Targeting Endothelial Cells Overexpressing VEGFR-2: Selective Toxicity of Shiga-like Toxin−VEGF Fusion Proteins

Marina V. Backer* and Joseph M. Backer

SibTech, Inc., Newington, Connecticut 06111. Received July 24, 2001

Growing endothelial cells at the sites of angiogenesis express high numbers of VEGF receptors and therefore may be particularly sensitive to VEGF-mediated drug delivery. To test this hypothesis we have constructed a protein containing the catalytic A-subunit of Shiga-like toxin I fused to $VEGF_{121}$ (SLT-VEGF/L). Wild-type A-subunit is a site-specific N-glycosidase of 28S rRNA that inhibits protein synthesis after being delivered into cells by separate cell-binding B-subunits. SLT-VEGF/L retains functional activities of both SLT and $VEGF_{121}$ moieties, since it inhibits protein synthesis in a cell-free translation system and induces VEGFR-2 tyrosine autophosphorylation. SLT-VEGF/L selectively inhibits growth of porcine endothelial cells expressing 2.5×10^5 VEGFR-2/cell with an IC_{50} of 0.2 nM and rapidly induces apoptosis at concentrations >1 nM. We found that sensitivity of VEGFR-2 transfected PAE cells to SLT-VEGF/L declined as the cellular VEGFR-2 density decreased; PAE cells expressing 25000 VEGFR-2/cell were as sensitive as parental cells lacking the receptor. Growth inhibition and induction of apoptosis by SLT-VEGF/L require intrinsic N-glycosidase activity of the SLT moiety, but take place without significant inhibition of protein synthesis. Selective cytotoxicity of SLT-VEGF/L against growing endothelial cells overexpressing VEGFR-2 suggests that it may be useful in targeting similar cells at the sites of angiogenesis.

INTRODUCTION

Angiogenesis is a tightly controlled process of growing new blood vessels that in the adult organism under normal circumstances takes place only during muscle or weight gains, development of the corpus luteum, and wound healing (reviewed in refs 1, 2). However, angiogenesis occurs in a large number of pathologies, such as solid tumor growth, various eye diseases, chronic inflammatory states, and ischemic injuries (reviewed in ref 3). Growing endothelial cells at the sites of angiogenesis are unique targets for treatment of several major pathologies, and a number of compounds targeting angiogenesis are currently at various stages of development (reviewed in ref 4). Several positive and negative regulators control angiogenesis, and the shift in equilibrium between these regulators is presumably responsible for angiogenesis in pathological situations (reviewed in refs 5, 6). The crucial positive regulator of angiogenesis is vascular endothelial growth factor (VEGF) also known as vascular permeability factor (reviewed in ref 7). VEGF is a secreted homodimeric glycoprotein that, as a result of alternative mRNA splicing, may consist of polypeptides with 121, 145, 165, 189, or 206 amino acid residues. VEGF is expressed by normal and tumor cells in response to hypoxia and nutritional deprivation, and its expression appears to be regulated on several levels (reviewed in refs 8, 9). The action of VEGF on endothelial cells is mediated by tyrosine kinase Flt-1 and KDR/Flk-1 receptors, also known as VEGFR-1 and VEGFR-2, with the latter playing the dominant role in angiogenesis (reviewed in refs 9, 10). These receptors are preferentially expressed on endothelial cells, and there is evidence that endothelial cells at the sites of angiogenesis express significantly higher numbers of VEGFR-2 than quiescent endothelial cells (*11*−*15*). VEGF binding to VEGFR-2 induces receptor dimerization followed by tyrosine phosphorylation of the SH2 and SH3 domains and internalization of the complexes (reviewed in refs 9, 10). Since VEGF binds specifically to endothelial cells, this growth factor provides a unique opportunity for targeted drug delivery to the sites of angiogenesis. Indeed, it has been shown that conjugation or fusion of the catalytically active fragments of diphtheria toxin (DT) to VEGF yielded proteins that inhibited growth of endothelial cells and displayed anti-angiogenic activity in in vivo models of angiogenesis (*16*−*18*).

To inhibit angiogenesis without damaging quiescent endothelium, the toxin−VEGF fusion proteins should selectively target endothelial cells at the sites of angiogenesis that overexpress VEGFR-2. We hypothesized that overexpression of VEGFR-2 receptor in the growing endothelial cells may endowed them with enhanced sensitivity to some toxin−VEGF fusion proteins. To test this hypothesis we have constructed a protein containing the catalytic A-subunit of Shiga-like toxin I fused to $VEGF_{121}$ (SLT-VEGF/L). SLT-1 produced by *E. coli* O157:H7 is a "natural killer" for endothelial cells. Damage to endothelial cells caused by SLT-1 plays a causative role in the pathogenesis of hemorrhagic colitis and hemolytic uremic syndrome induced by *E. coli* O157:H7 (*19*−*22*). SLT-1 is composed of a single copy of a 32 kDa A-subunit associated with a ring shaped pentamer of 7 kDa B-subunits that bind to the cellular receptor globotrioaosylceramide known as Gb_3/CD77. SLT-1 enters cells via CD77-mediated endocytosis, whereby the A-subunit is cleaved into disulfide bond linked 27.5 kDa A_1 and 4.5 kDa A_2 fragments (*22*, *23*). The processed A-subunit is a site-specific N-glycosidase that cleaves off A_{4324} in the 5′ terminus of 28S rRNA (*24*). Depurination of A_{4324} inhibits

* To whom correspondence should be addressed, at SibTech, Inc., 705 North Mountain Road, Newington, CT 06111. Phone: (860) 953−1753; Fax: (860) 953−1317; E-mail: mbacker@sibtech.com.

10.1021/bc015534j CCC: $20.00
Published on Web 10/18/2001

binding of the elongation factor EF-1/aminocyl-tRNA complex to ribosomes, resulting in the inhibition of the protein synthesis and initiation of apoptosis. Proteolytic processing of the A-subunit is not required for its enzymatic activity, since the full length A-subunit, and truncated A-subunits missing most of the A_2 region, retain significant N-glycosidase activity in vitro (*25*−*27*). Furthermore, fusion proteins containing the full length A-subunit as well as various truncated A-subunits fused to the N-terminus of CD4 retain N-glycosidase activity and are cytotoxic for cells expressing the HIV-1 gp120/gp41 complex (*26*, *27*).

Our preliminary data indicated that SLT-VEGF/L inhibits growing cells overexpressing VEGFR-2 (*28*). We report here that the number of VEGFR-2 receptors on endothelial cells determines their sensitivity to SLT-VEGF/L. Furthermore, contrary to a commonly held view that plant and bacterial toxin act via inhibition of protein synthesis we found that SLT-VEGF/L induces apoptosis without significant inhibition of protein synthesis.

MATERIALS AND METHODS

Plasmids. The pJB144 plasmid encoding VT1/SLT-1 holotoxin was kindly provided by Dr. A. Soltyk (Samuel Lunfield Research Institute, Toronto, Canada). The pBalPst/KDR mammalian expression plasmid containing the full-length human VEGFR-2 was kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, NY).

Construction of Expression Plasmids. Primers for cloning and mutagenesis of SLT-1 fragments (GenBank accession: AB015056) were synthesized by CeneLink (Thornwood, NY). The full-length (1−293 aa) and truncated (62−264 aa) fragments of the SLT-1 A-subunit were amplified by PCR using the pJB144 plasmid as a template. The "sense" primers for the full-length (5′-CCGAGATCTGAAGGAATTTACCTTAGAC) and for the truncated (5′-CCCAGATCTGCTACGGCTTATTGTTGAACG) SLT-1 fragments included *Bgl*II sites (underlined). The "antisense" primers for the full-length (5′-ATAGGTACCACTGCTAATAGTTCTGCG) and for the truncated (5′-ATAGGTACCATCTGCCGGACACATAGAAG) SLT-1 fragments introduced *Kpn*I sites (underlined). PCR-amplified full-length (L) and truncated (S) SLT-1 fragments were cloned separately into $VEGF_{121}$ encoding pET32-VEGF121 plasmid (constructed as described in *29*) in-frame with $VEGF_{121}$. The resulting plasmids, pET32/SLT-VEGF/L and pET32/SLT-VEGF/S, were confirmed by sequencing (CSU-Macromolecular Resources, Ft. Collins, CO). Site-specific mutagenesis of SLT-1 A-subunit encoded by pET32/SLT-VEGF/L was done using GeneEditor in vitro Site-Directed Mutaganesis System (Promega). Two mutagenic primers were designed to introduce three point mutations (underlined): Y114S (5′-ACGTGGTAGAGCTACTGTCACC), and E167Q and R170L (5′-TTGCCGAAAAAGTAAAGCTTGAGCTGTCACAG). The Y114S and R170L mutations were confirmed by sequencing of mutated DNA isolated from two clones. The E167Q mutation was not detected in either clone.

Expression of Fusion Proteins. $VEGF_{121}$ and SLT-VEGF fusion proteins were expressed in *E. coli* strain Origami(DE3)pLysS (Novagen). After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Life Technologies), bacteria were grown for 4 h at 30 °C, harvested, and lysed by freezing-thawing in 50 mM Tris-HCl pH 7.5, 0.1 mM $MgCl_2$, 1% NP-40, and all protease inhibitors. After DNase treatment inclusion bodies were pelleted at 5000*g* for 30 min, washed twice with 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, and solubilized in 8 M urea by sonication. The proteins were refolded by dialysis against 10 mM Tris-HCl pH 8.0, 150 mM NaCl. For dialysis of truncated form of SLT-VEGF, the buffer was supplemented with 0.01% Brij-35. All fusion proteins contained S-tags on the N-termini, making it possible to determine their molar concentrations by S−Tag Assay (Novagen).

Cell Cultures. Porcine aortic endothelial (PAE) cells and 293 human primary embryonic kidney cells were obtained from American Type Culture Collection (Rockville, MD). PAE cells expressing 2×10^5 VEGFR-2 per cell (PAE/KDR) and PAE cells transfected with the empty vector (PAE/V) were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, NY). 293 cells expressing 2.5×10^6 VEGFR-2 per cell (293/KDR) were described elsewhere (*29*). PAE cells expressing different numbers of VEGFR-2 (PAE/0.25-KDR and PAE/0.10-KDR clones) were constructed by transfection of PAE cells with the pBalPst/KDR plasmid using Trans*IT*-LT1 reagent (PanVera Corporation), followed by selection in the presence of 0.375 μg/mL puromycin. All cells and their derivatives were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine, and antibiotics at 37 °C, 5% CO_2.

VEGFR-2 Autophosphorylation. VEGFR-2 autophosphorylation was assayed as described (*28*). Briefly, subconfluent cells were incubated in serum-free DMEM at 37 °C for 4 h and then shifted to serum-free DMEM containing 0.1 mM orthovanadate, 100 ng/mL bovine serum albumin, 25 mM HEPES pH 7.2, for 20 min at 37 °C, followed by a 20-min incubation at 4 °C. After addition of SLT-VEGF cells were incubated for 1 h at 4 °C and then for 10 min at 37 °C, lysed, and analyzed by SDS−PAGE.

Western Blot Analysis. Cell lysates were separated by SDS−PAGE on 7.5% gels and transferred to nitrocellulose membranes (Life Technologies). After incubation with primary antibodies, phosphotyrosine RC20H (PharMingen), α-fodrin (Chemicon), or rabbit polyclonal anti-VEGFR-2 serum (kindly provided by Dr. B. Terman, Albert Einstein School of Medicine, NY), immune complexes were visualized by the *femto*Lucent Chemiluminescence System (Genotechnology).

Protein Synthesis Inhibition in Vitro. The protein inhibition activities of SLT-VEGF fusion proteins were measured in an in vitro translation system containing a rabbit reticulocyte lysate programmed with luciferase mRNA (Promega) according to the manufacturer's instructions. Protein synthesis in the presence of 1 μM recombinant $VEGF_{121}$ was taken as 100%.

^{35}S-Incorporation. PAE/KDR were plated onto 24-well plates, $(6-8) \times 10^3$ cells/well. Twenty hours later cells were shifted to fresh culture medium containing 2.5 nM SLT-VEGF/L, 1 mL/well. After 1−52 h of incubation at 37 °C cells from duplicate wells were counted in a Coulter Counter. Protein synthesis was measured at each time point by replacing media with methionine-free DMEM containing 0.45 μCi/ml [^{35}S]-Methionine (1000 Ci/mmol, Amersham Pharmacia Biotech). After a 30-min incubation at 37 °C cells were washed twice with ice-cold 10% TCA, lysed in 0.1 M KOH, and counted in a beta-counter. ^{35}S-incorporation assays were performed in triplicate.

DNA Laddering. PAE/KDR cells were plated onto six-well plates, 2×10^5 cells/well. Twenty-four hours later cells were shifted to fresh culture medium containing 2.5 nM SLT-VEGF/L, 1.5 mL/well. After 3−72 h of incubation at 37 °C, DNA was extracted and purified with Suicide-

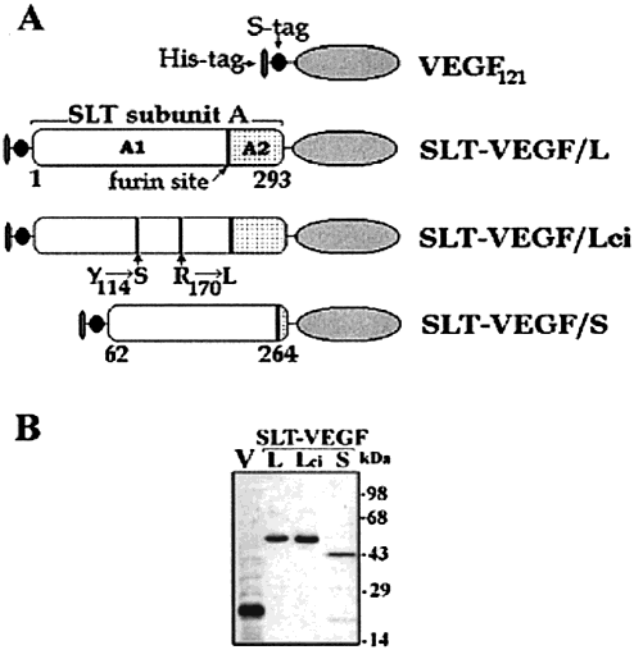

Figure 1. SLT-VEGF fusion proteins. All proteins were expressed in *E. coli* strain Origami(DE3)pLysS, solubilized from inclusion bodies, and refolded as described in Materials and Methods. A, Schematic representation of the proteins. B, SDS−PAGE analysis of the purified proteins (15% PAAG gel, Coomassie Blue staining). Lane V, $VEGF_{121}$.

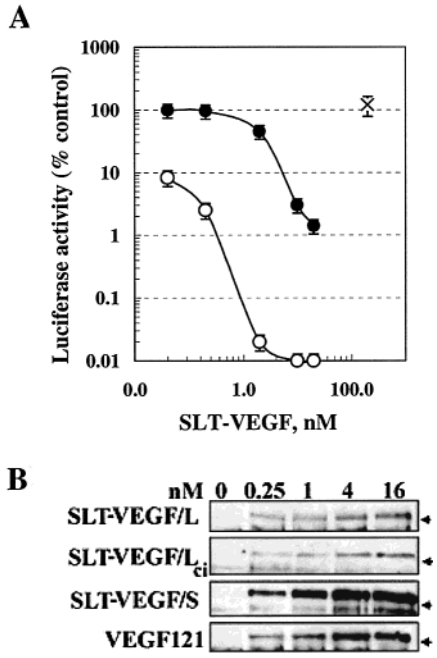

Figure 2. Functional activities of SLT-VEGF fusion proteins. A, Effects of SLT-VEGF/L (○), SLT-VEGF/S (●), or SLT-VEGF/Lci (X) on luciferase production in an in vitro translation system containing a rabbit reticulocyte lysate programmed with luciferase mRNA. Activity of luciferase translated in the presence of 1 μM recombinant $VEGF_{121}$ was taken as 100%. B, Dose-dependence of VEGFR-2 tyrosine autophosphorylation in 293/KDR cells induced by SLT-VEGF fusion proteins and $VEGF_{121}$. Arrows indicate positions of 205 kDa markers.

Track DNA Ladder Isolation kit (Oncogene) and fractionated on 1.5% agarose gels.

Cytotoxicity Assay. Cells were plated in 24-well plates, $5-10 \times 10^3$ cells/well. Twenty hours later 2.5 nM SLT-VEGF/L (unless otherwise indicated) in fresh culture medium was added to duplicate wells, 1 mL/well. After 72-h, treatments cells were detached with trypsin-EDTA and counted in a Coulter Counter. Cytotoxicity assays were performed in triplicate.

RESULTS

Expression of SLT-VEGF Proteins. SLT-VEGF fusion proteins (Figure 1A) were constructed, expressed, and purified as described in Materials and Methods. The $VEGF_{121}$ isoform was chosen for construction of these proteins because, unlike other VEGF isoforms, (1) it does not contain heparin-binding domain(s) that are involved in nonspecific electrostatic interactions with the cell surface and extracellular matrix components, and (2) it has a selective affinity to VEGFR-2 (*30*). Full-length and truncated SLT-1 A-subunits were fused to $VEGF_{121}$ with the expectation that the resulting SLT-VEGF/L and SLT-VEGF/S fusion proteins would display different N-glycosidase activities (*25*−*27*). To separate the effects of ribosome inactivation from other effects that might be induced by recombinant SLT-VEGF proteins we have constructed SLT-VEGF/Lci protein containing a double mutant A-subunit with the Y114S and R170L amino acid substitutions. Each substitution significantly decreases the enzymatic activity of SLT-1 A-subunit, while not affecting its antigenic properties (*31*, *32*). We therefore reasoned that simultaneous mutation of these residues might yield an SLT-1 moiety that lacks N-glycosidase activity. $VEGF_{121}$ and SLT-VEGF proteins were expressed in Origami(DE3)pLysS *E. coli*, recovered from inclusion bodies as 90−95% pure proteins (Figure 1B), and used in the following experiments without additional purification.

SLT and VEGF Moieties Are Active in SLT-VEGF. The fully activated A-subunit isolated from the AB_5 holotoxin inhibits protein synthesis in cell-free system with the IC_{50} value of 0.1 nM (*33*). We therefore tested catalytic activity of SLT-VEGF in a cell-free translation system programmed with luciferase mRNA. We found that SLT-VEGF/L and SLT-VEGF/S inhibited protein synthesis by 90% at concentrations 0.04 nM and 2 nM, respectively (Figure 2A). Thus, SLT-VEGF/L may be even more effective inhibitor of protein synthesis than the wild type A-subunit. A 50-fold difference in activities of SLT-VEGF/L and SLT-VEGF/S suggested that truncation affected either the intrinsic catalytic activity of SLT-1 moiety, or its ability to fold properly within the fusion protein (Figure 2A). SLT-VEGF/Lci did not inhibit protein synthesis (Figure 2A).

The ability of SLT-VEGF fusion proteins to interact with VEGFR-2 was tested in a VEGFR-2 tyrosine autophosphorylation assay with 293/KDR cells. All SLT-VEGF fusion proteins induced VEGFR-2 tyrosine autophosphorylation in a dose-dependent manner in the same concentration range as parental $VEGF_{121}$ (Figure 2B). Different plateau levels observed in this assay suggested that complexes of VEGF fusion proteins with VEGFR-2 possess different intrinsic efficacies in tyrosine autophosphorylation. SLT-VEGF/L and SLT-VEGF/Lci were similarly efficacious (Figure 2B), indicating that mutations in the A-subunit, which eliminated N-glycosidase activity, did not affect interactions with the VEGFR-2. SLT-VEGF/S was more efficacious than SLT-VEGF/L and SLT-VEGF/Lci (Figure 2*B*), suggesting that SLT-VEGF/S may be better suited for induction of VEGFR-2 mediated processes. The ability of SLT-VEGF/L and SLT-VEGF/S to induce VEGFR-2 tyrosine autophosphorylation in PAE/KDR cells was reported previously (*28*).

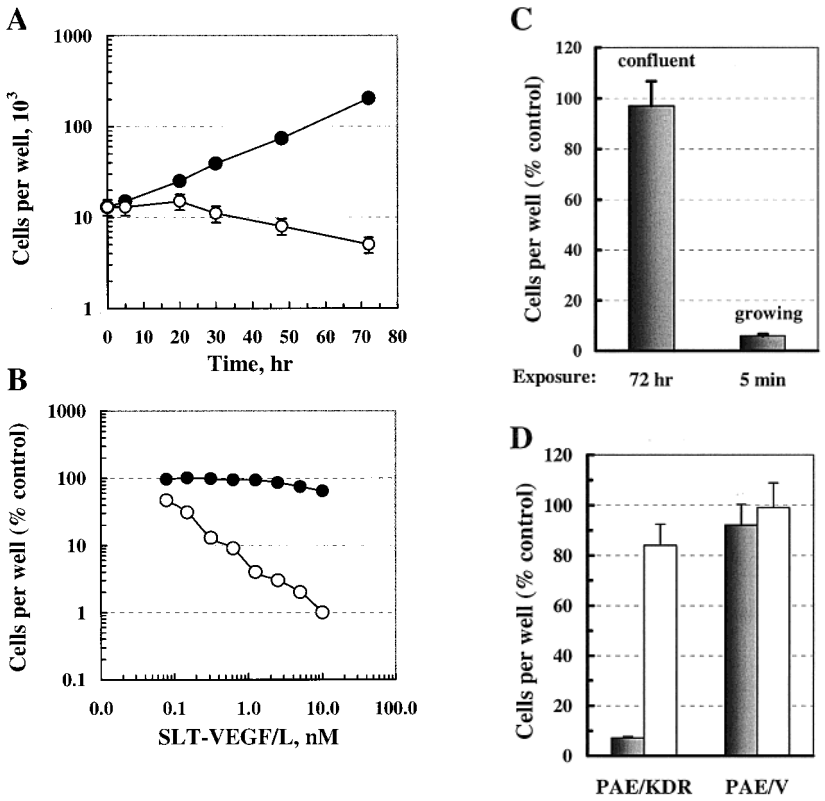

Figure 3. SLT-VEGF/L is selectively cytotoxic against growing endothelial cells overexpressing VEGFR-2. PAE/KDR cells were plated onto 24-well plates at densities of $5-10 \times 10^3$ cells/well and exposed to SLT-VEGF/L 20 h later. After a 72-h exposure cells were counted in a Coulter Counter. A, PAE/KDR cells treated with 2.5 nM SLT-VEGF/L (○) and untreated cells (●). B, PAE/KDR cells (○) and PAE cells lacking VEGFR-2 (●) exposed to varying concentrations of SLT-VEGF/L. C, Confluent PAE/KDR were maintained at 100% confluence for 3 days; then treated with 20 nM SLT-VEGF/L for 72 h. Growing PAE/KDR were exposed to 20 nM SLT-VEGF/L for 5 min and then shifted to fresh culture medium and counted after 72 h. D, effect of depletion of SLT-VEGF preparation. SLT-VEGF/L (0.5 mL of 25 nM solution in DMEM serum-free) was combined with 0.1 mL S-protein agarose (Novagen). After a 30-min incubation at 4 °C, the solution was cleared at 500*g* for 5 min, diluted 10-fold with complete culture medium, and added to cells (open bars). Control cells were treated with 2.5 nM SLT-VEGF/L (filled bars).

SLT-VEGF/L Is Selectively Cytotoxic for Growing Cells Overexpressing VEGFR-2. To test VEGFR-2-mediated effects of SLT-VEGF fusion proteins on endothelial cells we have used PAE/KDR cells engineered to express 2.5×10^5 VEGFR-2/cell (*29*). PAE/V or wild type PAE cells were used as controls. After an exposure to 2.5 nM SLT-VEGF/L, PAE/KDR, but not control cells, ceased to grow, and their number progressively decreased below the number of plated cells because of widespread death (Figure 3*A*). SLT-VEGF/L inhibited growth of PAE/KDR cells in a dose-dependent manner with an IC_{50} of 0.19 ± 0.13 nM (average ± STD for five independent preparations of SLT-VEGF/L) and was highly cytotoxic to PAE/KDR cells at concentrations ≥1 nM (Figure 3B for a typical SLT-VEGF/L preparation). These effects were mediated by VEGFR-2, since parental PAE cells lacking VEGFR-2 were inhibited by SLT-VEGF/L with an IC_{50} of 3.95 ± 0.95 (Figure 3B). This sensitivity of nontransfected PAE cells might be mediated by VEGFR-1 present on parental PAE cells (*34*), since several other cell lines without known VEGF receptors were not affected by SLT-VEGF/L at concentrations as high as 20 nM (*28*). Presence of VEGFR-2 appears to be necessary, but not sufficient for induction of endothelial cell death by SLT-VEGF/L. We found that quiescent PAE/KDR cells were not affected by a 72-h exposure to SLT-VEGF/L even at concentrations as high as 20 nM (Figure 3C). In contrast, growing PAE/KDR cells exposed to 20 nM SLT-VEGF/L for 5 min, and then shifted to fresh culture medium and counted after 72 h, were dramatically affected (Figure 3C). To confirm a causative role of SLT-VEGF/L in the observed effects, a preparation of SLT-VEGF/L was depleted of recombinant proteins by capturing them with S-protein agarose. The depleted preparation was ineffective against PAE/KDR and PAE/V cells (Figure 3D).

Effect of SLT-VEGF/L on Endothelial Cells Depends on the VEGFR-2 Density. To test effects of VEGFR-2 density on cytotoxicity of SLT-VEGF/L, we have constructed two clones of PAE cells expressing different numbers of VEGFR-2. The levels of VEGFR-2 expression were estimated by Western blot analysis with PAE/KDR cells (2.5×10^5 VEGFR-2/cell) used as a standard. Two clones of VEGFR-2 transfected PAE cells, PAE/0.25-KDR and PAE/0.10-KDR, expressed, respectively, four and ten times less VEGFR-2/cell then PAE/KDR (Figure 4). We found that sensitivity of VEGFR-2 transfected PAE cells to SLT-VEGF/L declined as the VEGFR-2 density decreased (IC_{50} on Figure 4). PAE/0.10-KDR cells expressing ~25000 VEGFR-2/cell were approximately as sensitive as PAE/V cells, indicating that only a high level of VEGFR-2 expression conferred sensitivity of growing endothelial cells to SLT-VEGF/L.

Requirement for Catalytic Activity of SLT Moiety in SLT-VEGF Fusion Proteins. To establish the role of N-glycosidase activity of SLT moiety in the observed effects, we tested SLT-VEGF/Lci containing a catalytically inactive A-subunit. As was described before, SLT-VEGF/Lci did not inhibit protein synthesis but retained the ability to bind and activate VEGFR-2 (Figure 2). We found that 2.5 nM SLT-VEGF/Lci did not affect growth of PAE/KDR or PAE/V cells (Figure 5*A*), establishing that the ability to damage ribosomes was the basis of the SLT-

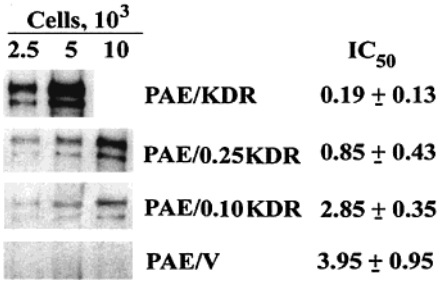

Figure 4. High level of VEGFR-2 is necessary to confer sensitivity of cells to SLT-VEGF/L. Three clones of VEGFR-2 transfected and control PAE/V cells were grown in six-well plates. Subconfluent cells were washed with PBS, detached with PBS containing 1 mM EDTA, counted in a Coulter Counter, and lysed in SDS−PAGE loading buffer. Cell lysates containing equal numbers of cells were analyzes by SDS−PAGE on 7.5% gels followed by Western blotting. IC_{50} for each clone was determined as described in the legend for Figure 3B. Each experiment was done in triplicate.

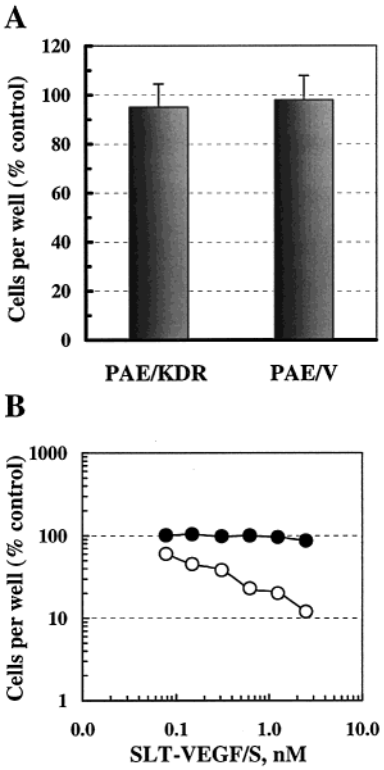

Figure 5. Cytotoxicity of SLT-VEGF proteins requires catalytic activity of SLT moieties. PAE/KDR and PAE/V cells were plated onto 24-well plates at densities of (6−8) × 10^3 cells/well. Twenty hours later cells were exposed to (A) 2.5 nM SLT-VEGF/Lci containing a catalytically inactive SLT A-subunit, or (B) varying concentrations of SLT-VEGF/S containing a truncated SLT A-subunit. (○), PAE/KDR; (●), PAE/V. After a 72-h exposure to the fusion proteins, cells were counted in a Coulter Counter.

VEGF/L cytotoxicity. The role of N-glycosidase activity of SLT moiety of SLT-VEGF proteins was further explored using SLT-VEGF/S that, in comparison with SLT-VEGF/L, was less active in inhibition of cell-free translation, but more active in induction of VEGFR-2 tyrosine phosphorylation (see, Figure 2A and 2B). SLT-VEGF/S inhibited growth of PAE/KDR cell, while PAE/V cells were marginally affected (Figure 5B), indicating the VEGFR-2-mediated mechanism of action. Surprisingly, despite ~50-fold lower activity in a cell-free translation assay (Figure 2A), SLT-VEGF/S inhibited PAE/KDR growth in the same concentration range as SLT-VEGF/L (compare Figure 3A and 5B). Perhaps, lower catalytic

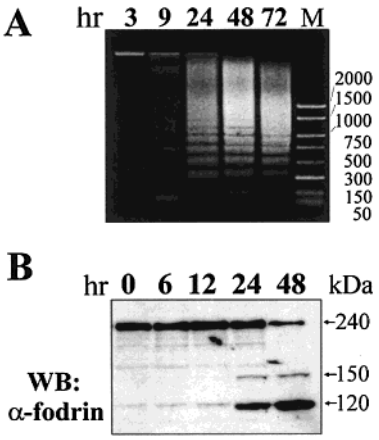

Figure 6. SLT-VEGF/L induces apoptosis in PAE/KDR cells. A, Apoptotic DNA fragmentation. Cells were plated onto six-well plates at a density of 2 × 10^5 cells/well and exposed to 2.5 nM SLT-VEGF 24 h later. After indicated periods of time DNA was isolated from cell lysates and fractionated on 1.5% agarose gel. B, Western blot analysis of α-fodrin. Cells were plated onto 24-well plates at a density of 4 × 10^4 cells/well and exposed to 2.5 nM SLT-VEGF 20 h later for indicated periods of time.

activity of SLT-VEGF/S was compensated by its ability to induce higher levels of VEGFR-2 tyrosine phosphorylation (Figure 2B) that might lead to a better uptake and/or processing of SLT-VEGF/S$(VEGFR\text{-}2)_2$ complexes. Unfortunately, a very low yield of SLT-VEGF/S prevented further experiments.

SLT-VEGF/L Induces Apoptosis in PAE/KDR Cells. We found that SLT-VEGF induces rapid apoptotic changes in PAE/KDR cells. After 5−10 h of exposure to 2.5 nM SLT-VEGF, PAE/KDR cells progressively underwent morphological alterations resembling apoptosis (data not shown). An apoptotic DNA laddering was observed after a 9-h exposure, and little or no high molecular weight DNA was detectable after 24 or 48-h exposures (Figure 6A). Another hallmark of apoptosis, cleavage of 240 kDa α-fodrin into 150 kDa and 120 kDa fragments (*35*) was readily detectable after a 24-h exposure (Figure 6B).

Effects of SLT-VEGF/L on Protein Synthesis. Native SLT-1, and presumably SLT-VEGF/L, inhibits protein synthesis by damaging ribosomes via depurination of A_{4324} in 28S rRNA. The resulting apoptosis may be initiated by collapse of protein synthesis. Alternatively, apoptosis may be initiated by ribosome damage through the recently discovered ribotoxic response mechanism that is mediated by activation of stress-response kinases and does not require significant inhibition of protein synthesis (*36*). To choose between these alternatives, we tested the effects of SLT-VEGF/L on protein synthesis in PAE/KDR cells. We found that ^{35}S-methionine incorporation per well with SLT-VEGF/L treated cells was progressively lower than that for untreated cells, with approximately 50% inhibition observed after a 20-h treatment (Figure 7*A*). Similar results have been reported recently for protein synthesis inhibition in SLT treated Caco-2 cells (*37*). However, this effect reflected mostly the difference in the cell numbers, since ^{35}S-methionine incorporation per cell varied only by 30−50% for both treated and control cells (Figure 7B). In contrast, cycloheximide treatment (0.1 mg/mL) rapidly inhibited protein synthesis on a per cell basis (Figure 7B). It was possible that even small alterations in protein synthesis might inhibit cell growth and be cytotoxic to PAE/KDR cells. However, this explanation appears unlikely since even an 80% inhibition of protein synthesis by cycloxe-

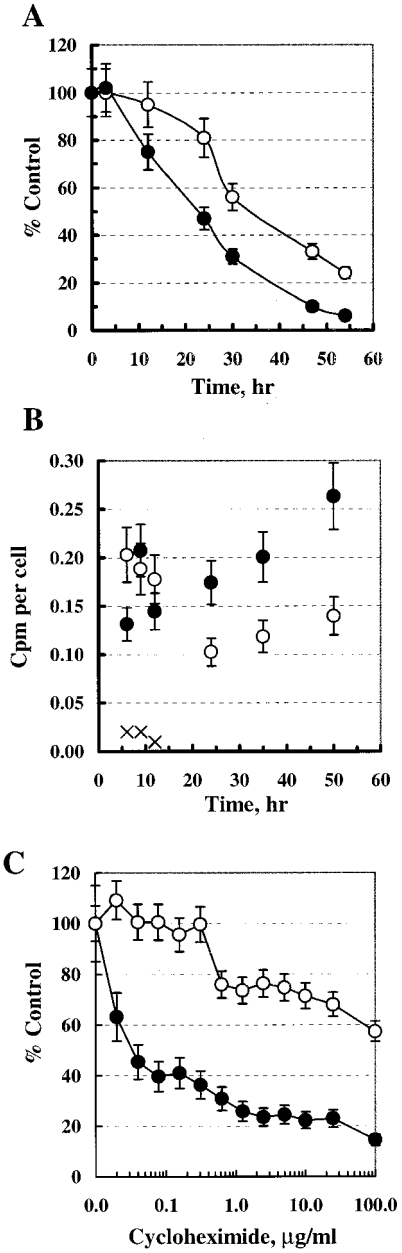

Figure 7. Protein synthesis inhibition in PAE/KDR. PAE/KDR cells were plated onto 24-well plates at densities of $6-10 \times 10^3$ cells/well and 20 h later exposed either to 2.5 nM SLT-VEGF/L or to varying amounts of cycloheximide. A, effects of SLT-VEGF/L on PAE/KDR growth (○) and ^{35}S-incorporation (●), B, effects of SLT-VEGF/L on ^{35}S-incorporation per cell for SLT-VEGF/L treated (○) and untreated cells (●). Effect of 0.1 mg/mL cycloheximide (X) is shown for comparison. C, Effects of cycloheximide on PAE/KDR growth (○) and ^{35}S-incorporation (●).

himide caused only a 40% inhibition of PAE/KDR cell growth (Figure 7*C*).

DISCUSSION

Growing endothelial cells at the sites of angiogenesis express high numbers of VEGF receptors (11−15) and therefore may be more sensitive to toxin−VEGF fusion proteins than quiescent endothelial cells. To test this hypothesis, we have constructed a protein containing the 293 amino acid catalytic A-subunit of Shiga-like toxin I fused to $VEGF_{121}$ (SLT-VEGF/L). Judging by its ability to inhibit protein synthesis in a cell-free system and to induce VEGFR-2 tyrosine autophoshorylation (Figure 2), both SLT and VEGF moieties in the fusion proteins are folded into functionally active conformations. It is somewhat surprising that VEGF isoform containing 121 amino acid residues can accommodate a 293 amino acid N-terminal extension and retain 3-D structure suitable for interactions with VEGFR-2 (*38*).

SLT-VEGF/L was tested on growing and quiescent endothelial cells and on endothelial cells expressing different levels of VEGFR-2. We report here that SLT-VEGF/L inhibits growth of PAE/KDR cells expressing 2.5×10^5 VEGFR-2 receptors/cell with an IC_{50} of ∼0.2 nM and induces massive apoptosis at concentrations ≥1 nM (Figure 3A). These effects are mediated by VEGFR-2, since parental cells lacking VEGFR-2 are only marginally affected by SLT-VEGF/L (Figure 3A).

However, the presence of VEGFR-2 appears to be necessary but not sufficient for the sensitivity of endothelial cells to SLT-VEGF/L. We found that quiescent PAE/KDR cells are not affected by a 72-h exposure to 20 nM SLT-VEGF/L, while growing PAE/KDR cells are killed by a 5-min exposure (Figure 3A and Figure 3C). Resistance of quiescent PAE/KDR to SLT-VEGF/L may be due to the lower metabolic rates in quiescent cells. Indeed, a lower sensitivity of quiescent endothelial cells to DT-VEGF conjugate was reported previously (*17*). Sensitivity to SLT-VEGF/L for growing endothelial cells expressing lower numbers of VEGFR-2 (∼ 6×10^4 and 2.5×10^4 receptors per cell) progressively declines to the level of PAE/V cells lacking VEGFR-2 (Figure 4). These results are in agreement with our previous finding that human umbilical vein endothelial cells (HUVEC) and mouse MS1 endothelial cells expressing $(3-5) \times 10^4$ VEGFR-2/cell are not sensitive to SLT-VEGF/L at concentrations as high as 20 nM (*28*). Taken together the requirements for growth and for high number of VEGFR-2 suggest that growing endothelial cells at the sites of angiogenesis cells would be more sensitive to SLT-VEGF/L than quiescent endothelium.

It should be noted that the lack of sensitivity of HUVEC to SLT-VEGF/L is in contrast to the reported high sensitivity of these cells to DT-VEGF proteins (IC_{50} of 36 pM) (*18*). The differential sensitivity of HUVEC may reflect differences in internalization, and/or intracellular processing of DT-VEGF and SLT-VEGF/L proteins (*39*−*41*). Furthermore, DT and SLT target different steps in protein biosynthesis, whereby DT catalyzes ADP-ribosylation of EF-2, while SLT damages ribosomes. However, the high activity of DT-VEGF against HUVEC raises a question whether this protein would discriminate between endothelial cells with low and high numbers of VEGFR-2 in a manner similar to SLT-VEGF/L.

Our finding that catalytically inactive SLT-VEGF/Lci does not affect PAE/KDR cells indicates that enzymatic inactivation of ribosomes is crucial for the cytotoxic activity of SLT-VEGF/L. However, we found that a collapse of protein synthesis is not required for SLT-VEGF/L induced onset of growth inhibition and apoptosis. Indeed, in the presence of 2.5 nM SLT-VEGF/L protein synthesis per PAE/KDR cell is inhibited marginally after 12 h, and only by 30% after 50 h (Figure 7B). Nevertheless, under the same conditions, apoptotic DNA laddering is readily detectable in treated cells after 9 h, and a 50-h treatment kills ∼30% of plated cells (Figure 3A).

There is evidence that bacterial toxins and immunotoxins may induce apoptosis not only through shortage of de novo synthesized proteins, but also through other mechanism(s) (*37*, *42*−*44*). Moreover, the inhibitory effect of toxins on protein synthesis may be overestimated

because many studies rely on comparison of ^{35}S-incorporation per well of treated vs control cells (see, for example *18*, *43*). In such comparison a decrease in ^{35}S-incorporation per well reflect both a decrease in a number of treated cell and a possible decrease in protein synthesis in each cells. Recently, Iordanov et al., discovered a ribotoxic stress response that can be initiated by site-specific damage of 28S rRNA by ricin and α-sarcin and by several other agents (*36*, *45*, *46*). This response includes activation of stress-activated protein kinases that initiate signal transduction cascades leading to apoptotic death (reviewed in *47*). Indeed, it was shown recently, that treatment of Caco-2 cells with SLT holotoxin induces expression of MAPK phosphatase (MKP-1) and activation of JNK (*37*). Alternatively, a surveillance mechanism may connect damage of ribosomes to transcription machinery that activate expression of pro-apoptotic cytokines. Indeed, it has been shown recently that treatment of monocytes, macrophages, and tumor cells with native SLT-1 induces cytokine production (*48*). Finally, existence of fine-tuned mechanisms for monitoring the integrity of ribosomes is suggested by the recent finding that ribosomal S6 protein is dispensable for growth but not proliferation (*49*). Regardless of molecular mechanism(s) of action, SLT-VEGF/L displays remarkably selective cytotoxicity against growing endothelial cells overexpressing VEGFR-2. Experiments are now in progress to determine efficacy of SLT-VEGF/L in animal angiogenesis models.

ACKNOWLEDGMENT

This work was supported in part by National Institutes of Health Grant # 1 R43 CA81832-01. We thank Dr. C. Hamby for numerous discussions and help in preparation of the manuscript.

LITERATURE CITED

(1) Folkman, J., and Shing, Y. (1992) Angiogenesis. *J. Biol. Chem. 267*, 10931−10934.

(2) Hanahan, D. (1997) Signaling vascular morphogenesis and maintenance. *Science 277*, 48−60.

(3) Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature, Med. 1*, 27−31.

(4) Gourley, M., and Williamson, J. S. (2000) Angiogenesis: new targets for the development of anticancer chemotherapies. *Curr. Pharm. Des. 6*, 417−439.

(5) Hanahan, D., and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell 86*, 353−364.

(6) Volpert, O. V., Dameron, K. M., and Bouck, N. (1997) Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene 14*, 1495−1502.

(7) Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999) Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J. 13*, 9−22.

(8) Claffey, K. P., and Robinson, G. S. (1996) Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. *Cancer Metast. Rev. 15*, 165−176.

(9) Veikkola, T., Karkkainen, M., Claesson-Welsh, L., and Alitalo, K. (2000) Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res. 60*, 203−212.

(10) Terman, B. I., and Dougher-Vermazen, M. (1996) Biological properties of VEGF/VPF receptors. *Cancer Metast. Rev. 15*, 159−163.

(11) Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Manseau, E. J., Dvorak, H. F., and Senger, D. R. (1993) Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *Am. J. Pathol. 143*, 1255−1262.

(12) Plate, K. H., Breier, G., Millauer, B., Ullrich, A., and Risau, W. (1993) Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res. 53*, 5822−5827.

(13) Detmar, M., Brown, L. F., Claffey, K. P., Yeo, K. T., Kocher, O., Jackman, R. W., Berse, B., and Dvorak, H. F. (1994) Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J. Exp. Med. 180*, 1141−1146.

(14) Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Guidi, A. J., Dvorak, H. F., Senger, D. R., Connolly, J. L., and Schnitt, S. J. (1995) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum. Pathol. 26*, 86−91.

(15) Couffinhal, T., Kearney, M., Witzenbichler, B., Chen, D., Murohara, T., Losordo, D. W., Symes, J., and Isner, J. M. (1997) Vascular endothelial growth factor/Vascular permeability factor (VEGF/VPF) in normal and artherosclerotic human arteries. *Am. J. Pathol. 150*, 1673−1685.

(16) Ramakrishnan, S., Olson, T. A., Bauch, V. L., and Mohanraj, D. (1996) Vascular endothelial growth factor-toxin conjugate specifically inhibits KDR/flk-1 positive endothelial cell proliferation in vitro and angiogenesis in vivo. *Cancer Res. 56*, 1324−1330.

(17) Olson, T. A., Mohanraj, D., Roy, S., and Ramakrishnan, S. (1997) Targeting the tumor vasculature: inhibition of tumor growth by a vascular endothelial growth factor-toxin conjugate. *Int. J. Cancer. 73*, 865−870.

(18) Arora, N., Maood, R., Zheng, T., Cai, J., Smith, L., and Gill, P. S. (1999) Vascular endothelial growth factor chimeric toxin is highly active against endothelial cells. *Cancer Res. 59*, 183−188.

(19) Obrig, T. G., Del Vecchio, P. J., Karmali, M. A., Petric, M., Moran, T. P., and Judge, T. K. (1987) Pathogenesis of haemolytic uraemic syndrome. *Lancet. 2*, 687.

(20) Richardson, S. E., Karmali, M. A., Becker, L. E., and Smith, C. R. (1988) The histopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infections. *Hum. Pathol. 19*, 1102−1108.

(21) Kaplan, B. S., Cleary, T. G., and Obrig, T. G. (1990) Recent advances in understanding the pathogenesis of the hemolytic uremic syndromes. *Pediatr. Nephrol. 4*, 276−283.

(22) Obrig, T., Louise, C., Lingwood, C., Boyd, B., Barley-Maloney, L., and Daniel, T. (1993) Endothelial heterogenecity in Shiga toxin receptors and responses. *J. Biol. Chem. 268*, 15484−15488.

(23) Olsnes, S., Reisbig, R., and Eiklid, K. (1981) Subunit structure of *Shigella* cytotoxin. *J. Biol. Chem. 256*, 48732−8738.

(24) Saxena, S. K., O'Brien, A. D., and Ackerman, E. J. (1989) Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA *N*- glycosideases of 28 S RNA when microinjected into *Xenopus oocytes*. *J. Biol. Chem. 264*, 596−601.

(25) Haddad, J. E., Al-Jaufy, A. Y., and Jackson, M. P. (1993) Minimum domain of the Shiga toxin A subunit required for enzymatic activity. *J. Bacteriol. 175*, 4970−4978.

(26) Al-Jaufy, A. Y., Haddad, J. E., King, S. R., McPhee, R. A., and Jackson, M. P. (1994) Cytotoxicity of a Shiga toxin A subunit-CD4 fusion protein to human immunodeficiency virus-infected cells. *Infect., Immun. 62*, 956−960.

(27) Al-Jaufy, A. Y., King, S. R., and Jackson, M. P. (1995) Purification and characterization of a Shiga toxin A subunit-CD4 fusion protein cytotoxic to human immunodeficiency virus-infected cells. *Infect., Immun. 63*, 3073−3078.

(28) Backer, M. V., Budker, V. G., and Backer, J. M (2001) Shiga-like toxin-VEGF fusion proteins are selectively cytotoxic to endothelial cells overexpressing VEGFR-2. *J. Controlled Release 74*, 349−355.

(29) Backer, M. V., and Backer, J. M (2001) Functionally active VEGF fusion proteins. *Protein Express. Purific. 23*, 1−7.

(30) Gitay-Goren, H., Cohen, T., Tessler, S., Soker, S., Gengrinovitch, S., Rockwell, P., Klagsbrun, M., Levi, B. Z., and Neufeld, G. (1996) Selective binding of VEGF121 to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. *J. Biol. Chem. 271*, 5519−5523.

(31) Deresiewicz, R. L., Austin, P. R., and Hovde, C. J. (1993) The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain. *Mol. Gen. Genet. 241*, 467−473.
(32) Cao, C., Kurazono, H., Yamasaki, S., Kashiwagi, K., Igarashi, K., and Takeda, Y. (1994) Construction of mutant genes for a nontoxic verotoxin 2 variant (VT2vp1) of *Escherichia coli* and characterization of purified mutant toxins. *Microbiol. Immunol. 38*, 441−447.
(33) Brigotti, M., Carnicelli, D., Alvergna, P., Mazzaracchio, R., Sperti, S., and Montanaro, L. (1997) The RNA-N-glycosidase activity of Shiga-like toxin I: kinetic parameters of the native and activated toxin. *Toxicon. 35*, 1431−1437.
(34) Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem. 269*, 26988−26995.
(35) Cohen, G. M. (1997) Caspases: the executioners of apoptosis. *Biochem. J. 326*, 1−16.
(36) Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L., and Magun, B. E. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol. Cell. Biol. 17*, 3373−3381.
(37) Kojima, S., Yanagihara, I., Kono, G., Sugahara, T., Nasu, H., Kijima, M., Hattori, A., Kodama, T., Nagayama, K. I., and Honda, T. (2000) mkp-1 encoding mitogen-activated protein kinase phosphatase 1, a verotoxin 1 responsive gene, detected by differential display reverse transcription-PCR in Caco-2 cells. *Infect Immun. 68*, 2791−2796.
(38) Muller, Y. A., Li, B., Christinger, H. W., Wells, J. A., Cunningham, B. C., and de Vos, A. M. (1997) Vascular endothelial growth factor: Crystal structure and functional mapping of the kinase domain receptor binding site. *Proc. Natl. Acad. Sci. U.S.A. 94*, 7192−7197.
(39) Umata, T., and Mekada, E. (1998) Diphtheria toxin translocation across endosome membranes. A novel cell permeabilization assay reveals new diphtheria toxin fragments in endocytic vesicles. *J. Biol. Chem. 273*, 8351−8359.
(40) Arab, S., and Lingwood, C. A. (1998) Intracellular targeting of the endoplasmic reticulum/nuclear envelope by retrograde transport may determine cell hypersensitivity to verotoxin via globotriaosyl ceramide fatty acid isoform traffic. *J. Cell Physiol. 177*, 646−660.
(41) Sandvig, K., and van Deurs, B. (1996) Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiol. Rev. 76*, 949−966.
(42) Chang, M. P., Bramhall, J., Graves, S., Bonavida, B., and Wisnieski, B. J. (1989) Internucleosomal DNA cleavage precedes diphtheria toxin-induced cytolysis. Evidence that cell lysis is not a simple consequence of translation inhibition. *J. Biol. Chem. 264*, 15261−15267.
(43) Bolognesi, A., Tazzari, P. L., Olivieri, F., Polito, L., Falini, B., and Stirpe, F. (1996) Induction of apoptosis by ribosome-inactivating proteins and related immunotoxins. *Int. J. Cancer 68*, 349−355.
(44) Keppler-Hafkemeyer, A., Brinkmann, U., and Pastan, I. (1998) Role of caspases in immunotoxin-induced apoptosis of cancer cells. Bi*ochem. 37*, 16934−16942.
(45) Shifrin V. I., and Anderson, P. (1999) Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *J. Biol. Chem. 274*, 13985−13992.
(46) Iordanov, M. S., and Magun, B. E. (1999) Different mechanisms of c-Jun NH(2)-terminal kinase-1 (JNK1) activation by ultraviolet-B radiation and by oxidative stressors. *J. Biol. Chem. 274*, 25801−25806.
(47) Chen, Y. R., and Tan, T. H. (2000) The c-Jun N-terminal kinase pathway and apoptotic signaling (review). *Int. J. Oncol. 16*, 651−662.
(48) Yamasaki, C., Nator, Y., Zeng, X. T., Ohmura, M., Yamasaki, S., Takeda, Y., and Natori, Y. (1999) Induction of cytokines in a human colon epithelial cell line by Shiga toxin 1 (Stx1) and Stx2 but not by nontoxic mutant Stx1 which lacks N-glycosidase activity. *FEBS Lett. 442*, 231−234.
(49) Volarevic, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., and George Thomas. (2000) Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science 288*, 2045−2047.

BC015534J