Hemolytic uremic syndrome (HUS) is a disease defined by nonimmune hemolytic anemia, thrombocytopenia, and acute renal failure (26). Its unique histological features are termed thrombotic microangiopathy, with endothelial cell injury and platelet thrombi being found in the microvasculature (28). HUS, especially in children, is often associated with infection by Shiga toxin (Stx)-producing Escherichia coli (STEC), and the clinical and histological abnormalities in these cases are mainly attributed to the action of Stx. Leukocyte infiltration and tubular damage are also noticed as early events in Stx-associated HUS (12, 13).

Stx produced by STEC is classified into two closely related subgroups, Stx1 and Stx2, and both consist of a single enzymatically active A subunit that inactivates 28S rRNA and inhibits protein synthesis and pentameric B subunits that bind to neutral glycolipid globotriaosylceramide (Gb3), a cell surface receptor. It has been demonstrated that cultured endothelial cells derived from the microvasculature of human tissues such as glomeruli contain high levels of Gb3 and are highly susceptible to Stx action of Stx on tubular epithelial cells (12). It has been shown that tubular epithelial cells are also highly susceptible to Stx in culture (8). Thus, these studies suggest the existence of a mechanism by which Stx causes HUS via its direct effect on renal cells.

On the other hand, other pathogenic mechanisms operating in HUS in which systemic changes are induced by Stx have also been observed. It was shown from in vitro studies that Stx binds to (2) and activates (16) platelets, implying that Stx may be directly involved in the prothrombotic state seen with HUS. It was also reported that Stx induced cytokine production in several types of cultured cells (34, 42) and that cytokine levels were higher in some patients with Stx-associated HUS (27) is not only secondary to Stx-induced glomerular and arteriolar injury but also induced by the direct action of Stx on tubular epithelial cells (12). It has been shown that tubular epithelial cells are also highly susceptible to Stx in culture (8). Thus, these studies suggest the existence of a mechanism by which Stx causes HUS via its direct effect on renal cells.

In contrast to clinical observations on Stx-associated HUS, rodent models induced by systemic administration of Stx or inoculation of STEC show only mild microvascular injury (33). This is probably due to the low level of Gb3 expression on endothelial cells of the rodent kidney (our unpublished observation). To assess the direct effect of Stx on the kidney, Shibot et al. (29) perfused isolated rat kidneys with Stx and demonstrated this perfusion to cause marked medullary tubu-
lar injury with no glomerular changes. Their study showed the direct effect of Stx on the kidney, but subsequent events in the Stx-injured kidney could not be analyzed.

The present study was conducted to test the hypothesis that Stx-induced renal damage causes platelet aggregation, tubular injuries, inflammation, and the deterioration of renal function without its systemic action. To this end, we performed Stx1 perfusion of a single kidney, followed by revascularization, in rats. This method allowed us not only to avoid the indirect effects of Stx1 via other tissues or blood but also to analyze interaction, such as platelet activation and leukocyte infiltration, between circulating blood cells and the Stx1-perfused kidney. In addition, because the toxin does not reach the brain in this procedure, we could avoid its fatal attack on the brain (20, 23) and thus administer higher doses of the toxin to cause more-severe damage to the kidney. Also, we could assess the renal function of the perfused kidney by removing the other, nonperfused, healthy kidney. The results of the present study indicate that Stx1 directly caused renal damage that resulted in platelet and monocyte accumulation in glomeruli and medullary interstitial, tubular injuries, and acute deterioration of renal function.

MATERIALS AND METHODS

Materials. Recombinant Stx1 was prepared by using DEAE-Sepharose column chromatography, chromatofocusing column chromatography, and high-performance liquid chromatography, as described previously (21). The 50% cytotoxic dose of the purified Stx1 on Vero cells was about 1 pg/ml. The endotoxin content of the Stx1 preparation was less than 0.016 endotoxin units per 1 mg, as determined by use of an HS-Limulus test (Wako Pure Industries, Ltd., Osaka, Japan).

Kidney perfusion. Wistar male rats (240 to 280 g) were obtained from Charles River Japan (Yokohama, Japan) and were allowed free access to chow and water. Handling of rats and experimental procedures were conducted in accordance with institutional guidelines for animal care and use. Kidney perfusion was performed as previously described (17), with slight modifications. Modified Tyrode buffer (17) saturated with 95% oxygen and 5% carbon dioxide and adjusted to pH 7.4 was used as a vehicle. Under anesthesia with pentobarbital, the left kidney of a rat was exposed. Polyethylene tubes were placed in the left renal artery and vein, and the proximal portions of the vessels were temporarily ligated. The left kidney was perfused at a rate of 2 ml/min by using a peristaltic pump. All of the perfusate was discarded through a cannula placed in the renal vein. To eliminate the blood contained inside the kidney, the kidney was perfused first with Tyrode buffer for 30 to 60 s and then with Stx1 (2 μg/ml in Tyrode buffer containing 1% rat serum) for 3 min. Finally, it was perfused again with the buffer only for 3 min to eliminate any unbound toxin. After the kidney perfusion, the tubes were removed, and the holes in the artery and vein were repaired by microsurgery. Blood circulation of the left kidney was reestablished by releasing the ligature. The average time required for the perfusion procedure was about 15 min. To assess the renal function (see Experiment 2 below), we removed the right kidney just after the perfusion procedure, the tubes were removed, and the holes in the artery and vein were ligated. The left kidney was perfused at a rate of 2 ml/min by using a peristaltic pump. The left kidney of a rat was exposed. Polyethylene tubes were placed in the left renal artery and vein, and the proximal portions of the vessels were temporarily ligated. The left kidney was perfused at a rate of 2 ml/min by using a peristaltic pump. All of the perfusate was discarded through a cannula placed in the renal vein. To eliminate the blood contained inside the kidney, the kidney was perfused first with Tyrode buffer for 30 to 60 s and then with Stx1 (2 μg/ml in Tyrode buffer containing 1% rat serum) for 3 min. Finally, it was perfused again with the buffer only for 3 min to eliminate any unbound toxin. After the kidney perfusion, the tubes were removed, and the holes in the artery and vein were repaired by microsurgery. Blood circulation of the left kidney was reestablished by releasing the ligature. The average time required for the perfusion procedure was about 15 min. To assess the renal function (see Experiment 2 below), we removed the right kidney just after the perfusion procedure, the tubes were removed, and the holes in the artery and vein were ligated.

Reverse transcription-PCR. Total RNA fractions were prepared from pieces of cortical and medullary tissue by the guanidinium thiocyanate-phenol-chloroform method (1), and 5 μg of RNA was reverse transcribed by use of oligo(dT) primers in 20 μl of buffer as described previously (25). For the quantitative measurement of the TNF-α mRNA levels, real-time PCR was performed by using an ABI PRISM 7700 sequence detector and TaqMan predeveloped assay reagents for gene expression quantification system (Applied Biosystems, Foster City, CA). We assayed the mRNA levels of TNF-α and the endogenous control (GAPDH [glyceraldehyde-3-phosphate dehydrogenase]) by using multiple reporter dyes (Applied Biosystems) and expressed the TNF-α mRNA levels as ratios to the levels of the endogenous control.

RESULTS

Histological changes were first examined by light microscopy. A few changes, such as small areas with detachment of tubular epithelial cells and irregularity of the brush border membranes, were seen for some samples from both sham-operated and Stx1-perfused groups at 6 and 24 h, which possibly resulted from ischemia during the operation; however, no obvious differences between the two groups were found at these time points. On day 3, however, the Stx1-perfused kidneys showed extensive tubular injuries, such as desquamation, cytoplasmic vacuolation, pyknosis, and loss of nuclei. Massive interstitial leukocyte infiltration was also observed to occur in the medulla. On day 9, severe tubular dilatation and degeneration were found to occur in both the cortex and medulla. Typical changes observed by light-microscopic examination are shown in Fig. 1. These abnormalities were not found to occur in the kidneys of sham-operated control rats. No obvious glomerular changes were observed for any animals at any time points. Tubulointerstitial changes in each animal were assessed semiquantitatively (Fig. 2). Statistically significant differences between the two groups were found from day 3 in all areas of the kidney.

Because some changes possibly derived from ischemia were observed to occur in both groups at 6 and 24 h as described above, light-microscopic examination was not appropriate for...
FIG. 1. Typical histological changes in tubulointerstitium of Stx1-perfused and revascularized kidneys seen under light microscopy. Cortex (A and B), outer stripe (C and D), inner stripe (E and F), and inner medulla (G and H) of Stx-perfused kidney on day 3 (A, C, E, and G) and day 9 (B, D, F, and H) postperfusion are shown. Asterisks, tubular dilation; black arrows, cytoplasmic vacuolation; white arrows, desquamation; black arrowheads, leukocyte infiltration; white arrowheads, pyknosis. In the Stx1-perfused kidney, massive infiltration of inflammatory cells is seen on day 3. Severe tubular dilatation and degeneration are notable on day 9. Hematoxylin-eosin staining. Bar, 100 μm.
analyzing specific effects of Stx1 on renal cells at these time points. Therefore, we examined the kidney sections by TUNEL staining instead. TUNEL-positive cells were detected in the renal medulla of Stx1-perfused animals on day 1 (Fig. 3). Only very few cells in the kidneys of sham-operated animals were positive for TUNEL staining. Almost no TUNEL-positive cells were found in the renal cortex, including glomeruli, for either group.

Activation of renal cells on day 1 was tested by measuring the mRNA levels of TNF-α, an inflammatory cytokine. As

Sham-operated control Stx1-perfused

FIG. 3. TUNEL labeling of rat kidneys on day 1. Some tubular cells in the kidneys of Stx-perfused rats were positive for TUNEL staining, but those in the kidneys of the sham-operated rats were not.

FIG. 4. Expression of TNF-α mRNA in renal cortex and medulla on day 1. Filled circles, Stx-perfused rats (S); open circles, sham-operated control rats (C). Data are expressed as the ratios of the levels of TNF-α mRNA to those of the internal control. Each symbol represents one animal. NS, not significant.
shown in Fig. 4, the levels of TNF-α mRNA in the medulla of Stx1-perfused kidneys were higher than those in the sham-operated kidneys. However, there was no difference in cortical TNF-α mRNA level between the two groups. These results indicate that the perfusion of Stx1 induced apoptosis as well as the gene expression of TNF-α in renal medullary cells.

Next, we tested whether the Stx1 perfusion and revascularization caused accumulation of blood cells in the kidney. On day 1, there was no increase in the number of monocytes/macrophages in the Stx1-perfused kidneys. By day 3, however, evidence of monocyte/macrophage infiltration was detected in both the glomeruli and the medullary interstitium but not in the cortical interstitium (Fig. 5).

As shown in Fig. 6, platelet aggregation in glomeruli in the Stx1-perfused kidneys but not in the sham-operated kidneys was also detected on day 3 by immunostaining with antiplatelet antibody. The level of platelet aggregation in glomeruli, evaluated by measuring the stained area by computer-analyzed densitometry, confirmed the increase in the glomerular deposition of platelets, suggesting that the injury induced by Stx1 perfusion of the kidney caused platelet aggregation in the glomeruli. Platelet aggregation was also observed to occur in the medullary capillaries.

Finally, to assess the effect of Stx1 on renal function, we removed the right kidneys of the animals just after the Stx1 perfusion. As shown in Fig. 7, levels of serum creatinine and BUN of Stx1-perfused rats were increased from day 2 postperfusion, and acute renal failure followed on day 3. This result clearly shows that Stx1 perfusion and subsequent revascularization caused renal dysfunction.

**DISCUSSION**

The present study demonstrates that the direct action of Stx1 on the kidney induced (i) apoptosis and TNF-α gene expression in the medulla, (ii) monocyte/macrophage infiltration, (iii) platelet aggregation, (iv) severe tubular damage, and (v) acute deterioration of renal function.

The first abnormalities induced by Stx1 were noticed to occur in the medulla: apoptotic cells, stained by the TUNEL method, and enhanced TNF-α gene expression were detected in the medulla 24 h after Stx1 perfusion. These results are consistent with a previous report (29) that perfusion of Stx in isolated rat kidneys for 160 min caused mainly medullary tubular injuries. Furthermore, apoptotic cells were detected in the kidneys of mice infected with STEC as well as in those of patients with Stx-associated HUS (15, 31). The present study demonstrated that Stx1 by itself induced apoptosis in medullary cells in vivo. Whereas apoptotic cells were detected in the renal cortex in the mouse infection study (15), the present study showed that they were found in the medulla but rarely in the cortex. The difference between these observations may have resulted from the involvement of some virulence factor(s) of the pathogen other than Stx in the mechanism of apoptosis, as suggested earlier (15). The induction of apoptosis by Stx has also been shown for various types of cultured cells, including renal tubular epithelial cells (11, 31).

The induction of TNF by Stx in vivo was also previously reported: an injection of Stx induced TNF biosynthesis in mouse kidney (7) and urinary TNF excretion in baboons (32). With mice, the induction of TNF synthesis by Stx1 was shown only for transgenic animals bearing a chloramphenicol acetyltransferase reporter gene that indicates TNF biosynthesis, as an increase of TNF itself could not be detected (7), suggesting that the cytokine-inducing activity of Stx is relatively weak in rodents. The study also showed that the induction of TNF was kidney specific. In the baboon model, the increase in TNF induced by Stx1 injection was observed with urine but not with serum, and so local TNF production in the kidney was speculated (32). Our results confirmed that speculation and directly showed that Stx1 has the ability to induce expression of the TNF-α gene in vivo, although we did not show it at protein level. In in vitro studies, it has been shown that Stx induced the production of inflammatory cytokines such as TNF-α and interleukin-1 in macrophages (34) and other cultured cells, including tubular epithelial cells (9). Because no increase in the
number of medullary macrophages was observed at 24 h after Stx1 perfusion, with the increase in TNF-α mRNA level detected in the medulla, it is conceivable that Stx1 directly induced TNF-α gene expression in intrinsic medullary cells, such as tubular epithelial cells and resident macrophages. In some patients with HUS, an increase in TNF level was observed with both serum and urine (14), but in these cases lipopolysaccharide, a well-known TNF inducer, may also have contributed to TNF production.

Although the induction of both apoptosis and cytokine biosynthesis by Stx has been shown for renal cells (9, 15), it was also reported that both phenomena in the cells can be induced by Stx1 perfusion, with the increase in TNF-α mRNA level detected in the medulla, it is conceivable that Stx1 directly induced TNF-α gene expression in intrinsic medullary cells, such as tubular epithelial cells and resident macrophages.

FIG. 6. Glomerular platelet aggregation on day 3. (a) Immunostaining of platelets in glomeruli and (b) quantitative analysis of the platelet aggregation. Glomerular platelet aggregation is expressed as a percentage of the area of glomeruli in cross-section that is occupied by platelets. Filled circles, Stx-perfused rats; open circles, sham-operated control rats. Each symbol represents one animal.

FIG. 7. Levels of serum creatinine and BUN in Stx-perfused rats with uninephrectomy. Filled circles, Stx-perfused rats; open circles, sham-operated control rats. Each symbol represents one animal. NS, not significant.
by ischemia (3, 22) and that tubular cells in the medulla, where these phenomena were observed with the present study, are particularly susceptible to ischemic injury (38). Because our light-microscopic examination showed a few changes in some samples of both sham-operated and Stx1-perfused groups until 24 h, suggesting that these changes potentially resulted from ischemia during the operation, we cannot exclude the possibility that ischemia is involved in the mechanism of the induction of apoptosis and TNF-α. However, the levels of both apoptosis and TNF-α mRNA were significantly higher in Stx1-perfused kidneys than in sham-operated kidneys, and so we consider that Stx1 mainly, if not entirely, contributes to the induction of these activities.

The Stx1 perfusion and subsequent revascularization resulted in monocye/macrophage infiltration on day 3. This result, together with the finding of TNF induction, suggests that Stx1 itself induces inflammation in the kidney. Although the importance of neutrophils in HUS has been mainly described so far (5), the accumulation of monocytes/macrophages and their role in the pathogenesis of HUS has also been suggested (36). In addition to inducing TNF-α, Stx has been shown to induce chemokine production in some types of cells (34, 42). For example, Simon et al. (30) reported that Stx1 induced monocyte chemoattractant protein 1 production in cultured human mesangial cells. Stx1 also induced the expression of adhesion molecules in endothelial cells (19). In addition to nonspecific, injury-mediated chemotraction and activation of monocytes, these activities of Stx toward intrinsic renal cells may have contributed to the monocyte infiltration into the kidney.

It has been unclear whether the platelet aggregation observed with HUS is induced directly by Stx or indirectly through Stx-mediated endothelial injury (2, 16, 39, 41). Although no obvious glomerular changes were observed by light microscopy, the present study demonstrates that Stx1 perfusion and revascularization of the rat kidney resulted in platelet aggregation in the glomeruli. This result indicates that the direct action of Stx on platelets is not essential for platelet aggregation and supports the hypothesis that the action of Stx on endothelial cells induces platelet activation in the glomeruli. Because the content of the receptor for Stx, Gb3, in endothelial cells is much lower in rodents than in humans (our unpublished observation), the level of platelet aggregation was relatively low, and so microthrombotic changes were not observed by light microscopy.

The results of the present study show that Stx directly caused renal damage that began with apoptosis and TNF induction in the medulla, resulting in acute renal failure. The histological changes in the tubulointerstitium were much more prominent than those in the glomeruli. Our result is consistent with previous studies showing that Stx causes much more severe damage to tubules than to glomeruli in rodent kidney (29, 33). Tubular cell damage, in addition to glomerular thrombotic microangiopathy, was observed to occur in patients infected by STEC (27). Although platelet aggregation in glomeruli was observed with the present study, the renal dysfunction may have resulted from the tubular damage rather than from microthrombotic lesions with this model. This may possibly be the case for humans as well.

In contrast to the rodent models, some other animal models have been shown to develop thrombotic microangiopathy, e.g., when baboons were injected with Stx (32) or when greyhound dogs (4), ferrets (40), or gnotobiotic piglets (6) were infected with STEC. As shown in the present study, rats did not develop typical thrombotic microangiopathy, even when perfused with a high dose of Stx. Therefore, this model does not seem to be appropriate for the study of the whole mechanisms of thrombotic microangiopathy, compared with the other models listed above. In addition, indirect effects of Stx through other cells and tissues that may modify the disease are not reflected in the kidney with this model. However, tubulointerstitial injuries, such as apoptosis, inflammation, and resulting renal dysfunction, clearly occurred by the direct action of Stx1 on the kidney, and so we consider that this model provides a new approach for the study of the mechanisms of these events directly caused by Stx in vivo.

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