

Shiga toxin 1 elicits diverse biologic responses in mesangial cells

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Shiga toxin 1 elicits diverse biologic responses in mesangial cells.

Background. Shiga toxin 1 (Stx1) is a causative agent in hemolytic uremic syndrome (HUS). Its receptor, the glycosphingolipid globotriaosylceramide (Gb₃), is expressed on cultured human endothelial and mesangial cells. Mesangial cell injury in HUS ranges from mild cellular edema to severe mesangiolysis and eventual glomerulosclerosis. We hypothesized that, in addition to endothelial cells, mesangial cells are targets of Stx1.

Methods. Human mesangial cells were exposed to Stx1. Protein synthesis was measured using [³⁵S]-methionine/cysteine. Cell viability was measured as the lysosomal uptake of Neutral Red. Monocyte chemotactic peptide (MCP-1) mRNA and protein were analyzed by Northern blotting and ELISA.

Results. Stx1 (0.25 to 2500 ng/ml) resulted in a dose-dependent inhibition of protein synthesis. This effect of Stx1 was potentiated by preincubation of the cells with interleukin-1 α (IL-1 α ; 2 ng/ml) or tumor necrosis- α (TNF- α ; 500 U/ml). Stx1 had little effect on mesangial cell viability during the first 24 hours of exposure to Stx1. However, prolonged incubation with Stx1 for 48 and 72 hours resulted in a 68% and 80% decrease in cell-viability, respectively. Stx1 elicited a dose and time dependent increase in the levels of MCP-1 mRNA, an effect that was potentiated by preincubation with IL-1 α .

Conclusion. These data indicate that mesangial cells are susceptible to the effects of Stx1 *in vitro*. Stx1 exerts a spectrum of biologic effects on mesangial cells ranging from activation of chemokine genes to a lethal toxic injury. Immunoinflammatory cytokines potentiate the effects of Stx1. Thus, glomerular pathology in HUS may also result from a direct effect of Stx1 on mesangial cells.

Hemolytic uremic syndrome (HUS) consists of thrombocytopenia, microangiopathic hemolytic anemia and acute renal failure, and was first described in 1955 by Gasser et al [1; reviewed in 2]. The disease is mostly seen in infants and young children, but it can also affect older children or adults, where the course often is more severe and associ-

ated with a higher incidence of complications. It is now known that there is an association between HUS and enteric infections with exotoxin producing bacteria, the most common being verotoxin producing *Escherichia coli* (VTEC) [3–5]. There are two types of verotoxins, also known as Shiga toxins, Stx1 (VT1) and Stx2 (VT2). Both toxins have been implicated in the pathogenesis of HUS. Approximately 90% of all children with HUS show evidence of VTEC infection, 70% of them with the *E. coli* serotype O157:H7, which is known to produce Stx1 and often Stx2 [6–8]. Both Stx1 and Stx2 consist of a 30 kD A-subunit and five noncovalently associated B-subunits, each about 7.5 kD [9]. The B-subunits are necessary for the receptor binding of the whole toxin, which leads to internalization of the toxin/receptor-complex via clathrin coated pits [10]. Within the cell, the subunits are dissociated and the A-subunit inhibits cellular protein-synthesis by removing one nucleotide from the 28S ribosomal RNA of the 60S ribosome subunit [11; reviewed in 12].

The functional receptor for Stx1, globotriaosylceramide (Gb₃), is a glycosphingolipid expressed on the cell membrane [13, 14]. Gb₃ has recently been identified in the human kidney cortex and medulla and it is also highly expressed on cultured human endothelial and mesangial cells [15–21]. The number of Gb₃ receptors found on the cell membrane determines the susceptibility of the cells to Stx1. It is thus not surprising that constitutive levels of Gb₃ are about 50 times higher in human renal endothelial cells (HRMEC), the most common target of Stx1, compared to human umbilical vein endothelial cells (HUVEC) [20]. In addition to endothelial cell injury, there is evidence of mesangial cell involvement in HUS. *In vitro* and *in vivo* studies indicate that mesangial cell injury contributes to progressive nephrosclerosis. The spectrum of pathologic changes involving mesangial cells in HUS ranges from mild cellular edema to mesangiolysis. In severe cases, these changes eventuate in glomerulosclerosis with irreversible distortion of the glomerular architecture and loss of renal function [22, 23].

Mesangial cell injury with consequent long-term effects on kidney function and histopathology may result from the

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hypoxic/ischemic insult that follows primary endothelial cell injury and the resulting thrombotic microangiopathy. However, it is also likely that Stx1 targets mesangial cells directly, and that the effect of Stx1 on mesangial cells contributes not only to the acute glomerular injury, but also participates in the long-term complication of this disease. In these studies, we investigated the biologic effects of Stx1 on human glomerular mesangial cells.

METHODS

Materials

Purified Stx1 was prepared as previously described [24]. Purified Stx1 migrated as two bands on SDS gel electrophoresis (30 kD and 7.5 kD), under reducing conditions. Purified protein was suspended in 10 mM phosphate buffered saline (PBS) at a concentration of 250 $\mu\text{g/ml}$ (equivalent of 3.53×10^{-6} M). Waymouth medium, modified Eagle's medium without methionine/cysteine, N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES), non-essential amino acids, sodium pyruvate and human tumor necrosis factor- α (TNF- α) were purchased from GIBCO (Grand Island, NY, USA). Insulin, salmon sperm DNA, EDTA, sodium dodecyl sulfate (SDS), trichloroacetic acid, 3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride (Neutral Red), 5,6-dichlorobenzimidazole-riboside (DRB) and sodium citrate (SSC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutamin, antibiotic/antifungal solution and fetal calf serum (FCS) were from Atlanta Biological, Inc. (Norcross, GA, USA). Human interleukin-1 α (IL-1 α) was from R&D Systems (Minneapolis, MN, USA). [^3H]-thymidine, [^{32}P]-dCTP and intensifying screens were from DuPont NEN (Boston, MA, USA). RNAzol was obtained from Tel-Test (Friendswood, TX, USA). [^{35}S]-Trans-Label was from ICN (Irvine, CA, USA). Megaprime kit was from Amersham (Arlington Heights, IL, USA). CytElisa MCP-1 was from CYTImmune Science, Inc. (College Park, MD, USA). X-OMAT AR X-ray films were from Eastman Kodak (Rochester, NY, USA).

Cell culture

Cultures of human mesangial cells were established from glomeruli isolated from human donor kidneys that were unsuitable for transplantation or normal portions of surgical nephrectomy samples. These cells had been extensively characterized by electron microscopy and immunohistochemistry [25, 26]. The cells were free of epithelial or endothelial contamination. Cells were used between the sixth to tenth passages. Cells were maintained in Waymouth's medium supplemented with 15 mM HEPES, 0.6 U/ml insulin, 2 mM glutamine, 100 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic/antifungal solution and 17% FCS. All assays were performed in triplicate or quadruplicate.

Protein synthesis assay

Human mesangial cells were plated in 24-well dishes at a density of 5×10^4 to 1×10^5 cells/ml in complete Waymouth medium. Near confluent cells were washed with PBS and preincubated in serum-free medium with or without IL-1 α [2 ng/ml] or TNF- α [500 U/ml] for 24 hours. Stx1 was then added in concentrations ranging from 0.25 ng/ml to 2500 ng/ml for 1 to 72 hours. At the end of the incubation, plates were washed twice with PBS to remove the toxin and incubated in methionine/cysteine free MEM containing 10 $\mu\text{Ci/ml}$ of [^{35}S]-Trans-S-Label for three hours at 37°C. The medium was then removed and the cells were washed twice in PBS and twice in 10% trichloroacetic acid on ice for 10 minutes each. Precipitated proteins were solubilized by adding 0.7 ml of 5% SDS and 0.1 N NaOH for 15 minutes at room temperature on a shaking plate. 0.3 ml of the precipitated radioactivity was neutralized with 50 ml of 6 M HCl and counted in a liquid scintillation counter. Results are expressed as percent protein synthesis in Stx1 treated cells compared with control non-treated cells.

Cytotoxicity assay

Human mesangial cells were plated in 24-well dishes at the same density as the protein synthesis assay and grown for 48 to 72 hours in complete Waymouth medium. The cells were washed in PBS and preincubated in 1 ml serum-free medium with or without IL-1 α [2 ng/ml] for 24 hours. Then Stx1 in concentrations ranging from 0.25 to 250 ng/ml was added for 24 to 72 hours. One milliliter of freshly diluted Neutral Red in PBS was added to a final concentration of 50 mg/ml and cells were incubated for additional two hours at 37°C. Cells were washed thoroughly (4 times) with PBS to remove excess Neutral Red; then 0.5 N HCl in 35% ethanol was added for five minutes at room temperature to release the dye from viable cells and the absorbance in each well was quantitated in a spectrophotometer at 570 nm. Results are expressed as a percentage of the absorbance of control cells or as optical density at 570 nm (OD_{570}).

[^3H]-thymidine assay

Human mesangial cells in 24-well dishes were rendered quiescent by placing them for 24 hours in serum-free medium containing IL-1 α [2 ng/ml]. Fifteen percent of FCS alone or with increasing doses of Stx1 ranging from 0.25 to 250 ng/ml was added for 24 hours at 37°C. Wells were then washed with PBS and serum-free medium containing 25 $\mu\text{Ci/ml}$ [^3H]-thymidine was added for four hours at 37°C. Cells were then washed twice in 5% trichloroacetic acid on ice. Precipitated DNA was solubilized in 0.1% SDS and 0.25 N NaOH. Then, 0.3 ml of the precipitated radioactivity was neutralized with 6 M HCl and counted in a liquid scintillation counter. Results are expressed as cpm of incorporated [^3H]-thymidine.

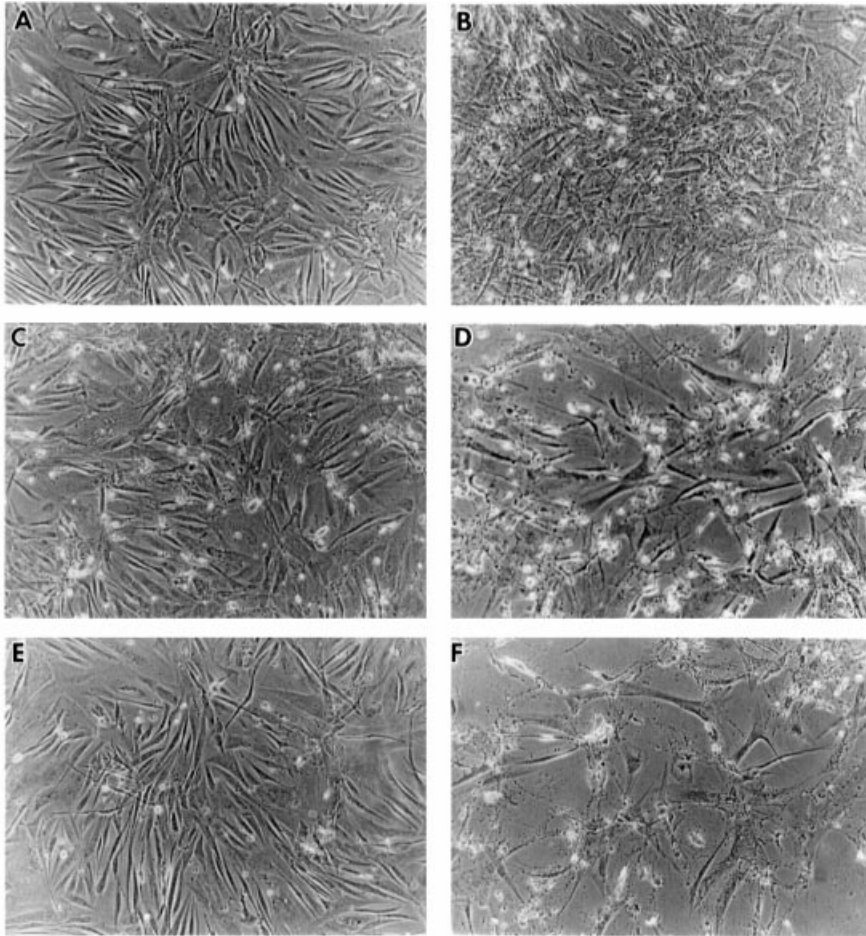


Fig. 1. Effect of Shiga toxin 1 (Stx1) on the morphology of human mesangial cells in culture. Cells were maintained without (A, C, E) or with (B, D, F) Stx1 (250 ng/ml) for 24 hours (A, B), 48 hours (C, D) and 72 hours (E, F). Cells were incubated in serum-free medium in the presence of IL-1 α (2 ng/ml) for 24 hours prior to the addition of Stx1. Note the viable cells after 72 hours of treatment with Stx1 (F).

RNA isolation and Northern blotting

Human mesangial cells were grown in 100 mm Petri dishes to near confluency. The medium was removed and the cells were washed with PBS and then placed in serum-free Waymouth medium with or without IL-1 α (2 ng/ml). After 24 hours, Stx1 (2.5 to 250 ng/ml), DRB (5 μ g/ml) or a combination of DRB and Stx1 was added for the time periods as indicated. The cells were harvested by trypsinization and total RNA was isolated by the modified method of Chomczynski and Sacchi using RNAzol [27]. Twenty micrograms of total RNA was electrophoresed through a formaldehyde (1%) agarose gel and transferred overnight to a GeneScreen membrane. A 715 bp *EcoRI/NotI* fragment of baboon MCP-1 cDNA (kind gift of Dr. A. Valente) was labeled with α -[³²P]dCTP (5000 Ci/mmol) using the Klenow fragment of *E. coli* [28]. As a control probe, a 114 bp *RsaI* fragment of human ribosomal RNA (36B4) was also labeled. Prehybridization and hybridization were carried out at 42°C in a buffer containing 50% formamide, 0.8 M NaCl, 0.02 M HEPES (pH 6.5), 20 mM EDTA, 100 mg/ml denatured salmon sperm DNA, and 0.5% SDS. After hybridization, blots were washed twice in 2 \times SSC, 0.1% SDS at 55°C for 15 minutes and finally in 0.5% SSC, 0.1%

SDS at 55°C for 20 minutes. The membranes were exposed to x-ray film for 8 to 24 hours at -70°C with intensifying screens. Variations in loading of RNA were tested by ethidiumbromide staining and examination under UV light.

Monocyte chemotactic peptide-1 ELISA

Human mesangial cells were plated in 24-well plates at a density of 2×10^4 cells/ml. Near confluent cells were preincubated in serum-free medium overnight and then Stx1 (1, 2.5 or 100 ng/ml) was added for 24 hours. Medium was collected and centrifuged to remove cell debris and stored at -20°C until assayed. The amount of human MCP-1 protein was determined using a sandwich enzyme immunoassay using a standard protocol provided by the manufacturer (CYTImmune Science, Inc., College Park, MD, USA). Samples were diluted 1:10 and 1:20 to ensure that values fall within the linear range of the standard curve. The sensitivity of the assay was 4.2 pg/ml with a range of detection from 8 to 500 pg/ml.

Statistical analysis

Data shown are mean \pm SD. The unpaired *t*-test was used to determine statistical significance with a two-tailed *P*

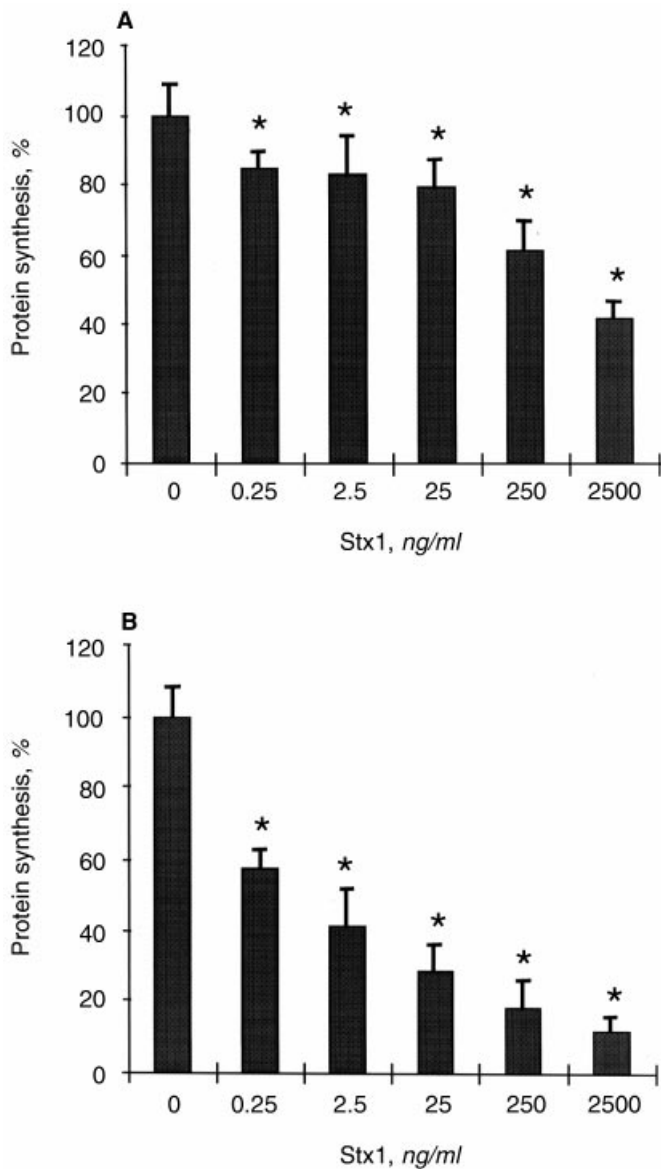


Fig. 2. Effect of Shiga toxin 1 (Stx1) on protein synthesis in human mesangial cells. Protein synthesis was measured as the incorporation of [³⁵S]-methionine/cysteine into total protein. Cells were preincubated in serum-free medium alone (A) or in the presence of IL-1 α (2 ng/ml) (B) for 24 hours prior to the addition of increasing doses of Stx1 (0.25 to 2500 ng/ml) for 24 hours. Data are expressed as % of the controls which represent cells incubated under the same conditions but without Stx1 (*two tailed *P* value < 0.05).

value of < 0.05 considered as significant difference between groups (Instat; GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cell morphology

Figure 1 shows human mesangial cells incubated without (A, C, E) or with (B, D, F) Stx1 for different time periods. After 24 hours of Stx1 incubation, there are minimal

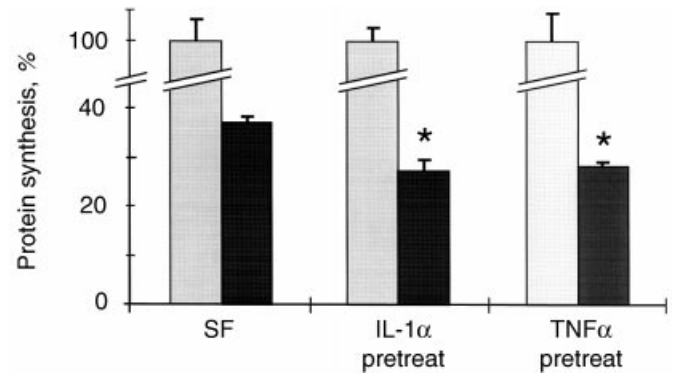


Fig. 3. Effect of cytokines on Stx1 induced inhibition of protein synthesis: Protein synthesis was measured as the incorporation of [³⁵S]-methionine/cysteine into total protein of human mesangial cells. Cells were preincubated in serum-free medium alone or in the presence of IL-1 α (2 ng/ml) or TNF- α (500 U/ml) for 24 hours prior to the addition of Stx1 (250 ng/ml) for an additional 24 hours. Symbols are: (□) without Stx1; (■) with Stx1. Data are expressed as percent and 100% represents the cells incubated under the same conditions but without Stx1 (*two tailed *P* value < 0.05).

changes in cell morphology; few cells detach and some cell blebbing can be seen. After 48 hours of Stx1 treatment there is a clear reduction in cell number and the remaining cells show an elongated shape and the cytoplasm starts to become more reticulated. This is more visible after 72 hours exposure to Stx1, when few viable attached cells remain.

Effect of Shiga toxin 1 on protein synthesis

Incubation of human mesangial cells with increasing doses of Stx1 for 24 hours results in inhibition of protein synthesis in a dose-dependent manner (Fig. 2A). This effect was significant at concentration of 0.25 ng/ml of Stx1. A 60% inhibition was achieved at 2500 ng/ml Stx1. Preincubation of the cells with 2 ng/ml IL-1 α for 24 hours, potentiated the inhibitory effect of Stx1 on protein synthesis. Under these conditions, approximately 60% inhibition could be seen with doses of Stx1 as low as 2.5 ng/ml. A 90% inhibition was achieved at 2500 ng/ml Stx1 (Fig. 2B). Similar to the effect of IL-1 α , incubation of mesangial cells with TNF- α enhanced the inhibitory effect of Stx1 on protein synthesis (Fig. 3).

Figure 4A shows the effect of cell density on the ability of Stx1 to inhibit protein synthesis. Subconfluent cells are more susceptible to the inhibitory effect of Stx1 than confluent cells. A time course for the effect of Stx1 is shown in Figure 4B. Within one hour of Stx1 treatment, protein synthesis was inhibited by approximately 50%. Maximum inhibition of 90% was achieved after 16 hours of Stx1 treatment. Longer incubations with Stx1 showed no further inhibitory effect.

Effect of Shiga toxin 1 on cell viability

To evaluate if protein-synthesis inhibition in response to Stx1 is associated with reduced cell viability, a Neutral Red

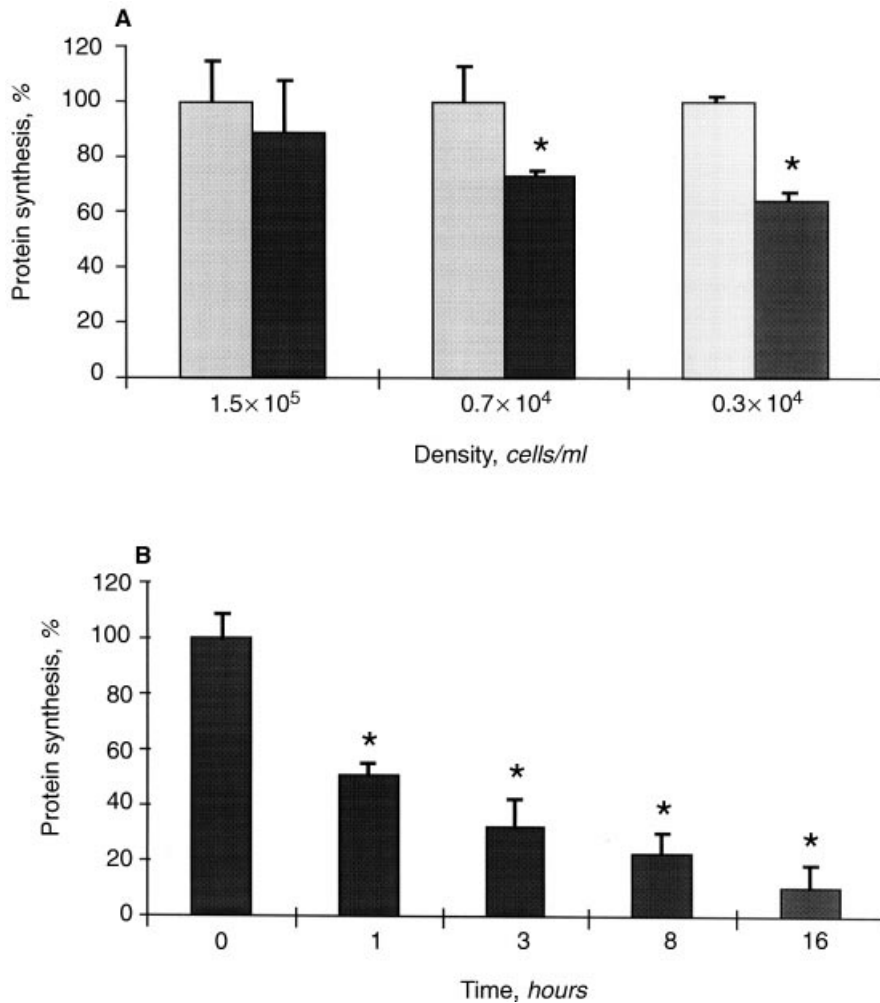


Fig. 4. (A) Effect of cell density on Stx1 induced inhibition of protein synthesis. Human mesangial cells were seeded at the indicated densities in 12-well dishes and incubated in serum-free medium in the presence of IL-1 α (2 ng/ml) for 24 hours prior to the addition of Stx1 (250 ng/ml) for an additional 24 hours. Data are expressed as percent, and 100% represents the cells incubated under the same conditions but without Stx1. Symbols are: (□) without Stx1; (■) with Stx1. (B) Time course for the effect of Stx1 on protein synthesis in human mesangial cells. Cells were preincubated in serum-free medium in the presence of IL-1 α (2 ng/ml) for 24 hours prior to the addition of Stx1 (250 ng/ml) for an additional 24 hours; 100% represents the cells incubated under the same conditions but without Stx1 (*two tailed *P* value < 0.05).

assay was used to assess cell viability. For these studies, cells were incubated under the same conditions as for the protein-synthesis assay. Figure 5A shows that after 24 hours of Stx1 incubation there is no significant reduction in the number of viable human mesangial cells. After 48 hours of Stx1 treatment, the number of viable cells was reduced to 35%, with further reduction in the number of viable cells to 25% after 72 hours. The cytotoxic effect of Stx1 on human mesangial cells is dose-dependent (Fig. 5B). The minimum concentration of Stx1 that resulted in significant cytotoxicity was 0.25 ng/ml. At this concentration, Stx1 caused a 40% reduction in viable cells. The highest concentration of Stx1 used was 2500 ng/ml and resulted in 85 to 90% reduction in the number of viable cells. It is noteworthy that some cells remained viable in spite of the high concentration of Stx1 and the prolonged incubation. This finding is in good agreement with the time course of the effect of Stx1 on protein-synthesis inhibition where some degree of protein synthesis persisted after long-term Stx1 treatment, albeit at a reduced level (Fig. 5). These data are also consistent with

the light microscopy studies showing attached viable cells after 48 and 72 hours of Stx1 treatment (Fig. 1 C-F).

Effect of Shiga toxin 1 on DNA synthesis

Since mesangial cells are partially resistant to the cytotoxic effect of Stx1 compared to reported data on endothelial cells, we next tested the effect of Stx1 on DNA synthesis in human mesangial cells treated with serum. Treatment of quiescent human mesangial cells with 15% fetal calf serum resulted in a five- to sixfold increase in DNA synthesis. The same response to 15% fetal calf serum was seen in cells treated with increasing doses of Stx1 (Fig. 6). Thus, Stx1 at concentrations that result in significant inhibition of protein synthesis did not alter DNA synthesis for at least 28 hours after exposure to Stx1. Only high concentrations of Stx1 reduced DNA synthesis by about 20 to 25%.

Effect of Shiga toxin 1 on monocyte chemotactic peptide-1 expression

We next tested the effect of Stx1 on MCP-1 mRNA abundance and MCP-1 protein production. Basal levels of

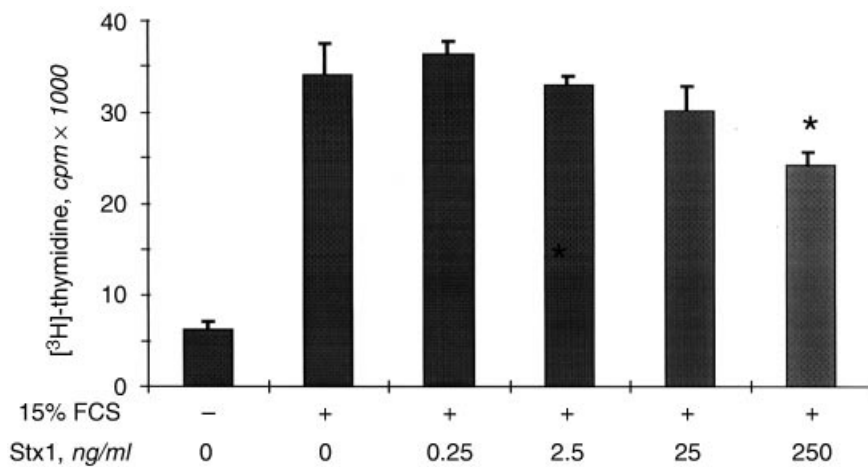
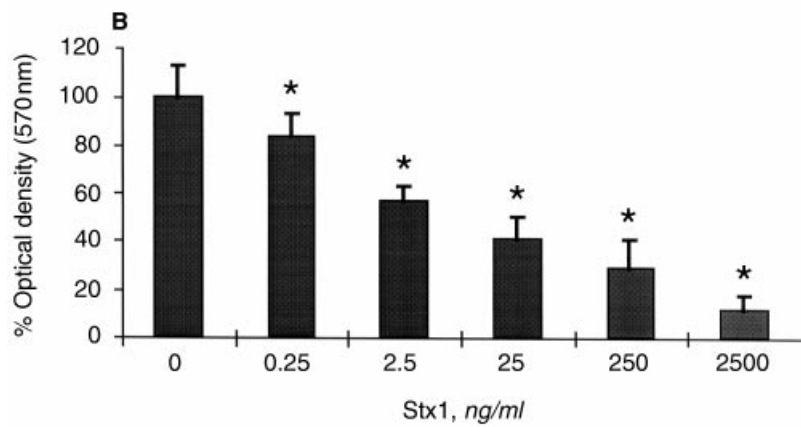
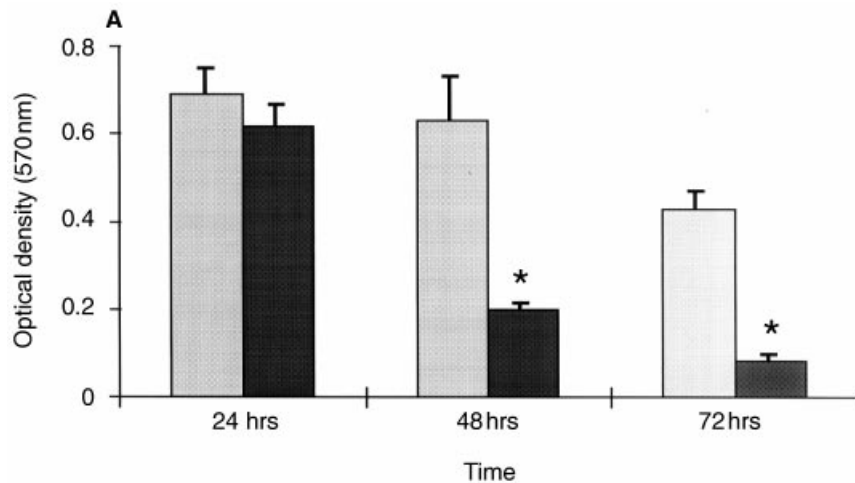


Fig. 5. (A) Time course for the effect of Stx1 on the uptake of Neutral Red into lysosomes of human mesangial cells. Cells were preincubated in serum-free medium in the presence of IL-1 α (2 ng/ml) for 24 hours prior to the addition of Stx1 (250 ng/ml) for the indicated time periods. Stx1 resulted in a time-dependent decrease in the optical density (OD) that represents the uptake of Neutral Red, a measure of cell viability. Symbols are: (□) without Stx1; (■) with Stx1. (B) Dose-response relationship for the effect of Stx1 on the uptake of Neutral Red into lysosomes of viable human mesangial cells. Cells were incubated in serum-free medium in the presence of IL-1 α (2 ng/ml) for 24 hours prior to the addition of Stx1 (0.25 to 2500 ng/ml) for an additional 48 hours. Data are expressed as percent. 100% represent cells incubated under identical conditions but without Stx1 (*two tailed P value < 0.05).

Fig. 6. Effect of Stx1 on DNA synthesis. DNA synthesis was measured as the incorporation of [³H]-thymidine into TCA precipitable material. Human mesangial cells were incubated in serum-free medium in the presence of IL-1 α (2 ng/ml) for 24 hours prior to the addition of 15% fetal calf serum with or without increasing doses of Stx1 (*two tailed P value < 0.05).

MCP-1 mRNA in growth arrested human mesangial cells is either undetectable or low. Treatment of mesangial cells with Stx1 alone resulted in an increase of MCP-1 mRNA abundance. This effect could be detected after 4 to 12 hours of exposure to Stx1 and lasted for at least 24 hours.

Concentrations of Stx1 as low as 2.5 ng/ml increase MCP-1 mRNA abundance (Fig. 7A). We next tested the effect of Stx1 on MCP-1 mRNA abundance in cells previously exposed to IL-1 α . Preincubation of mesangial cells with IL-1 α (2 ng/ml) for 24 hours resulted in an increase in

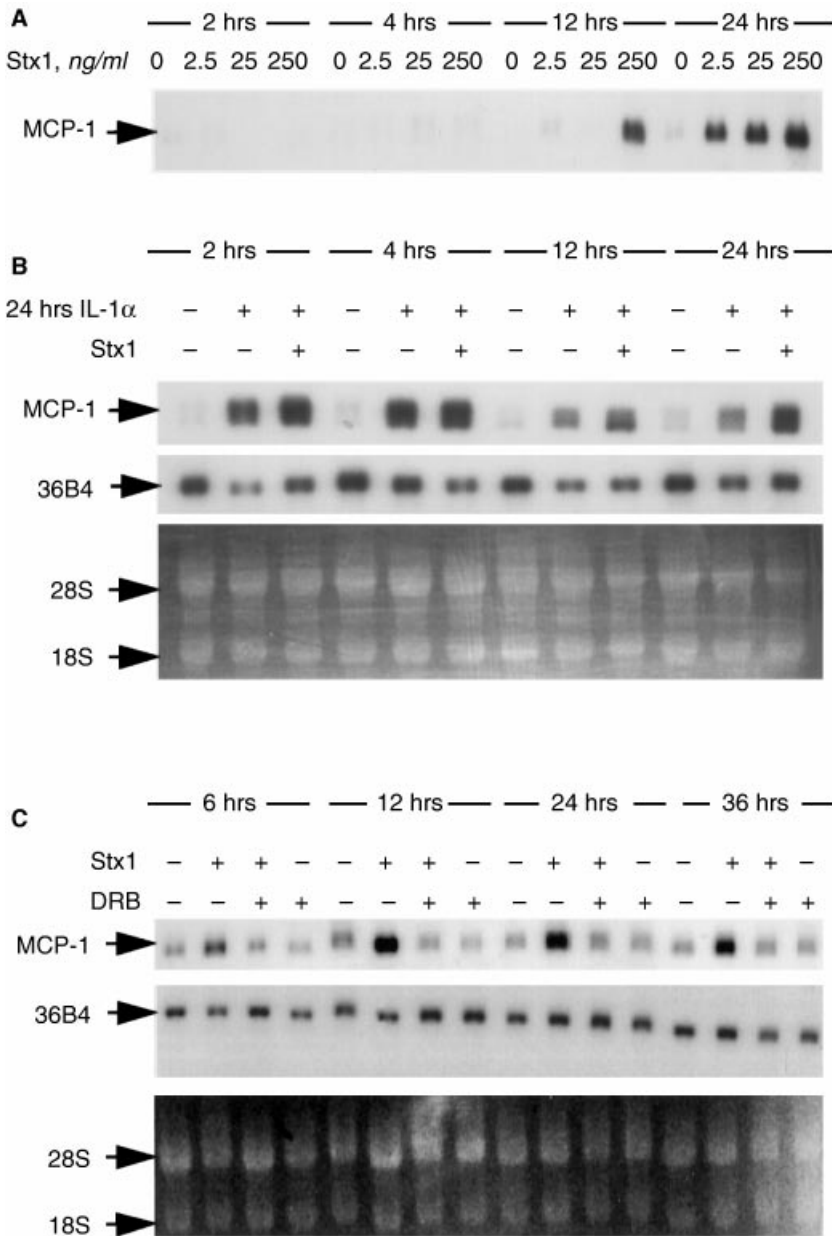


Fig. 7. Induction of MCP-1 mRNA in human mesangial cells by Stx1. Cells were plated in 100 mm dishes and grown to near confluency. (A) Cells were preincubated for 24 hours in serum-free medium prior to the addition of increasing concentrations of Stx1 for the time periods indicated. (B) Cells were preincubated with IL-1 α (2 ng/ml) for 24 hours prior to the addition of Stx1 (2.5 ng/ml) for the time periods indicated. RNA was analyzed by Northern blotting (Methods section) using a baboon MCP-1 cDNA. Lower two panels show the result of hybridization of the same blot with 36B4 cDNA as a housekeeping gene and the ethidium bromide staining of the membranes after RNA transfer. (C) Inhibition of Stx1 induced MCP-1 mRNA by the transcriptional inhibitor DRB. Human mesangial cells were plated in 100 mm dishes and grown to near confluency. Cells were preincubated in serum-free medium for 24 hours prior to the addition of Stx1 (2.5 ng/ml), DRB (5 μ g/ml) or both. RNA was analyzed by Northern blotting using a baboon MCP-1 cDNA. Lower two panels show the result of hybridization of the same blot with 36B4 cDNA and the ethidium bromide staining of the membrane after RNA transfer.

MCP-1 mRNA expression. The addition of Stx1 to cells preincubated with IL-1 α caused a further increase in MCP-1 gene expression (Fig. 7B). The rapid increase in MCP-1 mRNA abundance in response to Stx1 suggest a transcriptional mechanism of regulation. Coincubation of mesangial cells with Stx1 and the transcription inhibitor DRB (5 μ g/ml) prevented the increase of MCP-1 mRNA abundance by Stx1 (Fig. 7C). These data suggest that Stx1 induced MCP-1 gene induction is due to enhanced MCP-1 mRNA transcription. Concomitant to the increase in MCP-1 mRNA abundance, the levels of MCP-1 protein measured by an ELISA increased significantly from 11.96 ± 1.95 to 18.04 ± 1.04 ng/ml (control vs. Stx1 2.5 ng/ml). This

increase in MCP-1 protein was dose-dependent starting with a Stx1 concentration as little as 1 ng/ml (Fig. 8).

DISCUSSION

This study demonstrates that mesangial cells, which are commonly involved in the histopathologic manifestations of HUS, are direct targets of Stx1 and that Stx1 exerts diverse biologic effects on these cells. Stx1 inhibits protein-synthesis of human mesangial cells in a dose- and time-dependent manner. The proinflammatory cytokines IL-1 α and TNF- α markedly potentiate the inhibitory effect of Stx1. In spite of the rapid and significant inhibition of protein synthesis, Stx1 elicited no short term cytotoxic effect on mesangial

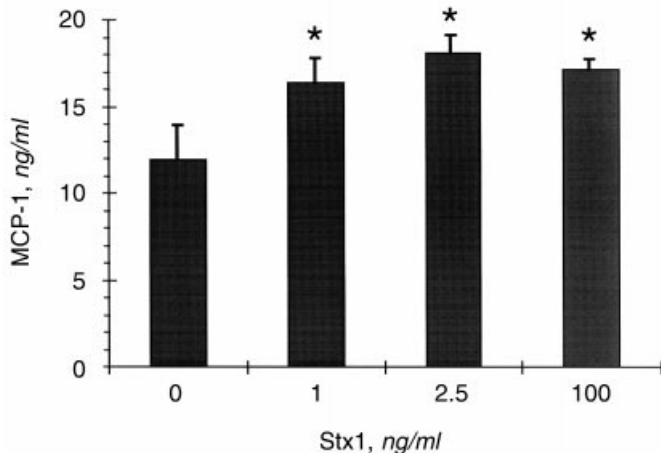


Fig. 8. Increased production of MCP-1 by Stx1 in human mesangial cells. Cells were grown to near confluency in 12-well plates. Cells were preincubated in serum-free medium for 24 hours prior to the addition of Stx1 in the indicated concentrations. The medium was collected after 24 hours, centrifuged and diluted in PBS (1:20) for measurement of MCP-1 by the sandwich ELISA technique.

cells. However, prolonged exposure of the cells to Stx1 results in significant cytotoxicity. Stx1, under conditions that do not result in significant protein synthesis inhibition or cytotoxicity, induces the expression of MCP-1 gene and protein.

The pathophysiology of HUS is not yet fully elucidated. While damage to the endothelium of the human kidney is thought to be the primary event in this disease, the factors that determine the severity of the acute injury as well as the long-term outcome are not known. The discovery of a bacterially-derived toxin as a major cause of HUS exerting its effect on a naturally occurring specific receptor primarily expressed on endothelial cells helped elucidate some of the pathogenic mechanisms of HUS. Microvascular endothelial injury results in a microthrombotic angiopathy that leads to a variable degree of renal ischemia. The presence of a specific ligand-receptor mediated process indicates that the injury to other renal cells may not simply result from a passive standby damage consequent to the ischemia. If other cells of the kidney, particularly cells that contribute to the histopathologic manifestations of the disease, are susceptible to the effect of Stx1, such an effect most likely represent a direct receptor-mediated action. Mesangial cells express the receptor for Stx1 [29], and our studies indicate that Stx1 exerts multiple biologic effects in these cells. Protein synthesis in mesangial cells is inhibited by Stx1 at concentrations in the picomolar to nanomolar range. These concentrations are somewhat higher than those required to inhibit protein synthesis in HRMEC. In the presence of cytokines, concentrations of Stx1 that are required to inhibit mesangial cell protein synthesis are comparable to those effective in HRMEC [20, 30]. On the other hand, HUVEC do not respond to Stx1 unless they are

primed with immune inflammatory cytokines [20, 31]. These data provide evidence that human mesangial cells similar to HRMEC are much more susceptible to protein synthesis inhibition by Stx1 than HUVEC, a non renal endothelial cell line. Immune inflammatory cytokines also potentiate the effects of Stx1 on mesangial cells. Pretreatment of mesangial cells with IL-1 α or TNF α increases their susceptibility to the effects of Stx1 to a level that approximates the response of HRMEC. Interestingly, these two cytokines also markedly alter the time course kinetics of mesangial cell response. These data indicate that the susceptibility of mesangial cells to the effect of the toxin is dependent on the availability of cytokines in the microenvironment. It should be emphasized that rather high concentrations of cytokines released by activated monocytes or macrophages can be achieved locally in the vicinity of mesangial cells.

Human mesangial cells are also more susceptible to the cytotoxic effects of Stx1 than HUVEC and are almost as susceptible as HRMEC. In HRMEC, Stx1 is cytotoxic at femtomolar range, concentrations that do not inhibit protein synthesis. On the other hand, HUVEC are susceptible at nanomolar concentrations and only after exposure to immune inflammatory cytokines [20]. We find that prolonged exposure of mesangial cells to Stx1 is required before significant cytotoxicity can be demonstrated. It is interesting to note that, in spite of significant inhibition of protein synthesis in response to Stx1, mesangial cells continue to synthesize DNA when incubated in the presence of serum. Only at high concentrations does Stx1 result in a small inhibition of DNA synthesis. Collectively, the data suggest that the differential response of the cells to Stx1 in terms of cell viability may be related to differential effects on protein synthesis. Recent evidence indicates that cell-specific responses to Stx1 are not necessarily dependent on the number of Gb₃ receptors. The type of cell surface glycolipids and length of fatty acid chain as well as the activity of specific intracellular lipid metabolizing enzymes are major determinants of the nature and severity of the biologic response of cells to Stx1 [32–37].

Van Setten et al have recently reported that Stx1 inhibits protein synthesis in human mesangial cells [38]. The concentrations required to inhibit protein synthesis (0.1 to 10 nmol/liter) was similar to the concentrations we used. However, the authors found that Stx1 does not decrease cell viability even after prolonged incubation in serum containing medium. It is likely that serum prevents or minimizes cytotoxicity since our viability assays were performed in the absence of serum. It is noteworthy that similar to their observation, we find that Stx1 inhibits protein synthesis even in the presence of serum (data not shown). It is likely, as van Setten et al suggested, that protein synthesis inhibition can be largely dissociated from cell viability; also of note is that they did not find that

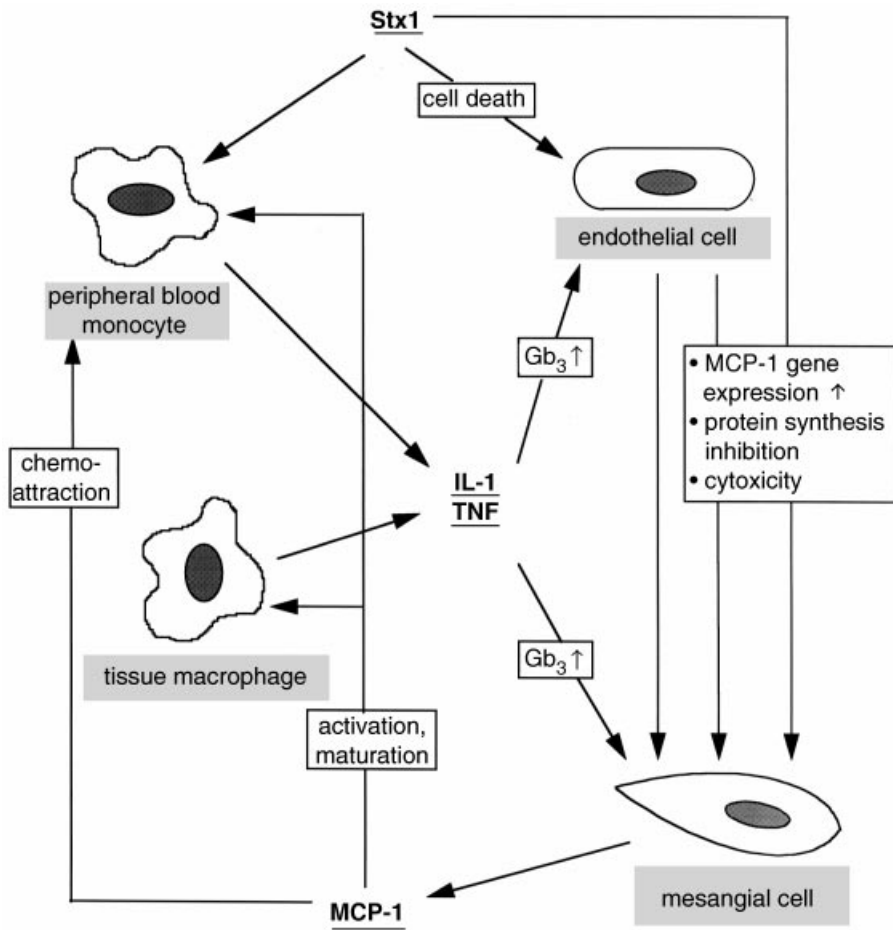


Fig. 9. Schematic hypothesis of the effect of Stx1 on human mesangial cells in HUS. Details are in the text.

preincubation with cytokines potentiates the cytotoxicity induced by Stx1.

Mesangial cells are a known source for MCP-1, a chemokine involved in a variety of glomerular inflammatory diseases due to its chemoattractant effect on monocytes and lymphocytes [39–41]. Infiltration by inflammatory cells, particularly monocytes, is now a recognized manifestation of HUS [22, 42]. We now demonstrate that Stx1 increases MCP-1 mRNA abundance and that this effect is potentiated in the presence of cytokines. The rapid increases in MCP-1 mRNA levels which can be prevented by DRB suggest a transcriptional mechanism of activation by Stx1. These data are at variance with a recent report demonstrating lack of effect of Stx1 on chemokine production [38]. These differences may be related to different responses of separate cell isolates to Stx1, or to the use of serum that can maximally activate the cells and render them unresponsive to Stx1. Activation of mesangial cells by Stx1 in HUS and production of MCP-1 may be responsible at least partially for the infiltration of peripheral blood mononuclear cells into the kidney. Monocytes may be activated by MCP-1 to produce cytokines including IL-1 and TNF- α , which in turn may enhance the effects of Stx1

on its renal target cells in HUS. It has been shown in a mouse model of HUS that the kidney is the sole source of excessive TNF- α production [43]. The specific cell type that produces TNF- α within the kidney is unknown, but the authors speculated that incoming monocytes/macrophages might be the source of TNF- α . This hypothesis is strengthened by findings in C3H/HeJ mice whose monocyte/macrophage function is deficient. These mice show a significantly longer mean time to death compared to normal controls when treated with lethal doses of Stx1 [44]. Furthermore, it has been demonstrated that human monocytes treated with Stx1 produce high amounts of TNF- α *in vitro* [45, 46].

While extrapolation from *in vitro* to *in vivo* data should be cautious, our studies suggest that activation of mesangial cells by Stx1 may be relevant to progressive renal injury that results from HUS. Activation of mesangial cells is one major factor that results in glomerulosclerosis [47]. Indeed, despite partial recovery of renal function in HUS, progressive glomerulosclerosis with or without segmental or diffuse mesangial proliferation in remaining glomeruli is a well recognized long-term complication [23, 48]. In studies looking at the outcome of kidney function ten years after

HUS, only 60% of the patients showed normal kidney function [49, 50]. Moreover, only 40% of the patients showed no renal abnormalities 15 to 25 years after HUS [51]. Our studies indicate that mesangial cells are relatively resistant to the cytotoxic effect of Stx1 unless the cells were exposed to immune inflammatory cytokines. Therefore, the severity and the duration of exotoxemia and consequent release of cytokines may play an important role in the outcome of the glomerular lesion in HUS, that is, complete resolution or sclerosis with residual impairment of renal function. The circulating or tissue levels of Stx1 are unknown. Concentrations of Stx1 that cause endothelial cell lysis may result in mesangial cell activation with subsequent production of MCP-1 and other cytokines. It is not difficult to conceptualize how Stx1 gains access to the mesangium. Mesangial cells may be exposed to plasma constituents and even more so after lysis of endothelial cells, the only barrier between blood circulating in the glomerular capillaries and the mesangium. Mesangial cell activation or injury may also be initiated or potentiated by material released upon endothelial cell injury or lysis. Activation of mesangial cells *in vivo* may be responsible for the incomplete resolution of glomerular lesions and the development of glomerulosclerosis observed in severe cases of HUS. A schematic illustration of this hypothesis is shown in Figure 9.

These data provide evidence that in addition to endothelial cells, human mesangial cells are susceptible to the effects of Stx1 *in vitro*. Stx1 exerts a spectrum of biologic effects in mesangial cells ranging from induction of chemokine expression to marked inhibition of protein synthesis and cytotoxicity. Glomerular pathology and long-term complications of HUS may therefore result from a direct effect of Stx1 on mesangial cells in addition to its effects on endothelial cells.

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APPENDIX

Abbreviations used in this article are: DRB, 5,6-dichlorobenzimidazole-riboside; Gb₃, glycosphingolipid globotriaosylceramide; HEPES, N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid; HRMEC, human renal endothelial cells; HUS, hemolytic uremic syndrome; HUVEC, human umbilical vein endothelial cells; IL-1 α , interleukin-1 α ; MCP-1, monocyte chemoattractant peptide; SDS, sodium dodecyl sulfate; SSC, sodium citrate; STX1 and STX2, Shiga toxin 1 and 2; TNF- α , tumor necrosis factor- α ; VTEC, verotoxin producing *Escherichia coli*.

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