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Fractalkine and CX3CR1 Mediate Leukocyte Capture by Endothelium in Response to Shiga Toxin

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Shiga toxins (Stx) are the virulence factors of enterohemorrhagic Escherichia coli O157:H7, a worldwide emerging diarrheal pathogen, which precipitates postdiarrheal hemolytic uremic syndrome, the leading cause of acute renal failure in children. In this study, we show that Stx2 triggered expression of fractalkine (FKN), a CX3C transmembrane chemokine, acting as both adhesion counterreceptor on endothelial cells and soluble chemoattractant. Stx2 caused in HUVEC expression of FKN mRNA and protein, which promoted leukocyte capture, ablated by Abs to either endothelial FKN or leukocyte CX3CR1 receptor. Exposure of human glomerular endothelial cells to Stx2 recapitulated its FKN-inducing activity and FKN-mediated leukocyte adhesion. Both processes required phosphorylation of Src-family protein tyrosine kinase and p38 MAPK in endothelial cells. Furthermore, they depended on nuclear import of NF-κB and other stress-responsive transcription factors. Inhibition of their nuclear import with the cell-permeating SN50 peptide reduced FKN mRNA levels and FKN-mediated leukocyte capture by endothelial cells. Adenoviral overexpression of IκBα inhibited FKN mRNA up-regulation. The FKN-mediated responses to Stx2 were also dependent on AP-1. In mice, both virulence factors of Stx-producing E. coli, Stx and LPS, are required to elicit hemolytic uremic syndrome. In this study, FKN was detected within glomeruli of C57BL/6 mice injected with Stx2, and further increased after Stx2 plus LPS coadministration. This was associated with recruitment of CX3CR1-positive cells. Thus, in response to Stx2, FKN is induced playing an essential role in the promotion of leukocyte-endothelial cell interaction thereby potentially contributing to the renal microvascular dysfunction and thrombotic microangiopathy that underlie hemolytic uremic syndrome due to enterohemorrhagic E. coli O157:H7 infection. The Journal of Immunology, 2008, 181: 1460–1469.

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2 C.Za. and C.Zo. contributed equally to this work.
3 Address correspondence and reprint requests to Dr. Carla Zoja, “Mario Negri” Institute for Pharmacological Research, Via Gavazzeni 11, 24125 Bergamo, Italy. E-mail address: zoja@marionegri.it
4 Abbreviations used in this paper: Stx, Shiga toxin; HUS, hemolytic uremic syndrome; D-HUS, diarrhea-associated HUS; Gb3, globotriaosyl ceramide; Tir, translocated intimin receptor; FKN, fractalkine; GEC, glomerular endothelial cell; PTK, protein tyrosine kinase; ODN, oligodeoxynucleotide; Ct, cycle threshold; BUN, blood urea nitrogen; vWF, von Willebrand factor; SRTF, stress responsive transcription factor; PMN, polymorphonuclear cell.

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syndrome protein. A pedestal-like structure, rich in polymerized actin and studded with Tir, provides a niche for intimin-anchored *E. coli* O157:H7 (13).

*E. coli* O157:H7 colonization of large intestine mucosa through formation of attaching and effacing lesions is followed by bloody diarrhea in ~90% patients (4). It is attributed to hemorrhagic lesions in submucosal microcirculation due to the noxious effect of Stx secreted by imbedded pathogen (10). Subsequently, 15% of affected children develop HUS characterized by microangiopathic lesions in the kidneys induced by Stx in circulating blood (4). Stx-induced thrombotic microangiopathy consists of swelling of endothelial cells and their detachment from the basement membrane associated with the leukocyte infiltration and formation of platelet thrombi that occlude the microcirculation of the intestines, kidneys, brain, and other organs (14). In fatal cases involving children, extensive thrombotic microangiopathic lesions are responsible for brain injury due to cerebral edema with hypoxic ischemic changes, medium vessel thrombosis, and cerebral infarction (15). Thus, Stx1 and 2 are associated with the development of microvascular injury in the intestinal submucosa, kidneys, brain, and other organs.

These microvascular compartments, which are particularly vulnerable to Stx action in children, are characterized by relatively high expression of Stx receptor Gb3 (CD77), with the renal microvasculature comprising the main target of the toxic effects of Stx1 and 2 (16). Stx1 and 2 bound to their receptor on endothelial cells trigger a cascade of signaling events which culminate in capturing leukocytes (17–19) and platelets (20, 21), thereby contributing to the development of microangiopathic lesions in the kidney during a full-blown HUS. Some of Stx–induced signaling pathways do not involve inhibition of protein synthesis by a subunit N-glycosidase activity (22), but may induce expression of genes encoding for adhesion molecules and chemokines (17–19). Ultrastructural studies of kidney specimens from children with D/HUS demonstrated infiltrating mononuclear and polymorphonuclear leukocytes within the glomeruli, along the zone of microvascular injury (23, 24).

Fractalkine (FKN; CX3CL1), a member of the CX3 family of chemokines, displays a unique role in the initiation and progression of intimate contacts of inflammatory cells with endothelium. It stands out from other chemokines because of its structure spanning the membrane of endothelial cells: an extracellular chemokine domain anchored to the cell surface through an extended mucine-like stalk fused to a transmembrane helix and an intracellular domain (25, 26). Its expression on the surface of endothelial cells and neurons can be up-regulated by proinflammatory agonists including LPS, IL-1, and TNF-α (25, 27, 28). Moreover, cell-free FKN can be produced by proteolytic cleavage of its extracellular segment by TNF-converting enzyme, a member of a family of proteins containing a disintegrin and metalloprotease domain (ADAM proteins) (29). The soluble form of FKN acts as a potent chemoattractant for monocytes, T lymphocytes, and NK cells that express the FKN receptor CX3CR1 (26, 30). The membrane-bound FKN plays a unique role as an adhesion molecule (counterreceptor) (31–33). By interacting with CX3CR1 on leukocytes, it directly mediates rapid and firm adhesion of leukocytes, independently of their integrin activation, a feature distinct from the other chemokines. Notably, video microscopy experiments showed that CX3CR1-expressing leukocytes were tethered more rapidly to immobilized FKN than to VCAM-1 (33).

The involvement of FKN pathway in the pathogenesis of D/HUS has been suggested by recent findings of a selective depletion of circulating mononuclear cells expressing CX3CR1 during the acute phase of the disease, in correlation with the severity of renal failure, and the presence of CX3CR1-positive monocytes and NK cells in the glomeruli of these patients (34). These intriguing observations raise questions about the mechanism of depletion of circulating monocytes that express FKN receptor, CX3CR1, during the acute phase of D/HUS. Does Stx2 induce FKN? If so, does FKN promote leukocyte adhesion to endothelial cells, especially those that are present in glomeruli thereby contributing to the development of D/HUS caused by enterohemorrhagic *E. coli* O157:H7? In the present study, we addressed these questions by investigating in cultured cells: 1) Stx2 induction of FKN in HUVECs and glomerular endothelial cells; 2) the functional role of FKN in Stx2-induced leukocyte adhesion to the endothelium under physiologic flow; and 3) the intracellular signaling pathways involved in FKN expression and FKN-mediated leukocyte adhesion in response to Stx2. Moreover, to validate these findings, we extended our study to the in vivo analysis of FKN expression and accumulation of CX3CR1-expressing cells in glomeruli of mice treated with either Stx2 alone or in combination with LPS. It has been previously recognized that these two virulence factors of enterohemorrhagic *E. coli* are required to elicit HUS-like lesions in relevant animal models (35–37). These combined findings demonstrate a key role for FKN in the development of glomerular endothelial cell injury induced by Stx2.

Materials and Methods

Endothelial cell culture and assays

HUVECs were cultured in medium 199 supplemented with 10% newborn calf serum and 10% human serum, antibiotics, heparin, and endothelial cell growth factor as previously described (19). Glomerular endothelial cells (GEC) were isolated from kidneys that were not suitable for transplantation by collagenase digestion (38) and cultured using the same medium as for HUVEC (19). FKN mRNA expression was evaluated in HUVEC exposed to medium 199 plus 10% FCS (HyClone, endotoxin free) in the presence of Stx2 (50 pM and 1 nM; Toxin Technology) for 3, 6, 15, and 24 h. GEC were incubated with Stx2 (50 pM) for 3 h. To exclude any possible effect of LPS contamination of Stx2 preparation, additional experiments were conducted in HUVEC incubated with Stx2 or LPS (1 ng/ml, *E. coli* serotype 026:B6; Sigma-Aldrich) plus the LPS inhibitor polymyxin B (10 μg/ml; Sigma-Aldrich) (39). Furthermore, to establish that FKN-inducing activity is solely due to Stx2, its commercial preparation was additionally processed in selected experiments on the LPS detoxification column (De-toxi-Gel Endotoxin Removing Columns; Pierce Biotechnology). The additional step yielded purified Stx2, which was determined to have <0.06 endotoxin units per milliliter by the Limulus amebocyte lysate assay (Cambrex Biogene). TNF-α (100 U/ml; Knoll) was used as a standard inducer of FKN gene expression.

FKN protein was evaluated by Western blot in HUVEC treated 6 h with Stx2 (50 pM). For adhesion experiments under physiologic flow, confluent HUVEC or GEC grown on plastic coverslips (Thermanox; Nunc) coated with 0.2% bovine gelatin (Sigma-Aldrich) were incubated 6 h with Stx2 (50 pM). Then, a leukocyte adhesion assay was performed using a parallel plate flow chamber (40). In selected experiments, HUVECs were exposed for 6 h to Stx2 plus polymyxin B, or to purified Stx2 alone or in combination with LPS (1 ng/ml, Toxin Technology) or irrelevant mouse IgG (20 μg/ml; Sigma-Aldrich). The role of FKN was evaluated by incubating Stx2-treated HUVEC or GEC with function-blocking anti-human FKN Ab (10 μg/ml; R&D Systems) or with isotype-matched IgG (normal goat IgG) as control, for 20 min before the adhesion assay. In other experiments, leukocyte suspension was incubated with polyclonal rabbit anti-rat CX3CR1 Ab (15 μg/ml; Torrey Pines Biolabs) or with isotype-matched IgG (normal rabbit IgG).

Phosphorylation of Src-family protein tyrosine kinases (PTK) and p38 MAPK was assessed by Western blot in HUVEC treated with 50 pM Stx2 for 15, 30, 60, 180 min. FKN mRNA expression and leukocyte adhesion were studied in HUVEC incubated with the Src-family PTK inhibitor PP1 (1 μM) (41) and the p38 inhibitor SB-202190 (20 μM) (39, 42) 1 h before and during Stx2 stimulation (50 pM, 6 h).

NF-κB and AP-1 activity was determined in nuclear extracts from HUVEC exposed for 1 h to Stx2 (50 pM and 1 nM). Then, the effect of NF-κB and AP-1 inhibition on Stx2-induced FKN gene expression and on leukocyte adhesion was evaluated. Cell-penetrating SN50 peptide which contains the nuclear localization sequence of NF-κB p50, and SM peptide...
which contains mutated nuclear localization sequence, were synthesized, purified, filter-sterilized, and analyzed as previously described (43, 44). Peptides (30 µM) were applied to HUVEC, 30 min before exposure to Stx2 (50 µM, 6 h), to inhibit nuclear translocation of NF-κB (43, 45). In separate experiments (see below), HUVEC were transfected for 3 h with recombinant adenovirus coding for IκBα, the natural inhibitor of NF-κB (46), before the challenge with Stx2. Finally, HUVEC were transfected with double-stranded phosphorothioate oligodeoxynucleotide (ODN) (47) that influence AP-1-binding capacity by a competitive reaction. dsODN were prepared as described (39). HUVEC were transfected for 2 h with 200 nM AP-1 decoy ODN or mutated control ODN in serum-free medium, using Oligofectamine Reagent (Invitrogen) before addition of 50 pM Stx2 (6 h).

Leukocyte adhesion assay under physiologic flow
For adhesion experiments, a parallel-plate flow chamber and a perfusion system were used as previously described (19, 40). Endothelial cells were perfused with leukocyte suspension (107 cells/ml) at the shear stress of 1.5 dynes/cm2 that mimics postcapillary venule circulation (48). This value may be also present within the glomerular microcirculation. In a three-dimensional model of glomerular capillary (49), we estimated that a large fraction of the glomerular capillary segment was exposed to a wall shear stress value <5 dynes/cm2.

Quantitative real-time PCR
Total RNA was extracted from HUVEC and GEC using the TRizol method, and then was treated with RNaSe-free DNase (Promega). Reverse transcription and quantitative real-time PCR was performed as described (39). The 2-ΔΔCt method was used to calculate relative changes in expression of target gene in respect to a calibrator sample serving as reference, where Ct is the cycle threshold. The following specific primers were used:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 18S</td>
<td>5'-CCAAGATGATTGCGCCTTT-3'</td>
</tr>
<tr>
<td>Reverse 18S</td>
<td>5'-GCGGTGACTCAGCCGGAA-3'</td>
</tr>
</tbody>
</table>

The following primers were used for CX3CR1 mRNA in response to Stx2 and LPS stimulation.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward CX3CR1</td>
<td>5'-GCCCTACCATCACCAAGGAA-3'</td>
</tr>
<tr>
<td>Reverse CX3CR1</td>
<td>5'-CCAAGGTGGGATATTGCCG-3'</td>
</tr>
</tbody>
</table>

Western blot analysis
For FKN detection, HUVEC were lysed and processed as described (50). Protein lysates (30 µg) were separated on 8% polyacrylamide gel by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight with goat anti-human FKN Ab (1/5000; R&D Systems) and then for 1 h with HRP-conjugated rabbit anti-goat IgG (1/200,000). Abs were diluted in PBS plus 1% BSA and 0.05% Tween 20. As positive control, 40 ng of recombinant human FKN (R&D Systems) were used. Protein bands were detected by Supersignal chemiluminescent substrate (Pierce).

For Src family PTKs and p38 MAPK protein detection, HUVEC were lysed and processed as described (30). Proteins (30 µg) were separated on 10% polyacrylamide gel by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with the following primary Abs: rabbit polyclonal anti-phospho-Src family (Tyr416, 1/1000) or mouse monoclonal IgM anti-phospho-p38 (1/300) in PBS plus 1% BSA, sections were incubated overnight with goat anti-rabbit Cy3 conjugated (60 µg/ml; Jackson ImmunoResearch Laboratories). Rabbit anti-vWF Ab (1/100; DakoCytomation) was incubated for 1 h at room temperature, followed by goat anti-rabbit Cy3 conjugated (60 µg/ml; Jackson ImmunoResearch Laboratories).

Statistical analysis
Results are expressed as mean ± SE. Data were analyzed by ANOVA followed by Tukey’s test for multiple comparison, or by the nonparametric Kruskal-Wallis test. Significance level was defined as p < 0.05.

Results

Stx2 induces FKN expression in HUVEC
We tested our hypothesis that Stx2 induces expression of endothelial chemokine FKN by measuring its mRNA transcripts and protein level in HUVEC at different time points. As documented in Fig. 1A, Stx2 at 50 pM caused an early induction of FKN mRNA, as indicated by 8.5-fold increase of transcripts level over the control at 3 h, followed by a gradual decline at 6, 15, and 24 h (6.8-, 2.3-, and 1.2-fold, respectively). The stimulatory effect of Stx2 was dose-dependent (results not shown). We verified that the increased endothelial FKN expression in response to Stx2 was not due to LPS contamination. In fact, the LPS inhibitor, polymyxin B (10 µg/ml), did not reduce FKN transcript level induced by Stx2, while it abrogated FKN mRNA expression in HUVEC incubated with LPS (data not shown). Moreover, in selected experiments (n = 3) the exposure of HUVEC to purified Stx2 (50 pM, 6 h) caused a 5.6- ± 1.2-fold increase of FKN mRNA over control. This effect was comparable to the original Stx2 preparation, which was not processed on LPS detoxification column before testing in the same experimental setting (6.1 ± 1.8-fold increase). TNF-α (100 U/ml), a known stimulus for endothelial FKN expression, caused a 9.6- ± 1.8-fold increase, respectively.
FIGURE 1. Stx2 induces FKN expression in HUVEC. A, FKN mRNA was assessed by quantitative PCR in HUVEC incubated for different times with Stx2 (50 pM). The results shown are mean ± SE of six independent experiments and are expressed as fold increase over control (considered as 1); *, p < 0.05; **, p < 0.01 vs control. B, Western blot analysis of FKN protein in cell lysates from HUVEC exposed for 6 h to Stx2 (50 pM and 1 nM). FKN electrophoretic mobility corresponded to the apparent molecular size of 95 kDa. Recombinant human FKN (rFKN, 40 ng) was used as positive control. Picture is representative of three experiments. Stx2 induces leukocyte adhesion to HUVEC under physiologic flow (1.5 dynes/cm²). C, HUVEC were treated for 6 h with medium alone or Stx2 (50 pM) and exposed for 20 min to anti-FKN-neutralizing Ab, or to isotype-matched IgG (irrelevant). D, Before the adhesion assay, leukocyte suspension was incubated with anti-CX3CR1 Ab or with isotype-matched IgG (irrelevant). Data are mean ± SE (n = 3 experiments). **, p < 0.01 vs control; ○, p < 0.01 vs Stx2; #, p < 0.05; ##, p < 0.01 vs corresponding irrelevant Ab.

0.4-fold increase of FKN mRNA levels over control. Induction of FKN mRNA by Stx2 resulted in increased synthesis of FKN protein as shown by Western blot analysis performed in lysates of HUVEC treated with Stx2 for 6 h (Fig. 1B). These results indicate that Stx2 induces time-dependent expression of FKN mRNA and protein in human endothelial cells.

FKN promotes Stx2-induced leukocyte adhesion by HUVEC under physiologic flow

We investigated the functional role of endothelial FKN expressed in response to Stx2 in a leukocyte adhesion assay under physiologic flow conditions. As shown in Fig. 1C, a significant increase in the number of captured leukocytes as compared with unstimulated control was observed following 6 h exposure of HUVEC to Stx2 (128 ± 8 vs 25 ± 4 leukocytes/mm², p < 0.01). This adhesion was dependent on interaction of endothelial FKN with its cognate leukocyte receptor CX3CR1. Hence, the treatment of HUVEC with anti-FKN-neutralizing Ab significantly (p < 0.01) decreased leukocyte adhesion (73 ± 4 leukocytes/mm²) in respect to cells exposed to Stx2 alone or Stx2 plus the isotype-matched IgG (irrelevant) Ab. A more profound 4-fold reduction of leukocyte adhesion to Stx2-challenged HUVEC was observed when the FKN receptor, CX3CR1, expressed on leukocytes was functionally blocked by its cognate Ab, as compared with leukocytes incubated with the irrelevant Ab (Fig. 1D).

We ruled out the effect of LPS, which potentially can contaminate Stx2 preparations in the following experiments. First, by adding the LPS inhibitor, polymyxin B (10 μg/ml), to culture medium we demonstrated that the stimulatory effect of Stx2 on leukocyte adhesion to HUVEC remained unchanged while the known stimulatory effect of LPS on HUVEC was suppressed by polymyxin B (Stx2 with polymyxin B: 106 ± 2 vs Stx2 without polymyxin B: 117 ± 3 leukocytes/mm²; LPS with polymyxin B: 59 ± 4 vs LPS without polymyxin B: 177 ± 3 leukocytes/mm², p < 0.01). Second, we found that HUVEC stimulation with purified Stx2 induced the adhesion of a number of leukocytes similar to that observed after endothelial cell challenge with the initial Stx2 preparation, which was not processed on LPS detoxification column before testing (Table I). Moreover, anti-Stx2 mAb abrogated the stimulatory effect of both Stx2 preparations (Table I). Cumulatively, these results indicate that Stx2-evoked endothelial FKN promotes adhesion of CX3CR1-positive leukocytes to HUVEC and that this leukocyte-endothelial cell adhesion is not due to potential contamination of Stx2 with LPS.

GEC respond to Stx2 by expressing FKN and capturing leukocytes

We expanded our analysis of FKN induction by Stx2 to GEC, which constitute the primary target of Stx2 (16, 51). Treatment of GEC with Stx2 for 3 h (50 pM) significantly increased FKN mRNA expression (4-fold increase over control, p < 0.05) (Fig. 2A). As in HUVEC, Stx2 promoted leukocyte adhesion to GEC under physiologic flow (100 ± 6 vs 21 ± 1 leukocytes/mm² in control, p < 0.01) (Fig. 2B). Treatment of GEC with anti-FKN Ab significantly (p < 0.01) reduced the number of adherent leukocytes (52 ± 2 leukocytes/mm²) (Fig. 2B), thereby indicating the potential role of FKN in the inflammatory response triggered by Stx2 in the glomerular circulation.

Table I. Effect of purified Stx2 on leukocyte adhesion to endothelial cells under physiologic flow

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adherent Leukocytes/mm²</th>
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<tbody>
<tr>
<td>Control</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Stx2 50 pM</td>
<td>99 ± 3*</td>
</tr>
<tr>
<td>Purified Stx2 50 pM</td>
<td>108 ± 9*</td>
</tr>
<tr>
<td>Stx2 50 pM + anti-Stx2 Ab</td>
<td>37 ± 4*</td>
</tr>
<tr>
<td>Purified Stx2 50 pM + anti-Stx2 Ab</td>
<td>35 ± 3*</td>
</tr>
<tr>
<td>Purified Stx2 50 pM + irrelevant Ab</td>
<td>110 ± 4*</td>
</tr>
</tbody>
</table>

* Purified Stx2 was obtained by processing Stx2 commercial preparation on LPS detoxification column. HUVEC were exposed to both Stx2 preparations in the absence or presence of anti-Stx2 antibody (11E10) or irrelevant antibody. Data are expressed as mean ± SE (n = 4 experiments).

* Value of p < 0.01 vs control, and p < 0.01 vs corresponding Stx2 preparation.
Intracellular signals involved in Stx2-induced endothelial FKN activation and leukocyte adhesion

Stx binding to its cognate receptor glycosphingolipid Gb3/CD77 activates Src family PTKs and p38 MAPK in human renal tubular-derived ACHN cells (41, 52). We analyzed the role of these signal transducers in FKN expression in HUVEC challenged with Stx2. As shown in Fig. 3A, Stx2 induced Src PTK phosphorylation within 15 min, which further increased at 30 min and persisted up to 180 min. Reprobing the blot with an Ab against the non-phos-Src family revealed that overall expression of Src PTKs did not change. A rapid and persistent phosphorylation of p38 MAPK was also observed in response to Stx2 challenge. In turn, inhibitors of Src PTKs and p38 MAPK, PP1, and SB-202190, respectively, reduced the expression of FKN mRNA in response to Stx2, with statistically significant inhibition by SB-202190 (Fig. 3B).

This analysis of signal transducers was extended to leukocyte adhesion evoked by Stx2. HUVEC were stimulated with Stx2 in the presence or absence of PP1 or SB-202190, before leukocyte perfusion. As shown in Fig. 3C, inhibition of Src family PTKs or p38 MAPK significantly (p < 0.01) reduced the number of captured leukocytes on Stx2-stimulated HUVEC under physiologic flow. Thus, Stx2 evokes stimulus-response coupling that involves Stx receptor-proximal signal transducers, Src PTKs and p38 MAPK, which participate in signaling pathways that result in FKN expression and leukocyte capture.

NF-κB, AP-1, and other stress-responsive transcription factors mediate Stx2-induced expression of endothelial FKN and its leukocyte capturing function

The gene that encodes FKN is under the control of proinflammatory stress-responsive transcription factors (SRTFs), including NF-κB and AP-1 (53–55). Following their import from the cytoplasm to the nuclear compartment, SRTFs act in concert to stimulate transcription of multiple genes encoding cytokines, chemokines, and other mediators of inflammation (56). The induction of these genes by SRTFs requires their translocation from cytoplasm to the nucleus by the nuclear import machinery. We reasoned that effective targeting of this machinery might suppress Stx2-induced FKN expression. To examine the role of NF-κB and other SRTFs in Stx2-induced FKN expression, we first analyzed nuclear translocation of NF-κB. Nuclear extracts from HUVEC were assayed for NF-κB DNA-binding activity by EMSA. Consistent with our previous study (19), unstimulated cells displayed low basal levels of NF-κB-binding activity, whereas a 1-h incubation with Stx2
elicited a dose-dependent rise in NF-κB activity (Fig. 4A). Densitometric analysis of NF-κB complexes showed 2.7- and 6.5-fold increase over control (expressed as 1) after stimulation with Stx2 at 50 pM and 1 nM, respectively. The specificity of the binding reaction was confirmed in competition experiments by using an excess of unlabeled (cold) NF-κB oligonucleotide to inhibit binding. 

Subsequently, we tested whether inhibition of nuclear import of NF-κB and other SRTFs would interfere with Stx2-induced expression of FKN. The cell-penetrating peptide inhibitor of nuclear import, SN50, contains a nuclear localization sequence derived from NF-κB1 (as a cargo) attached covalently to the membrane-translocating motif based on signal sequence hydrophobic region of fibroblast growth factor 4 (43). Upon reaching the cytoplasm of HUVEC and other cell types through the receptor/transporter-independent mechanism, SN50 targets nuclear import adapter Rch1 (importin/karyopherin α1) thereby preventing inducible translocation of NF-κB and other SRTFs (AP-1, NFAT, and STAT1) to the nucleus (44, 57). HUVEC were preincubated with SN50 (30 μM), or control cell-penetrating peptide SM (30 μM) (with mutated cargo) for 30 min and then challenged with Stx2 (50 pM, 6 h). 

FKN gene expression was almost completely suppressed when SN50 was added to Stx2-treated HUVEC, whereas the SM peptide was ineffective (Fig. 4B). The lack of Stx2-induced FKN mRNA transcripts in SN50-treated HUVEC was coupled to their inability to capture leukocytes. As shown in Fig. 4C, leukocyte adhesion induced by Stx2 was significantly (p < 0.05) reduced in HUVEC treated with SN50 as compared with Stx2-stimulated cells that were treated with control cell-penetrating SM peptide. The SN50 peptide-directed inhibition of FKN expression was compared with the effect of a recombinant adenovirus encoding the NF-κB-specific inhibitor IκBα (46). The inhibitory IκBα protein sequesters the p65-containing NF-κB complex in the cytoplasm by masking its nuclear localization sequence. Overexpression of IκBα resulted in a significant (p < 0.01) reduction of FKN mRNA as compared with Stx2-treated cells infected with the control adenovirus (Ad.null) (Fig. 4D). Thus, adenoviral delivery of IκBα significantly decreased FKN mRNA levels in endothelial cells.

AP-1 along with NF-κB and other SRTFs is involved in combinatorial regulation of FKN gene transcription (55). We determined that AP-1 DNA-binding activity was increased in HUVEC exposed to Stx2 (Fig. 5A). Densitometric analysis showed a 2.7- and 4-fold increase over control (expressed as 1) when cells were incubated with Stx2 at 50 pM and 1 nM, respectively. Competition assay with an excess of unlabeled probe completely abolished the complexes formed with labeled probe, indicating complex specificity. To assess whether Stx2-induced FKN gene transcription involved AP-1, HUVEC were transfected, before exposure to Stx2, with synthetic double-stranded phosphorothioate decoy ODN containing the specific consensus sequence for AP-1. The decoy ODN competes with the promoter region of AP-1-regulated genes for binding of the transcription factor, thereby suppressing gene expression (47). As shown in Fig. 5B, the expression of FKN gene was significantly (p < 0.05) reduced in cells transfected with AP-1 decoy ODN as compared with the control ODN. Consistent with reduced FKN gene expression, Stx-induced leukocyte capture was significantly (p < 0.01) decreased in cells transfected with AP-1 decoy ODN as compared with control ODN (Fig. 5C). Thus, AP-1 together with NF-κB and other SRTFs regulates Stx2-induced FKN gene expression. By blocking nuclear import of NF-κB, AP-1, and other SRTFs or by inhibiting transcriptional activity of AP-1, we demonstrated that suppression of FKN expression in response to Stx2 prevents potentially harmful adhesion of CX3CR1-positive leukocytes to FKN-expressing endothelial cells.

Glomerular FKN expression in a murine model of HUS

Recent studies have established that two virulence factors of enterohemorrhagic E. coli, Stx and LPS, are required to elicit cardinal signs of clinical HUS in mice (36, 37). Therefore, we extended our analysis to this relevant murine model of human HUS. We examined FKN and CX3CR1 expression in the glomeruli of C57BL/6 mice i.p. injected with either Stx2 and LPS alone or their combination. Mice were also evaluated for blood platelet count,
renal function, and glomerular ultrastructural changes at 24 h. As shown in Fig. 6A, platelet count remained within the normal range in mice administered Stx2 alone. LPS induced a 43% reduction in platelet count in respect to control mice. A more severe thrombocytopenia was observed in mice given the combination of Stx2 plus LPS, with a 74% reduction of platelet count being achieved. Likewise, as shown in Fig. 6B, renal function was significantly impaired only after coinjection of Stx2 and LPS, as indicated by abnormally high serum BUN levels (p < 0.01 vs saline, Stx2 or LPS). Consistent with these results, combined administration of Stx2 and LPS caused glomerular ultrastructural changes characteristic of focal endothelial swelling, platelet clumps in the capillary loops, marked infiltration of polymorphonuclear cells (PMNs) (Fig. 6C). Mice given each agent alone displayed mild endothelial swelling and few PMNs within the glomeruli (data not shown).

Importantly, the expression of FKN was virtually absent in glomeruli of control mice that received saline (0.03 ± 0.02 FKN-positive cells/glomerular section) based on immunohistochemical analysis (Fig. 7A). In striking contrast, FKN was detected in mice

FIGURE 5. AP-1 regulates expression of FKN mRNA and leukocyte adhesion to HUVEC in response to Stx2. A, Left, AP-1 DNA-binding activity in nuclear extracts from HUVEC exposed for 1 h to medium alone or Stx2 (50 pM and 1 nM). The specificity of the binding reaction was assessed by addition of an excess unlabeled (cold) nucleotide. Picture is representative of three independent experiments. Right, Densitometric analysis of autoradiographic signals of AP-1. Data are mean ± SE (n = 3); *, p < 0.05 vs control. B, FKN mRNA expression in HUVEC transfected for 2 h with AP-1 decoy ODN or control ODN before the exposure to Stx2 (50 pM; 6 h). The results are mean ± SE of five independent experiments. *, p < 0.05 vs control, ○, p < 0.05 vs Stx2; #, p < 0.05 vs control ODN + Stx2. C, Leukocyte adhesion in HUVEC transfected with AP-1 decoy ODN or control ODN before the incubation with Stx2. Data are mean ± SE (n = 5 experiments). ***, p < 0.01 vs control; ○○, p < 0.01 vs Stx2; ###, p < 0.01 vs control ODN + Stx2.

FIGURE 6. Blood platelet count (A) and serum BUN levels (B) measured 24 h after i.p. injection of saline (control), Stx2 (200 ng), LPS (75 μg), or Stx2 plus LPS in C57BL/6 mice (n = 5 each group). ○, p < 0.01 vs control and Stx2; *, p < 0.01 vs control and each agent alone. C, Electron micrographs of glomeruli from C57BL/6 mice given saline (control) or Stx2 plus LPS. Focal endothelial swelling (arrows), platelet clumps in the capillary loops, PMN were evident after coinjection of Stx2 plus LPS. P, platelets. Magnification, ×5600.

FIGURE 7. FKN expression and accumulation of CX3CR1-positive cells within the glomeruli of mice treated with Stx2 alone or in combination with LPS. A, Immunohistochemical staining of FKN in kidney sections of mice i.p. injected with saline (control), Stx2 (200 ng), Stx2 (200 ng) plus LPS (75 μg) at 24 h. Magnification, ×1000. B, Section of kidney from a Stx2 + LPS-treated mouse labeled for FKN (green) and vWF (red). Yellow color in the merge reflects localization of FKN to glomerular endothelial cells. Magnification, ×1000. C, Immunohistochemistry for CX3CR1 in kidney sections of mice treated with saline (control), Stx2 (200 ng), Stx2 (200 ng) plus LPS (75 μg) at 24 h. Arrows point to CX3CR1-positive PMN. Magnification, ×1000.
treated with Stx2 (0.53 ± 0.10 FKN-positive cells/glomerular section, p < 0.01), and to a lesser extent with LPS alone (0.31 ± 0.08 FKN-positive cells/glomerular section, p < 0.05). Combined administration of Stx2 and LPS further increased FKN staining (2.27 ± 0.54 FKN-positive cells/glomerular section, p < 0.01 vs saline and each agent alone). FKN was detected in glomerular endothelial cells, as shown by dual labeling with anti-vWF Ab (Fig. 7B). Significantly, increased FKN expression within glomeruli of infected mice was associated with accumulation of CX3CR1-expressing cells, identified as PMNs by typical morphology (Fig. 7C). Thus, these experimental results provide an important pathogenetic link for human HUS.

Discussion

We demonstrated that Stx2, a virulence factor of E. coli O157:H7 evokes expression of chemokine FKN, which mediates a capture of CX3CR1-positive leukocytes by human endothelial cells under physiologic flow. Our analysis of the mechanism of Stx2-induced expression and leukocyte capturing function of FKN provides a missing link to clinical observations of children with D+HUS who displayed a striking decline of CX3CR1-positive leukocytes in blood and their reciprocal accumulation in damaged kidneys (34). Endothelial injury by noxious Stx signaling constitutes a cardinal feature of microangiopathic lesions in D+HUS (10). Therefore, it cannot escape our attention that FKN preponderance in the kidneys and brain along with Stx glycolipid receptor Gb3/CD77 parallels the predominance of microangiopathic lesions in these two organs in fatal cases of D+HUS (1, 3, 15). By establishing a pathogenetic link between Stx, endothelial FKN, and CX3CR1-positive leukocytes, we offer a new plank for better understanding of D+HUS. Moreover, by addressing the mechanism of FKN induction by Stx2, we provide a launch pad for the development of new therapeutic approaches to the leading cause of acute renal failure in children caused by enterohemorrhagic E. coli O157:H7. The management of this potentially devastating syndrome is hampered by the potentially harmful effect of currently available antimicrobial agents (4).

The unique tripartite, membrane-spanning structure of endothelial FKN constitutes the basis for CX3CR1-positive leukocyte capture, firm adhesion, and activation (31). Our findings, based on using FKN- and CX3CR1-neutralizing Abs, document that Stx2-induced adhesion of leukocytes to endothelial cells is dependent on FKN recognition and binding of CX3CR1-positive leukocytes. That activation of the FKN pathway may be relevant to the inflammatory process occurring in the glomeruli during D+HUS is suggested by the finding that Stx2 caused expression of FKN mRNA in human glomerular endothelial cells. Moreover, leukocyte adhesion to these cells, in response to Stx2 exposure, was substantially reduced after treatment with anti-FKN Abs. Importantly, we validated in vivo our findings in cultured human and glomerular endothelial cells by demonstrating enhanced FKN expression in glomerular endothelial cells of mice challenged with Stx2 and, to a greater extent, with the combination of Stx2 and LPS. As a functional consequence of the increased FKN expression, CX3CR1-positive cells accumulated within the glomeruli of infected mice. The role of E. coli LPS has been recognized in the molecular pathogenesis of D+HUS. Endotoxemia as documented by the elevated level of LPS in blood (58, 59) or Abs to LPS have been demonstrated in patients with D+HUS (60). Colonic vascular damage by enterohemorrhagic E. coli may facilitate the entry of LPS and other bacterial products to the circulation, thereby promoting an inflammatory response that contributes to the pathogenesis of renal injury. In a baboon model of HUS, LPS augmented Stx toxicity by up-regulating renal expression of the Gb3 receptor (61). Consistent with these findings in nonhuman primates, both Stx2 and LPS were required to elicit a HUS-like response in mice (35–37). Notably, in agreement with previous studies (62, 63), infiltration of neutrophils, in the face of rare macrophages, was observed in glomeruli of mice coinjected with Stx2 and LPS. Moreover, our findings demonstrate that endothelial FKN by acting as a cell adhesion molecule promotes the capture of CX3CR1-positive leukocytes to Stx-activated microvasculature in the kidney. Thus, our experiments highlight FKN expression as a key rate-limiting step in capturing neutrophils by GEC. This pathogenetic process may be amenable to therapeutic intervention in children with D+HUS. Of note, a recently identified mutant form of the FKN receptor CX3CR1, termed CX3CR1-M280, is defective in mediating adhesive and chemotactic activity. This mutated form of CX3CR1 is linked to lower risk of atherosclerosis, acute coronary events, and coronary artery endothelial cell dysfunction (64–66). It is plausible that a protective effect of CX3CR1-M280 in inflammation-mediated vascular injury could allow some of the children that bear CX3CR1-M280 and became infected with E. coli O157:H7 escape from a full-blown D+HUS.

Induction of endothelial FKN by proinflammatory agonists depends on signaling pathways involving several signal transducers such as the Src PTK upstream regulator of p38 MAPK (41, 55, 67). Inhibition of both Src PTK and p38 MAPK reduced Stx2-induced leukocyte adhesion on HUVEC, albeit incompletely. The redundancy of signal transducers accounts most likely for this partial inhibition. However, cell-penetrating peptide SN50 simultaneously inhibits the nuclear import of multiple SRTFs, including NF-κB, AP-1, NFAT, and STAT1 (44). Because these SRTFs afford combinatorial induction of genes that encode several proinflammatory cytokines and chemokines, the nuclear import blockade provided more complete suppression of Stx2-induced endothelial FKN expression and its leukocyte-capturing function. Based on the evidence that the transcriptional activity of NF-κB is regulated by src PTK and p38 MAPK pathways (68, 69), our data that both inhibitors PP1 and SB-202190 limited FKN mRNA up-regulation in response to Stx2 would imply the involvement of both kinases in NF-κB-dependent FKN gene regulation.

Leukocyte adhesion on cell-bound FKN can be facilitated by recruitment of leukocytes stimulated by chemokines as MCP-1 and IL-8 produced by endothelial cells. Their expression in response to Stx2 is transcriptionally regulated by NF-κB (19). Adenovirus-mediated gene transfer of IκBα down-regulated IL-8 and MCP-1 mRNA and inhibited the adhesion and transmigration of leukocytes induced by Stx2 (19). NF-κB signaling pathway is tightly coupled to TLR4 that transduces LPS-evoked signals (70). The same TLR4-initiated signaling pathway activates p38 MAPK and JNK after bifurcation at the TAK1/TAB2-signaling complex formed on ubiquinated TRAF6. JNK activates transcriptional complex AP1 that regulates, in tandem with NF-κB, expression of proinflammatory cytokines and chemokines. Alternatively, JNK and p38 MAPK are activated by apoptosis signal-regulating kinase 1 (71). Apoptosis signal-regulating kinase 1 responds to inducers of cellular stress that encompass Stx1 and Stx2 due to their capacity for the ribotoxic stress response (72). The p38 MAPK also mediates LPS and TNF-α-enhanced sensitization of HUVEC to Stx2 by increasing the expression of its receptor, Gb3/CD77 (73). Cumulatively, inhibition of NF-κB-, AP-1-, and p38 MAPK-mediated intracellular signaling abrogates direct and indirect cytotoxic effects of Stx2 by reducing expression of its receptor as well as membrane-bound FKN and other (MCP-1 and IL8) chemokines. Thus, endothelial targeting and injury by activated leukocytes is attenuated.
In conclusion, we demonstrated that Stx2-induced expression of FKN plays an essential role in capturing CXCR3-positive leukocytes to GEC and delineated the role of Stx2-induced signaling to the nucleus in FKN expression and function. This new pathogenic network among Stx2-induced endothelial FKN expression and capture of CXCR3-positive inflammatory leukocytes may contribute to the renal microvascular dysfunction and thrombotic microangiopathy that underlie D+HUS due to enterohemorrhagic E. coli O157:H7 infections. Moreover, it highlights potential targets in host cells for therapeutic interruption of nosignaling by Stx of enteropathogenic E. coli O157:H7.

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Disclosures

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