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Human Microvascular Endothelial Cells Are Strongly Sensitive to Shiga Toxins

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We show here that the susceptibility of endothelial cells to Shiga toxin (Stx)s differs remarkably depending on their cellular origins. The concentration of Stx-1 required to reduce cell viability by 50% as measured by MTT assay was 30 and 300 fM for neonatal and adult human microvascular endothelial cells (HMVEC), respectively, and 30 pM for human coronary artery endothelial cells (HCAEC). Human umbilical venous endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) showed no sensitivities to Stx-1. Surprisingly, Stx-2 was approximately 10–100 times more toxic to HMVEC than Stx-1. Moreover sodium butyrate sensitized HMVEC by 100-fold to the cytotoxic activity of Stxs. These results were found to reflect the amount of Gb3/CD77 on the cell surface on a per cell basis using flow cytometrical analysis. The high sensitivity of HMVEC to Stxs suggests their involvement in the pathogenesis of organ failure induced by Stx-producing *Escherichia coli.*

Infections with Stxs producing *Escherichia coli* (STEC) are frequently associated with two important human diseases, hemorrhagic colitis (1) and hemolytic uremic syndrome (HUS) (2). Pathologic findings of these two diseases are similar, exhibiting microvascular endothelial cell damages which lead to vessel occlusion and infarction (3).

Several members of Stx family have been characterized. Stx-1 is essentially identical to Shiga toxin (4), and Stx-2 has an approximately 60% sequence identity to Stx-1 (5). All these toxins are composed of two subunits: a single polypeptide A subunit is surrounded by multiple copies of B subunit (6). These toxins specifically recognize globotriaosylceramide (Gb3/CD77) via B subunit to mediate cell cytotoxicity (7-9). Human umbilical venous endothelial cells (HUVEC) are not very sensitive to Stxs in their original culture, but can be induced to express Gb3/CD77 and become sensitive to Stxs by sodium butyrate, lipopolysaccaride (LPS) or LPS-inducible cytokines (10,11). Susceptibility of HSVEC to Stxs is also enhanced by similar pretreatments (10). In contrast, human glomerular epithelia (12,13) and renal tublar epithelia (14,15) appear to constitutively express higher levels of Gb3/CD77 and might be considerably more responsive to Stxs than HUVEC.

A major concern regarding the use of HUVEC as a model for studying the pathogenesis of thrombotic microangiopathy induced by Stxs is that they are obtained from large blood vessels rather than arteriolar or capillary endothelial cells, i. e. the cells which are frequently affected by STEC infection. Streamlines of evidence have indicated significantly different properties of endothelial cells depending on the sites they reside (16–18). This heterogeneity of endothelial cells may typify the different response to Stxs as suggested by the observation that HUVEC and human saphenous vein endothelial cells respond quite differently to Stxs (10). Therefore, use of other endothelial cells, especially those of microcapillaries, will be appropriate to study the effect of Stxs.

Maintanance of microvascular endothelial cells have been thought to be difficult. However, various types of endothelial cells including those of microcapillaries, are now commercially available for examination. The present study was thus designed to further assess the heterogeneity of readily available endothelial cells and smooth muscle cells in their susceptibilities to Stxs. We show that they are heterogenous regarding their sensitivities to Stxs.

MATERIALS AND METHODS

Stxs and cell culture. Stx-1 and -2 were prepared as described before (19,20). Primary cultures of human umbilical vein endothelial

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cells (HUVEC, lot No. 16197, and 15361 from new born), human cutaneous adult microvascular endothelial cells (adultHMVEC, lot No. 14512 from 30 year old adult), human cutaneous neonatal endothelial cells (neoHMVEC, lot No.14700 and 15354), and human coronary artery endothelial cells (HCAEC lot No.6F0712 from 52 year old adult) were purchased from Clonetics Co., Walkersville, USA through Sanko Junyaku Co., Ltd. Japan. These cells were positive for factor VIII. Cells were cultured on collagen type I-coated dish (BIOCOAT, Nippon Becton Dickinson Co., Ltd., Japan) and grown in EBM-2 medium supplemented with 2% (v/v) fetal bovine serum (FBS), 0.04% (v/v) hydrocortisone, 0.1% (v/v) heparin, 0.1% (v/v) human epidermal growth factor, 0.1% (v/v) long R insulin-like growth factor-1, 0.1 (v/v) ascorbic acid, 0.4% (v/v) h-fibroblast growth factor-B, 0.1% (v/v) vascular endothelial growth factor and 0.1% (v/v) gentamicin sulfate amphotericin-B according to the manufacture's protocol. When they were reached to confluency , they were passaged by detaching with trypsin-EDTA. Bovine aorta endothelial cells (BAEC) were purchased from Cellsystems Co., USA through Dainihon Seiyaku Co., Ltd., Japan and were grown in Dulbecco's modified Eagle medium(DMEM, GIBCO BRL, life technologies Co., Ltd., Japan) supplemented with 10% FBS. Mouse aorta smooth muscle cell line (AC01) was prepared as described (21). These cells were grown in DMEM supplemented with 10% FBS.

Cell viability assay. Cells were removed from substratum with trypsin-EDTA and plated at 1×10^4 cells/cm^2 in collagen type-I coated 96-well plates (BIOCOAT, Nippon Becton Dickinson Co.). After cultivation with or without 1 mM sodium butyrate for two days, medium was replaced by a fresh one containing Stxs at various concentrations. Cells were further incubated for two days and cell viability was assessed by a modified MTT assay (22). Briefly, medium in each well was replaced with 100 μl of fresh medium plus 20 μl of MTT solution (5mg/ml in phosphate buffered saline(PBS), Sigma-Aldrich Fine Chemicals, St.Louis, MO, USA) and incubated for 2h at 37°C. One hundred μl of lysing buffer (20% (w/v) SDS in 50% n,n-dimethyl formamide, pH4.7) was added to lyse the cells for overnight and the absorbance at 630 nm was measured with a microplate reader. Results represent the mean and standard deviation of triplicate determinations and are expressed as a percentage of absorbance of control (untreated) cells. The concentration of the toxin which reduced viability by 50% was determined and expressed as IC50.

Flow cytometrical analysis. Cells were removed from substratum with trypsin-EDTA and plated at 1×10^4 cells/cm^2 in collagen type-I coated 100 mm dish (BIOCOAT, Nippon Becton Dickinson Co.). After cultivation with or without 1 mM sodium butyrate, cells were trypsinized, counted, centrifuged, washed in medium. Then cells were incubated for 30 min at 4°C in 100 μl medium containing with or without anti monoclonal Gb3/CD77 antibody (1A4, a gift from Dr. K-I., Hakomori, University of Washington) (23), washed two times, incubated with FITC-conjugated goat anti mouse IgG+M(Jackson) for 60 min at 4°C, and again washed two times with PBS. Analysis of the cells was performed using a flow cytometer (EPICS XL). To avoid artifacts arising from autofluorescence of dead cells and cellular debris, data aquisition was electrically gated for large particles presumably representing intact cells by forward and side scatter. Routinely, data from green fluorescence of 10,000 cells were subjected to histogram and dot-plot analysis.

RESULTS AND DISCUSSION

First we compared HUVEC, adultHMVEC, neoHMVEC, HCAEC, BAEC, and AC01 for their susceptibilities to purified Stx-1 by MTT assay. As dramatic morphological change was observed after culture with Stx-1 for 2 days, MTT assay was started at this point. As shown in Fig. 1A, neoHMVEC and adult HMVEC were most susceptible among the cells tested and Stx-1 exhibited toxicity on these cells in a dose dependent manner. IC50 for neoHMVEC and adultHMVEC were approximately 30 fM (30×10^{-15} M) and 300 fM (300×10^{-15} M), respectively. HCAEC were weakly susceptible to Stx-1, with IC50 being approximately 30 pM (30×10^{-12} M) (Fig. 1B). In contrast, HUVEC, BAEC, and AC01 were unaffected by Stx-1 even at concentration as high as 15 pM (Fig. 1). As described above, both HMVEC were susceptible to very low concentrations of Stx-1. In the case of Shiga toxin, which is virtually identical to Stx-1 with only one amino acid substitution in A subunit (4), IC50 to cultured renal endothelial cells was reported to be 1×10^{-12} M (24). Although side-by-side comparison was not done in this study, it is likely that HMVEC are more sensitive to Stx-1 than renal endothelia. It was recently suggested, based on the laboratory data indicating renal tubular dysfunction, that primary targets of Stxs in the progress of HUS may be renal tubular epithelia as well as vascular endothelia (25–28). Thus the data described here may well explain clinical findings. HUVEC have been used as a model to study the effect of Stxs.

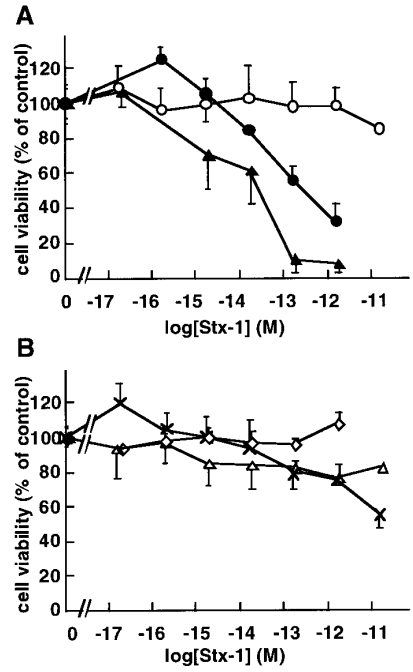

FIG. 1. Different sensitivity of cultured endothelial cells and smooth muscle cells to Stx-1. HUVEC(○), adultHMVEC(●), neoHMVEC(▲), HCAEC(×), BAEC(△) and AC01(◇) were treated with Stx-1 for 2 days at the concentrations indicated. After removal of toxin, cell viability was determined as described under Materials and Methods. Results are expressed as a percentage of control (untreated) cells. Similar results were obtained in repeated experiments.

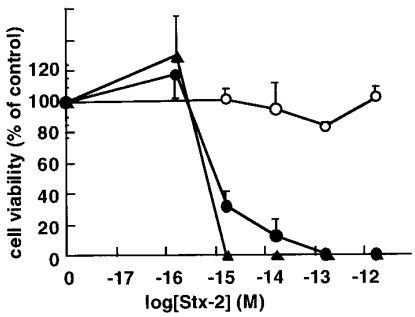

FIG. 2. Differential sensitivity of cultured endothelial cells to Stx-2. HUVEC(○), neoHMVEC(▲), and adultHMVEC(●) were treated with Stx-2 for 2 days at the concentrations indicated. After removal of toxin, cell viability was determined as described under Materials and Methods. Results are expressed as a percentage of control (untreated) cells. Similar results were obtained in repeated experiments.

Little or weak susceptibility to Stx-1 as described above and by others suggests that HUVEC may not be suitable for this purpose. Indeed, pathologic changes of such large vessels are reported to be less prominent as compared with those of small vessels in the case of HUS (2). BAEC similarly did not show any sensitivity to Stx-1. This observation may explain the fact that STEC colonizes the intestine of healthy cattle but does not cause any disease.

We next examined the effect of Stx-2 on HMVEC comparing with HUVEC. As shown in Fig. 2, both neoHMVEC and adultHMVEC were highly sensitive to Stx-2 with approximately 1~10 fM (1~10 $\times$ 10^{-15} M) IC50, indicating 10 to 100 times more sensitive than Stx-1. In contrast, HUVEC was unaffected at concentration as high as 1.5 pM (1.5 $\times$ 10^{-12} M). As HMVEC responded to Stx-1 and -2 quite differently, a possibility remained that activity of Stx-1 might have been lost during purification process. However, this is unlikely because same Stxs preparations exhibit cytotoxicities toward Stx-sensitive Burkitt's cell lines (unpublished observations). Most data describing the cytotoxic effect of Stxs has been performed using Stx-1, and thus no information is available concerning the different cytotoxicities of Stx-1 and -2 on the same cells. Stx-2 is less similar to Shiga toxin than Stx-1 and has approximately 50% to 60% homology on the basis of the deduced amino acid sequence (5). Whether this structural diversity accounts for the different cytotoxicity must await further study, but clinical findings that HUS caused by Stx-2 or Stx-1/2 producing E.coli occasionally becomes more serious than that by Stx-1 (Takeda, T., personal communication) may coincide with our results. The sensitivity of a few cell types to Stxs has been attributed to the expression of the glycolipid receptor for Stxs, Gb3/CD77. We examined the expression of Gb3/CD77 on HUVEC and neo HMVEC by flowcytometry. On HMVEC, Gb3/CD77 was expressed at low level, but it was not detectable on HUVEC (Fig. 3). Permealization of cell membrane resulted in the weak positivity of Gb3/CD77 in

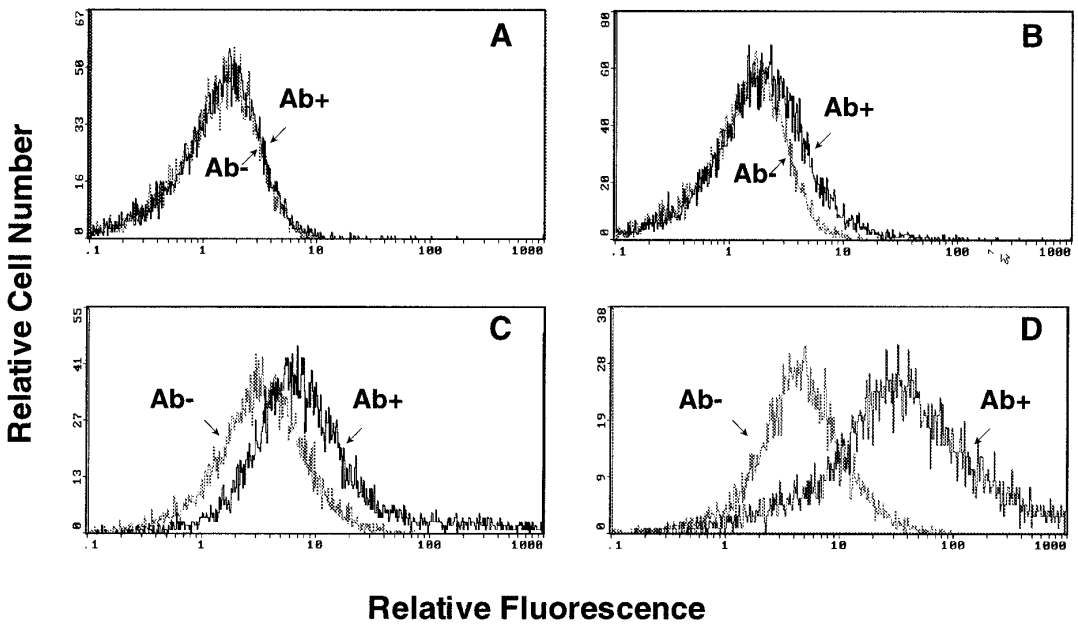

FIG. 3. Flow cytometrical analysis of HUVEC and neoHMVEC. HUVEC (A), HUVEC (B) treated with 1 mM sodium butyrate, HMVEC (C), and HMVEC (D) treated with 1 mM sodium butyrate were stained with anti-Gb3/CD77 monoclonal antibody (Ab+) or medium alone (Ab−) followed by FITC-labeled secondary antibody.

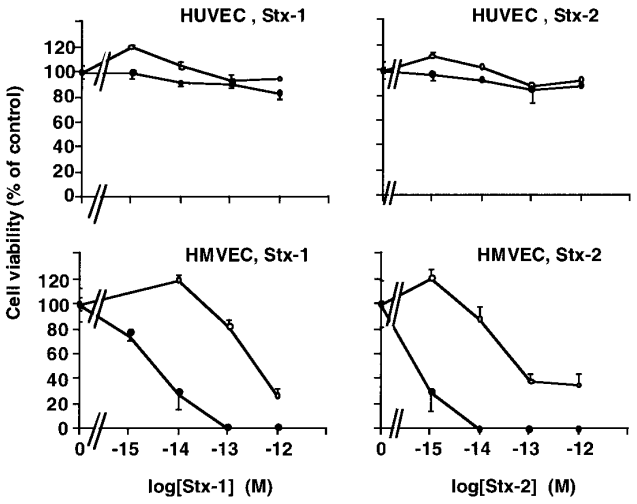

FIG. 4. Effect of sodium butylate on the sensitivity of HUVEC and HMVEC to Stx-1 and -2. HUVEC or HMVEC were grown and treated with (●) or without (○) 1 mM sodium butyrate for 2 days and exposed to the indicated concentration of STXs. After 2 days of incubation, cell viability was determined as described under Materials and Methods. Results are expressed as a percentage of control (untreated) cells. Similar results were obtained in repeated experiments.

HUVEC, suggesting that HUVEC produce Gb3/CD77 but do not express on cell membrane (data not shown). This higher level of Gb3/CD77 expression on the cell surface in HMVEC versus HUVEC might correspond to the greater sensitivity of HMVEC to Stxs.

Previous report demonstrated that HUVEC and HSVEC could be sensitized to Stx by pretreatment of cells with 1 mM sodium butyrate for seven days, although the concentration of Stx-1 was significantly high (15 nM)(10). Similar treatment of HMVEC with 1 mM sodium butyrate for two days was found to sensitize HMVEC to cytotoxic activity Stxs, at extremely lower concentrations (<100 fM) (Fig. 4). Thus, sensitivities of sodium butyrate treated cells increased by approximately 100-fold for both toxins as compared with those untreated cells. In addition sensitization of HMVEC to Stx by sodium butylate was accompanied by an great increase in Gb3/CD77 expression (Fig. 3). Previous work demonstrated that Gb3/CD77 is the receptor for Stx-1 and -2 (7–9). Therefore, it appears that Gb3/CD77 expression is one important factor in defining susceptibility of individual cell types to toxic effects of Stxs. In our experiments, HUVEC was not sensitized with sodium butyrate (Fig. 4) although surface expression of Gb3/CD77 became faintly positive (Fig. 3B). Higher amount of toxins may be required to induce HUVEC cell damage, but this result further indicate the difference between HUVEC and HMVEC with regard to their sensitivities to Stxs.

In conclusion, we showed that human vascular endothelial cells exhibited different susceptibilities to Stxs depending on their origins, cutaneous microvascular endothelial cells prepared from both adult and neonate being most sensitive. Usage of various lines, especially HMVEC, should serve to better understanding of cytotoxic mechanism of Stxs.

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