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Shiga Toxin 2 Reduces Complement Inhibitor CD59 Expression on Human Renal Tubular Epithelial and Glomerular Endothelial Cells

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Infections with enterohemorrhagic *Escherichia coli* (EHEC) are a primary cause of hemolytic-uremic syndrome (HUS). Recently, Shiga toxin 2 (Stx2), the major virulence factor of EHEC, was reported to interact with complement, implying that the latter is involved in the pathogenesis of EHEC-induced HUS. The aim of the present study was to investigate the effect of Stx2 on the expression of membrane-bound complement regulators CD46, CD55, and CD59 on proximal tubular epithelial (HK-2) and glomerular endothelial (GEnC) cells derived from human kidney cells that are involved in HUS. Incubation with Stx2 did not influence the amount of CD46 or CD55 on the surface of HK-2 and GEnC cells, as determined by fluorescence-activated cell sorter analysis. In contrast, CD59 was significantly reduced by half on GEnC cells, but the reduction on HK-2 cells was less pronounced. With increasing amounts of Stx2, reduction of CD59 also reached significance in HK-2 cells. Enzyme-linked immunosorbent assay analyses showed that CD59 was not present in the supernatant of Stx2-treated cells, implying that CD59 reduction was not caused by cleavage from the cell surface. In fact, reverse transcription-quantitative PCR analyses showed downregulation of CD59 mRNA as the likely reason for CD59 cell surface reduction. In addition, a significant increase in terminal complement complex deposition on HK-2 cells was observed after treatment with Stx2, as a possible consequence of CD59 downregulation. In summary, Stx2 downregulates CD59 mRNA and protein levels on tubular epithelial and glomerular endothelial cells, and this downregulation likely contributes to complement activation and kidney destruction in EHEC-associated HUS.

Shiga toxins (Stxs) were described to represent the most potent virulence factors of enterohemorrhagic *Escherichia coli* (EHEC) (1). Among the Shiga toxin types, Shiga toxin type 1 (Stx1) and Stx2, the latter was shown to correlate significantly more with severe illness in humans, such as typical hemolytic-uremic syndrome (HUS) (2). HUS is characterized by the triad of hemolytic anemia, thrombocytopenia, and acute renal failure (1). After oral ingestion, EHEC colonizes the intestine and Stxs are translocated into the circulation, allowing them to reach the main target organs responsible for HUS, the kidney and the brain (1, 3). In the target organs, Stxs bind to glycosphingolipids of the globo series, which are abundantly expressed on both glomerular and brain microvascular endothelial cells (4, 5).

Besides the virulence factors of the pathogen, host factors are involved in the development of EHEC-associated HUS, as demonstrated by the fact that only 5 to 15% of patients suffering from EHEC infection progress to develop HUS (6). We have previously shown that complement plays an essential role in the pathogenesis of EHEC-associated HUS (7), and this finding has been corroborated by other studies (8, 9). These reports encouraged Lapeyraque and colleagues to employ the licensed terminal complement C5 inhibitor eculizumab for the treatment of severe EHEC-associated HUS in three 3-year-old children with devastating prognoses (10). Due to its success in these three patients, eculizumab was used to treat more than 300 severe cases in the recent EHEC O104:H4 outbreak in Germany in May 2011 (11). However, data on the outcomes of these patients are still equivocal (12–14). Despite the widespread use of eculizumab, its effectiveness in the therapy and the role of complement in the pathogenesis of EHEC-associated HUS have not been elucidated so far.

The complement system is an important part of innate immunity, and the balance between acceleration and inhibition of complement activation is crucial for the host, determining whether it results in host defense or tissue damage. For the regulation of the complement cascade, the membrane-bound proteins CD46, CD55, and CD59 play an important role (15, 16). CD46 (membrane cofactor protein [MCP]) is a glycoprotein which protects the cell from complement damage by inducing factor I-mediated cleavage of the complement factors C3b and C4b, essential proteins in the complement-activating cascade (17–19). CD55 (decay accelerating factor [DAF]) accelerates the decay of C3 and C5 convertases and therefore downregulates complement to protect cells from self-destruction (16, 20). DAF is anchored to the plasma membrane by a carboxy-terminal glycosylphosphatidylinositol (GPI) linkage. CD59 (protectin) is another GPI-anchored glyco-

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protein that binds to C8 and C9, preventing formation of a lytic lesion by limiting incorporation of C9 into the membrane attack complex (C5b-C9) (21).

The presence of all three membrane-bound complement regulatory proteins (CD46, CD55, and CD59) in kidney cells has been reported, although with discrepancies for DAF in glomerular endothelial and proximal tubular epithelial cells and for CD59 in the latter (22).

The aim of the present study was to evaluate the role of membrane-bound complement regulators in EHEC-associated HUS by investigating the effect of Stx2 on CD46, CD55, and CD59 cell surface expression on renal target cells, using cells of the two immortalized human cell lines HK-2 and GEnC.

MATERIALS AND METHODS

Reagents. Purification of Stx2 was done as described previously (23). Labeling of Stx2 was done by use of an Oyster-488 antibody (Ab) labeling kit (Luminartis, Münster, Germany), and the degree of labeling (DOL) was determined to be 1.36 following the instructions in the manual provided with the kit. This control ensures that the labeling worked properly and, thus, that the amount of unlabeled Stx2 within the sample may be assumed to be negligible. Tetramethylrhodamine methyl ester (TMRM), streptavidin, and 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (NBT) substrate were all purchased from Sigma-Aldrich (St. Louis, MO). A Live/Dead fixable dead cell stain kit was purchased from Live Technologies (Carlsbad, CA). Dulbecco modified Eagle medium (DMEM) and Ham's F-12 cell culture medium and supplements were from PAA Laboratories (Pasching, Austria). EBM-2 medium with a growth supplemental kit was from Lonza (Cologne, Germany). Mouse IgG1-fluorescein isothiocyanate (FITC) (GM4992) and mouse IgG2a-FITC (MG2A01) were used as isotype controls (Invitrogen, Carlsbad, CA). Mouse anti-human CD46, mouse anti-human CD55, mouse anti-human CD59, and mouse anti-human terminal complement complex (TCC) Abs were from Hycult Biotech (Uden, Netherlands). Polyclonal goat anti-mouse FITC-labeled Ab was from Dako (Glostrup, Denmark). The mouse anti-human CD59 Ab used in enzyme-linked immunosorbent assay (ELISA) was a kind gift from Claire L. Harris and B. Paul Morgan.

Cell culture. HK-2 cells (ATCC CRL-2190) were maintained in DMEM and Ham's F-12 medium supplemented with penicillin-streptomycin (1:100), insulin-transferrin-selenium (10 ng/ml), GlutaMAX supplement (2 mM), epidermal growth factor (10 ng/ml), and hydrocortisone (36 ng/ml) at 37°C in 5% CO_2. Conditionally immortalized GEnC cells were cultured in EBM-2 medium using a growth factor kit, as described previously (24).

Time curve of Stx2 uptake and morphological analyses of HK-2 and GEnC cells by confocal microscopy. Cells of both cell lines, HK-2 and GEnC, were cultured in 8-well chambered cover glasses (Nalge Nunc International, Rochester, NY) to 90% confluence. Oyster-488 antibody-labeled Stx2 (20 pg/μl) was added to the cells, and the mixture was incubated for up to 32 h at 37°C. Cells were counterstained with 100 nM TMRM, which is a noncytotoxic fluorescent dye staining active mitochondria.

Confocal laser scanning microscopy was performed on a TCS SP5 microscope from Leica equipped with an HCX PL APO lambda blue ×63.0 water-UV objective (numerical aperture, 1.20). Pictures were processed with LAS AF software from Leica and a microlens-enhanced Nipkow disk-based confocal system (UltraVIEW RS) from PerkinElmer mounted on an IX-70 inverted microscope by Olympus. Images were taken using UltraVIEW RS software from PerkinElmer at a ×60 magnification with oil.

Stx2 binding and penetration into HK-2 and GEnC cells and its impact on CD46, CD55, and CD59 expression and on complement deposition by flow cytometry. Cells of both cell lines, HK-2 and GEnC, were grown in 75-cm^2 filter-top flasks and for TCC determination were grown in 25-cm^2 filter-top flasks (Greiner Bio-One, Kremsmünster, Austria) to 90% confluence of the cell monolayer. Different concentrations of Stx2 (200 pg/μl, 20 pg/μl, 2 pg/μl, 200 fm/μl, 20 fm/μl) were added, and the mixture was incubated for 4 h at 37°C. For binding/penetration assays, labeled Stx2 was used. For determination of TCC deposition, normal human serum (NHS, 10%) was added to the cells together with Stx2 (200 pg/μl, 20 pg/μl) for 4 h at 37°C. Phosphate-buffered saline (PBS) or HEPES buffer with NHS (10%), in the case of TCC determination, served as a negative control. From every incubation group (Stx2 and control), 10^6 cells were frozen for subsequent analyses by reverse transcription-quantitative PCR (RT-qPCR) to determine also the expression levels in all samples that showed a significant reduction in any of the complement regulators within the same cells.

The cells were harvested with trypsin-EDTA from PAA Laboratories. Centrifugation was done at 200 × *g* for 7 min, and the washing solution was PBS. CD46, CD55, and CD59 antibodies were diluted at 1:100, TCC antibody was diluted at 1:250, and anti-mouse IgG-FITC antibody was diluted at 1:50. The antibody incubation time was 30 min each. A Live/Dead fixable dead cell stain kit was used for detection and quantification of dead cells. Measurement of 10,000 events for 150,000 cells per sample was carried out by use of a BD FACSCanto II apparatus.

Isotype controls were used to take the background signals of the secondary antibody into account. Results are given as the ratio of the mean fluorescence intensity (MFI) of the sample and the MFI of the isotype control to avoid biases due to background fluorescence.

Determination of CD59 in supernatant of cells treated with Stx2. Cell culture supernatant from previous fluorescence-activated cell sorter (FACS) experiments was measured for its CD59 contents. Mouse anti-human CD59 (1 μg) was immobilized in 100 μl of coating buffer onto the wells of a microtiter plate. Following blocking with 1% (wt/vol) gelatin and 1% (wt/vol) bovine serum albumin at room temperature for 30 min, 100 μl of cell culture supernatant was added for 1 h at 37°C. A suspension (100 μl) of CD59-positive cells served as a positive control; PBS served as a negative control. After washing, bound CD59 was detected with a mouse anti-CD59 Ab (1:500), which was biotinylated using an AbD Serotec rapid biotin antibody conjugation kit (AbD Serotec, Oxford, United Kingdom). Detection was done with streptavidin (1:1,000) and 5-bromo-4-chloro-3-indolyl phosphate–NBT substrate, and measurement was performed on a Bio-Rad 680 microplate reader.

Impact of Stx2 on the level of expression of complement regulators investigated by RT-qPCR. RNA was isolated from the cells frozen in every FACS experiment with an RNeasy kit purchased from Qiagen (Hilden, Germany). Transcription of mRNA to cDNA was done with reagents purchased from Invitrogen. For RT-qPCR, a QuantiFast SYBR green PCR kit from Qiagen was used. Gene-specific primers were ordered from Metabion (Martinsried, Germany). The β-actin housekeeping gene was used for relative quantification of the qPCR values of the target gene. Primer sequences are shown in Table 1 (25, 26).

RT-qPCR was carried out on a Roche LightCycler (version 2.0) real-time PCR detection system after sample preparation by following the protocol of the Qiagen QuantiFast SYBR green PCR kit. The threshold cycle (C_T) values were calculated by relative quantification in a CFX96 real-time system with a C1000 thermal cycler. Furthermore, C_T values

TABLE 1 Primer sequences of sample (CD59) and housekeeping gene (β-actin) used for qPCR

Primer[a] (reference)	Sequence
β-Actin (25)	
F	5′-GAGCTACGAGCTGCCTGACG-3′
R	5′-GTAGTTTCGTGGATGCCACAGGACT-3′
CD59 (26)	
F	5′-ATGCGTGTCTCATTAC-3′
R	5′-TTCTCTGATAAGGATGTC-3′

[a] F, forward; R, reverse

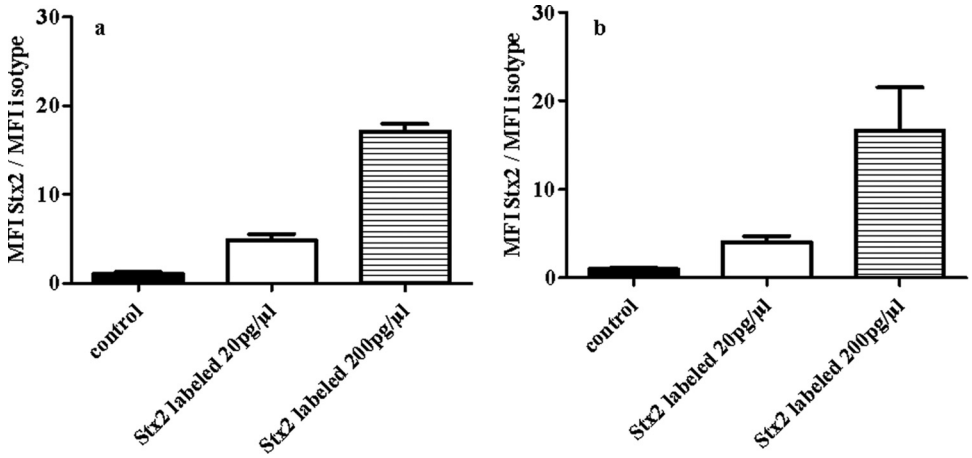

FIG 1 (a and b) Concentration-dependent Stx2 binding and/or penetration into tubular epithelial (HK-2) (a) and glomerular endothelial (GEnC) (b) cells. The cells were incubated with 20 or 200 pg/μl Oyster-488-labeled Stx2 or PBS as a control for 4 h at 37°C. Binding to and penetration into the cells by Stx2 were investigated by FACS analyses. The results are given as the ratio of the MFI of the sample and the MFI of the control. Stx2 binds to and/or penetrates into HK-2 and GEnC cells to a similar extent. With increasing concentrations of Stx2, binding to and penetration into both cell types increased. The results are the means ± SDs of 5 independent experiments.

were analyzed by the $\Delta\Delta C_T$ method in Bio-Rad CFX manager software (version 2.1) from Bio-Rad.

Statistical analyses. The results were analyzed by the use of GraphPad Prism (version 5) software. Student's *t* test was performed to compare the paired means of the two measurement groups. *P* values of <0.05 were considered significant. *P* values of <0.01 were considered highly significant.

RESULTS

Stx2 penetrates into HK-2 and GEnC cells within a few minutes, and apoptotic effects occur after 30 h. To evaluate the optimum incubation time of Stx2 and to exclude apoptosis-related effects (e.g., decrease of membrane standing proteins due to membrane flip-flop effects) that could bias the study, we used confocal laser scanning microscopy to monitor cell viability. The time curve of Stx2 uptake and the morphological changes of the cells caused by Stx2 were comparable for both cell lines. After 60 min, the majority of the added Stx2 was taken up into the cell. Morphological changes demonstrating apoptosis of cells (i.e., rounding of cells) were observed after 30 h. The mitochondrial membrane potential, which is shown by TMRM counterstaining, also decreased after approximately 30 h, pointing toward a loss of mitochondrial activity as a hint toward the beginning of apoptosis.

In addition, microscopic analyses showed differences between different cells of the same population with respect to Stx2 uptake as well as with regard to the effect of Stx on morphological changes, once Stx entered the cell, yielding a heterogeneous picture (data not shown).

The amount of Stx2 binding to and penetrating into HK-2 and GEnC cells is comparable and dose dependent. To compare the amount of Stx2 bound to the cell surface and finally taken up into the cell, both cell lines were incubated with different amounts of labeled Stx2 for 4 h. FACS analyses showed similar amounts of Stx2 on the plasma membrane and/or in HK-2 and GEnC cells. Binding to and/or uptake into both cells increased with increasing amounts of Stx2 (Fig. 1).

Stx2 significantly reduces CD59, but not CD46 and CD55, on HK-2 and GEnC cells. The presence of the membrane-bound complement regulators CD46, CD55, and CD59 and the effect of Stx2 on these were evaluated by flow cytometry (FACS) analyses.

All three membrane-bound complement regulators, CD46, CD55, and CD59, were present on human renal tubular epithelial (HK-2) and glomerular endothelial (GEnC) cells. CD46 and CD55 levels were almost identical in both cell lines. The level of CD59 expression in GEnC cells was approximately twice as high as that in HK-2 cells (Fig. 2a to f).

Incubation with Stx2 did not influence the amount of complement regulators CD46 and CD55 on the surface of HK-2 (Fig. 2a and b) and GEnC (Fig. 2d and e) cells. For CD59, a significant reduction by half was found on GEnC cells, while the reduction on HK-2 cells was not significant at an Stx concentration of 20 pg/μl. With increasing amounts of Stx2 (200 pg/μl), the reduction of CD59 was more prominent and reached significance compared to the baseline expression in HK-2 cells (Fig. 2c and f). For both cell lines, the lowest concentration of Stx2 showing a reduction in CD59 levels was 20 pg/μl. A concentration of 2 pg/μl or lower (200 fg/μl and 20 fg/μl) did not show any effect on CD59 expression for both cell lines (data not shown). Quantification of dead cells showed about 15% dead cells in both the Stx2 and the PBS groups for both cell lines (Fig. 3).

CD59 is not cleaved off from the cell surface by Stx2. To determine whether the reduction of CD59 levels in Stx2-treated HK-2 and GEnC cells was caused by cleavage of the complement receptor from the cell surface, cell supernatant was investigated for the presence of CD59 by ELISA. CD59 was not detected in the supernatant of HK-2 or GEnC cells incubated with Stx2 (data not shown).

Stx2 causes a decrease in CD59 mRNA expression in HK-2 and GEnC cells. To investigate whether the reduction of CD59 on HK-2 and GEnC cells seen by FACS analysis after incubation with Stx2 was caused by downregulation of CD59 mRNA, quantification of mRNA was done by RT-qPCR. The mRNA of the complement regulator CD59 was significantly reduced in HK-2 cells after incubation with Stx2 (200 pg/μl for 4 h 37°C) and was highly significantly reduced by half in GEnC cells after incubation with

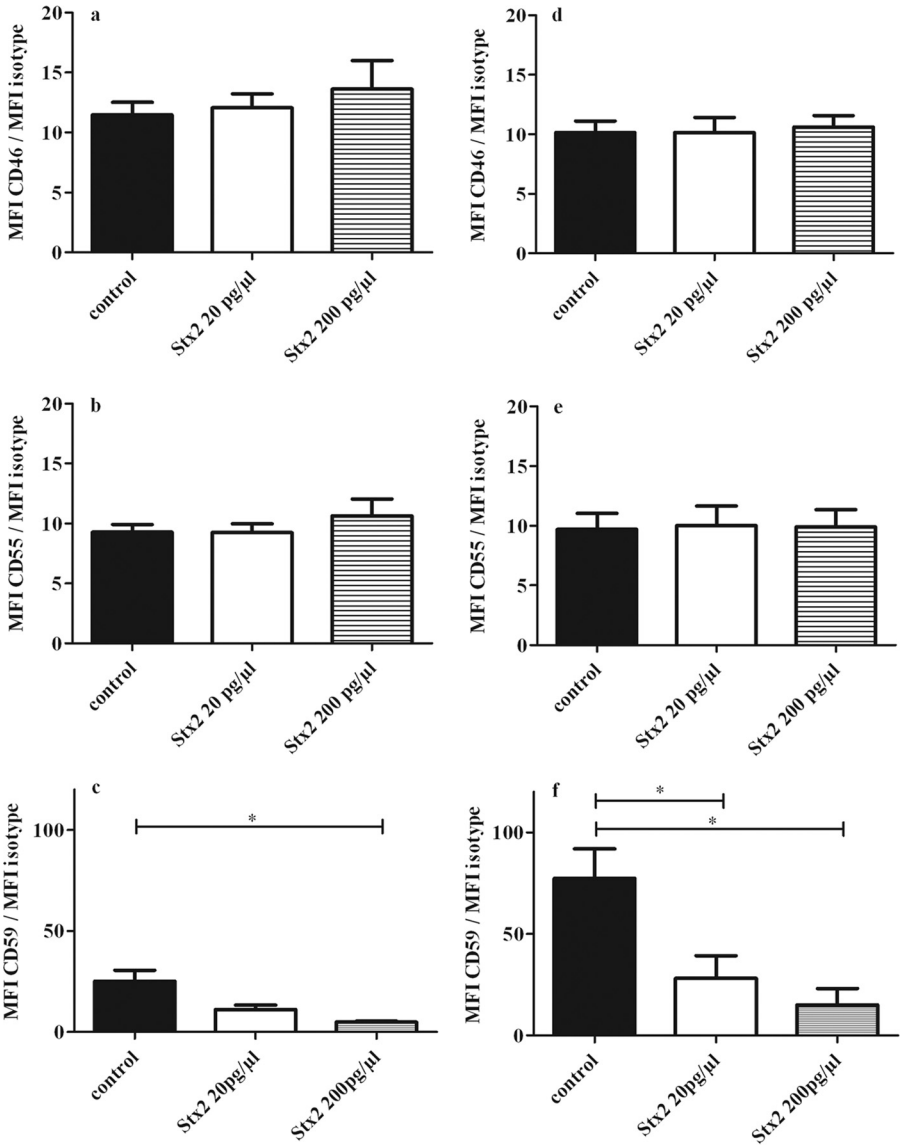

FIG 2 (a to f) Expression of complement regulators CD46, CD55, and CD59 on tubular epithelial (HK-2) and glomerular endothelial (GEnC) cells after Stx2 treatment. Expression of complement control protein CD46 (a, d), CD55 (b, e), or CD59 (c, f) on HK-2 cells (a to c) or GEnC cells (d to f) incubated with Stx2 for 4 h at 37°C is shown. Incubation of cells with PBS served as a control. Expression of complement regulators was detected by FACS analyses using mouse-anti-human CD46, CD55, or CD59 Abs and a polyclonal goat anti-mouse FITC-labeled secondary Ab. The results are given as the ratio of the MFI of the sample and the MFI of the isotype control. Panel c shows a significant reduction of CD59 expression on HK-2 cells after 4 h of treatment with 200 pg/μl Stx2. Panel f demonstrates a significant reduction of CD59 on GEnC cells after treatment with only 20 pg/μl Stx2. The results are the means ± SDs of 5 independent experiments. *, $P < 0.05$.

Stx2 (20 pg/μl for 4 h, 37°C) compared to the levels for the PBS control (Fig. 4a and b). In GEnC cells, the lowest mRNA level was observed at 100 pg/μl, whereas at higher Stx concentrations (e.g., 200 pg/μl), mRNA levels were in the range of those observed after application of very low Stx concentrations (<5 pg/μl), and at a concentration of 500 pg/μl, the mRNA level was comparable to that of the control without Stx (data not shown).

Stx2 increases the deposition of TCC on HK-2 cells. To analyze whether the reduction of CD59 expression by Stx2 results in increased complement activation, deposition of TCC on the cell surface was measured by FACS analyses. For HK-2 cells, a highly significant increase in TCC deposition was observed after treatment with 200 pg/μl Stx2 compared to the amount for the control (HEPES with 10% NHS). Incubation with a lower Stx2 concentration (20 pg/μl) did not show any differences (Fig. 5a). On GEnC cells, a dose-dependent but not significant increase in TCC deposition was detected under the influence of Stx2 (Fig. 5b).

DISCUSSION

Recent data have indicated that complement activation occurs in EHEC-associated HUS: Stx2 has been demonstrated to activate complement in the fluid phase and to bind to factor H (FH), delaying its cofactor activity on Chinese hamster ovary cells (7). Thurman and colleagues have corroborated the involvement of

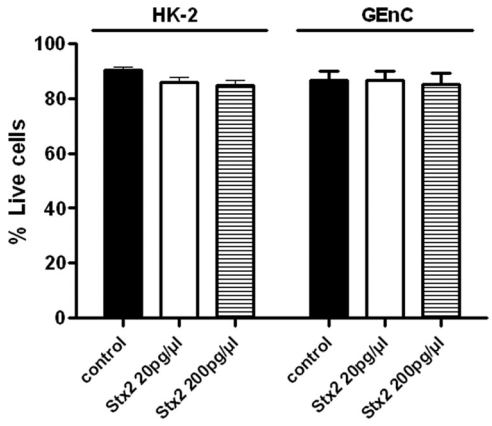

FIG 3 Percentages of living cells after incubation with Stx2. Tubular epithelial (HK-2) and glomerular endothelial (GEnC) cells were incubated with increasing concentrations of Stx2 or with PBS as a control for 4 h. The percentages of living cells were analyzed by flow cytometry. The results are the means ± SDs of 4 independent experiments.

complement by reporting on elevated levels of complement products Bb and C5-C9 in 17 children with EHEC-associated HUS (8). Furthermore, Ståhl and coworkers have found surface-bound C3 on 30% of platelet-monocyte complexes and monocyte microparticles bearing C3 and C9 in HUS patients (9). When incubating blood with Stx2 *in vitro*, they detected platelet-monocyte and platelet-neutrophil complexes with surface-bound C3 and C9. In addition, they have demonstrated that Stx2 triggered the release of C3- and C9-bearing microparticles from platelets and monocytes (9). Likewise, Morigi and coauthors have found by *in vitro* and *in vivo* experiments with a mouse model that Stx induced expression of P-selectin on microvascular endothelial cells, followed by complement activation through the alternative pathway and C3 deposition on the cell surface (27). Moreover, they have suggested that C3a is a key component for further endothelial P-selectin expression, thrombomodulin loss, and thrombus formation (27). Thus, these studies confirm that Stx activates complement in the fluid phase and leads to complement deposition on cells.

In the present study, we show that Stx2 leads to a significant increase in the deposition of terminal complement complex on human tubular epithelial cells, whereas on glomerular endothelial cells, this increase did not reach significance. TCC deposition on Stx2-treated HK-2 cells and its biological significance have yet to be shown and extended to other cell lines. To show a more prominent effect, it may be necessary to use a dynamic cell culture model, which, in addition, would resemble a more physiological situation, instead of a static cell culture model, as used in the present study. So far, there are several reports on the ability of Stx to trigger complement activation; however, the point of attack by which Stx initiates complement activation has not been extensively investigated.

Here, we show that Stx2 reduced the level of CD59 expression on the surface of human tubular epithelial and glomerular endothelial cells. Other membrane-bound complement regulators, namely, CD46 and CD55, were apparently not affected by Stx2. Unfortunately, we could not include a control where Stx2 is preincubated with a neutralizing antibody prior to use in the assay, because all commercially available neutralizing anti-Stx and anti-CD59 antibodies are of murine origin, resulting in cross-reaction and thus no valid results. Former studies have suggested that CD59 plays an important role in protecting glomeruli from complement attack. Cultured glomerular epithelial and endothelial cells have been shown to exhibit increased susceptibility to complement-mediated lysis in the presence of a neutralizing antibody to CD59 *in vitro* (28–31). Rat models of complement-dependent glomerulonephritis have also demonstrated a more severe glomerular damage in the presence of anti-CD59 neutralizing antibodies (32, 33).

In addition, there are several diseases which have been associated with impairment of membrane-bound complement regulators. In paroxysmal nocturnal hemoglobinuria, deficient expression of GPI-anchored complement inhibitors CD55 and CD59 leads to excessive destruction of red cells and leukocytes upon complement activation (34). Another disease is hepatitis B, where downregulation of CD59 expression in hepatocyte cell lines sensitizes cells to complement-dependent lysis (35). Furthermore, atypical HUS (aHUS), which is a hereditary form of HUS not dependent on EHEC infection, has been associated with muta-

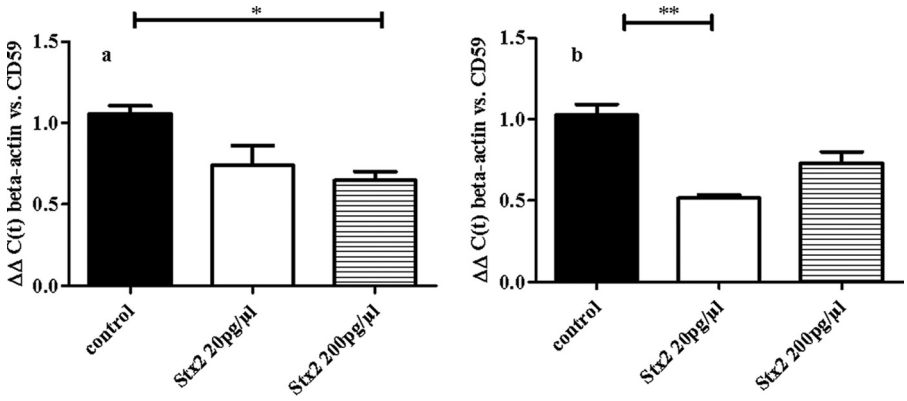

FIG 4 (a and b) CD59-specific mRNA reduction after incubation with Stx2 in tubular epithelial (HK-2) (a) and glomerular endothelial (GEnC) (b) cells. The cells were incubated with Stx2 (20 pg/µl or 200 pg/µl) for 4 h at 37°C or with PBS as a control. Quantification of mRNA was done by RT-qPCR. The β-actin housekeeping gene was used for relative quantification of qPCR values of the target gene. The mRNA of CD59 was significantly reduced in HK-2 cells after incubation with 200 pg/µl Stx2 and highly significantly reduced by half in GEnC cells after incubation with only 20 pg/µl Stx2 compared to the results for the PBS control. The results are the means ± SDs of 4 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

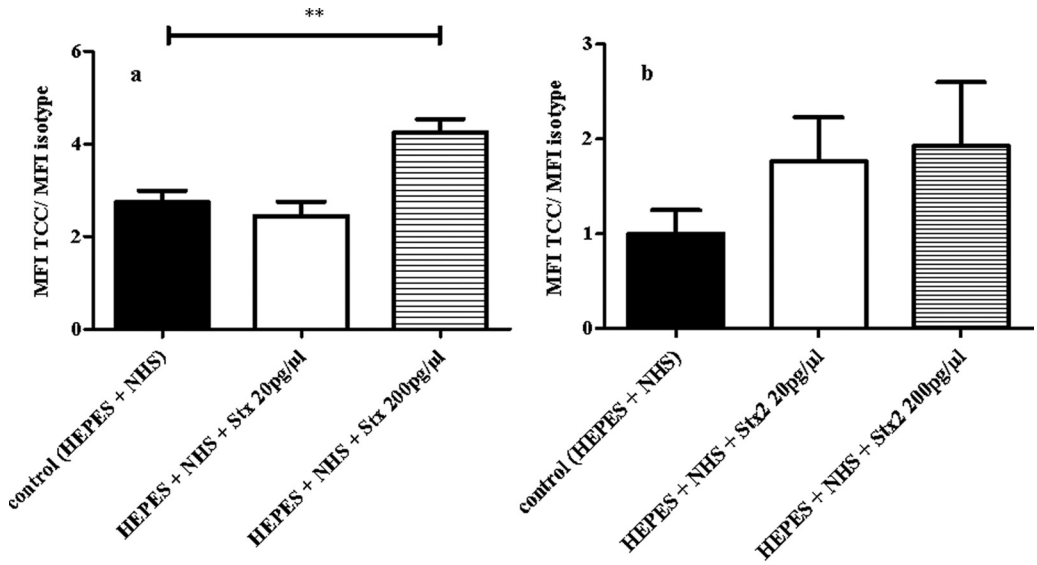

FIG 5 (a and b) TCC deposition on tubular epithelial (HK-2) (a) and glomerular endothelial (GEnC) (b) cells after incubation with Stx2. Deposition of TCC on HK-2 (a) and GEnC (b) cells after treatment with NHS (10%) and Stx2 (20 pg/μl or 200 pg/μl) for 4 h at 37°C or with HEPES buffer plus 10% NHS as a control is shown. TCC deposition was detected by FACS analyses using mouse anti-human TCC Ab and a polyclonal goat anti-mouse FITC-labeled secondary Ab. The results are given as the ratio of the MFI of the sample and the MFI of the isotype control. Panel a shows a highly significant increase in TCC deposition on HK-2 cells after treatment with 200 pg/μl Stx2 compared to the control. Panel b shows a dose-dependent but not significant increase in TCC deposition on GEnC cells under the influence of Stx2. The results are the means ± SDs of 3 separate experiments. **, $P < 0.01$.

tions in complement regulators (36, 37). Mutations in the gene encoding CD46 have been found in 5 to 10% of aHUS patients (38); however, impairment of CD55 or CD59 has not been described for aHUS patients so far.

There are several reports regarding the reduction of CD46, CD55, and CD59 on various cell lines after triggering of apoptosis by chemicals (39–41). From our experiments, we hypothesize that the CD59 reduction is caused by a direct effect of Stx2 and exclude the possibility that the reduction is caused by apoptotic effects triggered by Stx for two reasons. First, we found a reduction of CD59 only and not that of other membrane-bound regulators, particularly not the other GPI-anchored regulator, CD55. Second, a period of 4 h for incubation of the cells with Stx2 was chosen, as microscopic investigations have demonstrated an absence of apoptotic changes in both cell lines within that period.

We have also demonstrated that the reduction of CD59 by Stx2 was not caused by cleavage from the cell surface, as CD59 was not detected in the supernatant of Stx2-treated cells. In fact, RT-qPCR analyses showed that downregulation of CD59 mRNA is a likely reason for the reduction of CD59 on the cell surface. In glomerulum endothelial cells, higher toxin concentrations (500 pg/μl) did not show a reduction in mRNA levels; however, these toxin doses are probably not physiological. Interaction of Stxs with rRNA is already well-known (42). Stxs have been reported to inactivate the eukaryotic ribosome by removal of a single adenine base from 28S rRNA within the large (60S) ribosomal subunit (42), resulting in a ribotoxic stress response on the one hand and in an inhibition of protein synthesis on the other hand. Alternatively, there is evidence that Stx-globotriaosyl ceramide (Gb3) can be transported directly to nuclei (43, 44). However, the function of Stx in the nucleus has yet to be determined. Petruzziello-Pellegrini and colleagues have shown that low concentrations of Stx (10 fM) that had relatively minor effects on overall protein synthesis in human dermal neonatal microvascular endothelial cells altered endothelial gene expression in a highly gene-specific manner, as expression of only 2.5% of the 14,500 genes represented on their microarray were modified (45). Of these, 86.2% were upregulated but only 13.8% were downregulated. In addition, they observed that the increase in chemokine receptor CXCR4 mRNA by Stx was caused by a combination of both nuclear events and posttranscriptional mechanisms, such as increased association of the transcript with the polyribosome fractions and stabilization of mRNA transcripts (45). Whether downregulation of CD59 mRNA by Stx2 is caused by a direct effect of Stx on DNA or mRNA or by posttranscriptional modifications or whether this downregulation is indirectly caused by Stx2 via signal transduction has to be elucidated in a further study.

The fact that only 5 to 15% of children with EHEC infection eventually develop HUS suggests that, in addition to virulence factors of the pathogen, host factors also contribute to this development (6). Several studies in the past have investigated Gb3 expression in adults and children in an attempt to find an explanation for the predominance of HUS among children (46, 47); however, the results were controversial. It may be possible that CD59 expression varies with age, and this could be addressed in a further study.

In summary, Stx2 downregulates CD59 mRNA and protein levels in or on both human tubular epithelial and glomerular endothelial cells, likely contributing to complement activation and kidney destruction. Taking our former findings into account, where we postulated that Stx2 interacts with the complement system at several stages, we can now corroborate that view and conclude that binding to fluid-phase regulators (such as factor H) and downregulation of cell-associated ones (such as CD59) appear to be important points of attack.

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