports this notion. However, it has been shown in Burkitt’s lymphoma cells that the binding subunit of Stx (B subunit), which by itself does not inhibit protein synthesis, is sufficient to induce apoptosis [22]. This observation suggests that the binding of a toxin to the cell membrane, via Gb3/CD77, may transduce an apoptotic signal even before the toxin becomes internalized and may inhibit protein synthesis in this manner. Thus, it is possible that 2 independent pathways of transducing cell death signalling occur in the process of Stx-mediated cell death. The precise mechanism(s) by which Stx induces apoptosis in lung cancer cells is of interest for future research.

The contribution of Stx-mediated apoptosis in lung epithelium to the pathogenesis of HUS-associated lung insufficiency is currently unclear, and precise retrospective and prospective analyses of HUS patients are needed to elucidate the molecular mechanism. However, our findings have suggested a new approach for better understanding of the pathogenesis of lung insufficiency in STEC-mediated HUS. Furthermore, it could explain why cold symptoms are seen during the initial stage of illness in many patients with STEC infection [7, 8].

Considering the present and previous studies, it is speculated that vascular endothelial cells are not the only target for Stx: Multiple cell lineages, including B lymphocytes, renal epithelial cells, and lung epithelial cells, are other candidates. These findings should facilitate our understanding of the pathogenesis of HUS, which seems to involve multicell damage and vascular endothelial cell damage.

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References


