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Human aortic endothelial cells compare favourably with macrophages for the study of anthrax toxins

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Abstract: Anthrax is an infectious disease caused by *Bacillus anthracis*. Although *B. anthracis* is rare in the natural environment, anthrax spores can be produced in vitro and potentially used as a biological weapon. Thus, understanding the molecular pathogenesis of anthrax is a critical concern for national security. The aim of this study was to compare the effects of anthrax toxins, lethal toxin (LeTx), and edema toxin (EdTx) on human aortic endothelial cells (HAECs) and the J774A.1 murine macrophage cell line. We analysed cell viability, adhesion, morphology, and lipopolysaccharide (LPS)-stimulated cytokine production after incubation of cells with varying concentrations of the toxins. Both LeTx and EdTx markedly inhibited LPS-induced transcription of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 in J774A.1 cells. In contrast, EdTx synergised with LPS to increase the transcription of IL-6 and IL-8 in HAECs. We showed that HAECs are suitable for anthrax toxin research and express higher levels of the two anthrax toxin receptors – tumour endothelial marker 8 (TEM8/ANTXR1) and capillary morphogenesis protein 2 (CMG2/ANTXR2) – than do J774A.1 cells. Collectively, our results suggest that HAECs may be superior to macrophages for the study of anthrax pathogenesis.

Keywords: anthrax toxins; protective antigen; lethal toxin; edema toxin; macrophages; human aortic endothelial cells.

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1 Introduction

*Bacillus anthracis* is a gram-positive, aerobic, spore-forming, and rod-shaped bacterium that is the causative agent of the virulent disease, anthrax. The three main infection routes for anthrax are ingestion (gastrointestinal), skin contact (cutaneous), and inhalation (pulmonary) [1]. Among these, inhalational anthrax is generally considered the most life-threatening form of the disease, which commonly presents with flu-like symptoms, chest pain, irregular breathing, tachycardia, hypotension, headache, and disorientation, and ultimately results in death [2–4].

The toxin secreted by *B. anthracis* is a tripartite toxin, comprising protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa) [5,6]. PA binds to and forms a complex with 1 of 2 widely expressed cell surface receptors, namely, tumour endothelial marker 8 (TEM8/ANTXR1) or capillary morphogenesis protein 2 (CMG2/ANTXR2), and is cleaved by cellular furin-like enzymes to generate an active 63-kDa product [7]. The receptor-bound 63-kDa PA combines with LF or EF to form the lethal toxin (LeTx) or edema toxin (EdTx), respectively. This multi-protein toxin complex is then internalised by the host cell within endosomal vesicles, after which EF and LF translocate from the toxin complex into the cytoplasm [8–10]. Once within the cytosol, LF cleaves several members of the mitogen-activated protein kinase kinase family (MAPKKs), thereby blocking activation of the major MAPKs, including extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK), and p38 MAPK [11–14]. EF is a calcium/calmodulin-dependent adenylate cyclase and increases intracellular cyclic AMP (cAMP) levels [6,15]. Knockout of EF or LF in the anthrax genome reduces the bacterial virulence by 10- or 1000-fold, respectively [16].

Once internalised, the anthrax toxins cause cell death by inducing apoptosis and necrosis or by exerting a cytolytic effect [17,18]. LeTx can infect and kill macrophages in certain inbred mice [6], and high concentrations of LeTx induce apoptosis in endothelial cells [19]. Because cardiovascular collapse is the ultimate cause of death of the infected host [4,20–22], we wished to evaluate the effect of the anthrax toxins on endothelial cell function. Here, we identified the potential utility of human aortic endothelial cells (HAECs) as a tool to investigate the molecular mechanisms underlying anthrax pathogenesis. Our study revealed that LeTx and EdTx have distinct effects on murine macrophages and human endothelial cells.
2 Materials and methods

Macrophage and endothelial cell culture

The murine macrophage cell line J774A.1 was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea), and cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (Hyclone, Grand Island, NY). HAECs were purchased from PromoCell (Heidelberg, Germany) and grown in endothelial cell basal medium-2 (EGM-2; Lonza, Basel, Switzerland) supplemented with 0.1% recombinant human epidermal growth factor, 0.4% recombinant human fibroblast growth factor, 0.1% gentamycin-amphotericin B (GA-1000), 0.1% recombinant insulin-like growth factor, 0.1% ascorbic acid, 0.1% vascular endothelial growth factor, 0.1% heparin, 0.04% hydrocortisone, and 2% FBS. Cells were grown in 100-mm dishes coated with 0.1% gelatin in PBS to facilitate stable cell attachment and incubated at 37°C in a humidified 5% CO₂ incubator. HAECs and J774A.1 cells were harvested by treatment with 0.25% trypsin/EDTA (Hyclone) or accutase (Innovative Cell Tech, San Diego, CA), respectively. Experiments were performed with HAECs between the fifth and eighth cell passage.

Toxin treatment

The three toxins were supplied by the Agency for Defense Development (ADD, Korea). The concentrations of purified PA, LF, and EF were 1.7, 1.8, 1.6 mg/mL, respectively. Toxins were diluted in culture medium before addition to cells.

Water-soluble tetrazolium assay

Cells were seeded in 6-well plates and incubated overnight at 37°C in a 5% CO₂ humidified atmosphere. Following treatment with the indicated concentrations of anthrax toxins, cell viability was measured using the water-soluble tetrazolium (WST) colorimetric assay (Ez-Cytox, Daeil Lab Service, Seoul, Korea). Absorbance was measured at 450 nm using a microplate reader (Tecan, Männedorf, Switzerland).

Cell adhesion assay

Cell adhesion was measured using the label-free xCELLigence System for real-time cell analysis (Roche Diagnostics, Rotkreuz, Switzerland). Cells were seeded into 96-well plates containing embedded gold microelectrodes, and the assay was performed according to the manufacturer’s instructions. Attachment of cells to the bottom of the plates was measured by the electrical impedance, which was quantified as the Cell Index (CI). To control for well-to-well variation in cell number, data are presented as CI normalised to the signal recorded just before toxin treatment. Cell adhesion was monitored every 15 s for 4.5 h and then, every 15 min for the remainder of the incubation.

Real-time live cell imaging

Cells were cultured in 96-well plates. Growth curves were determined using the IncuCyte live cell imaging system (Essen Bioscience, Ann Arbor, MI), which is an automated and non-invasive method for long-term monitoring of live cells in culture. The morphology of toxin-treated cells was observed in specific areas of the wells at 2-h intervals over a 72-h period.
Albumin permeability assay

Cells were seeded on porous polyester membrane inserts of Transwell plates (12-mm diameter, 0.4-µm pore size; Transwell, Corning, Cambridge, MA). The upper and lower chambers contained 0.5 and 1.5 mL of medium, respectively. The indicated concentrations of EdTx were added to the upper chamber, and the plates were incubated for 48 h. The culture medium in the upper chamber was then replaced with an equal volume of medium containing FITC-coupled albumin (final concentration, 0.5 mg/mL; A7016, Sigma-Aldrich, Saint Louis, MO). After 2 h, samples were drawn from the lower chamber to measure FITC-albumin content. Fluorescence measurements were obtained using a microplate reader (Tecan) with excitation and emission filters of 485 and 530 nm, respectively. Albumin content was quantified using FITC-conjugated human serum albumin (HSA) as a standard.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from HAECs and J774A.1 cells with Hybrid-R (GeneAll, Seoul, Korea) according to the manufacturer’s instructions. cDNA synthesis was performed with the SuperScript First Strand Synthesis System (Invitrogen, Grand Island, NY), and quantitative real-time polymerase chain reaction (qRT-PCR) of anthrax toxin receptors and cytokines was performed using the primer sequences listed in Table 1. Specific transcript levels were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression for mouse and human genes, respectively. qRT-PCR was performed on a LightCycler 2.0 system (Roche Diagnostics). PCR cycling conditions consisted of 10 min at 95°C followed by 45 cycles of 10 s at 95°C and 30 s at 55°C.

Table 1  Primers sequences for qRT-PCR

| Human primer sequences | Mouse primer sequences |
|------------------------|------------------------|
| **Name**: Sequence 5’-3’UPL | **Name**: Sequence 5’-3’UPL |
| **ANTXR1 F**: TGGGTCTCTCTGAGGAAGG-76 | **ANTXR1 F**: TGACCGAGTTGCTTGATGAG-76 |
| **ANTXR1 R**: GACCCTGTTGAAGTGTGC | **ANTXR1 R**: TCACCTGTTGGAAGTGATGC |
| **ANTXR2 F**: GAGGAAAAACTGTGCTATTCAAGA-5 | **ANTXR2 F**: GGGAATGTGCTGCTGAAGA-5 |
| **ANTXR2 R**: GCTGGCGATCCCCTGTTAGAA | **ANTXR2 R**: GATGCGCTCAACTCCCAT |
| **IL-6 F**: CAGGAGCCACCTAGGA-77 | **IL-6 F**: GCACACCTAATGATAGA-77 |
| **IL-6 R**: AGGGCCGCAACCAGGAG | **IL-6 R**: CCAAGTAGCTATGGACTCAGA |
| **IL-8 F**: AGACGACGACACAAGC-72 | **IL-8 F**: TAGGAAGGGACCGCAGAACC-78 |
| **IL-8 R**: ATGTGTCCTCCCGTGGT | **IL-8 R**: TCTTCTGCTTCTGCTTG |
| **HPRT F**: CTCAACTTTAACTGGAAAGAATGTC | **HPRT F**: CTGTAAGCCCCACGTCGTCG |
| **HPRT R**: TCCCTTTCCAGCAAGC | **HPRT R**: CACATAGTCGCTTG |
| **GAPDH F**: GAGCCCAACCGGGTCTACA-29 | **GAPDH F**: CATATTTCTCGTGTCGAAC |
| **GAPDH R**: CATATTTCTCGTGTCGAAC | **GAPDH R**: CATATTTCTCGTGTCGAAC |
Whole-cell protein extraction and western blot analysis

Cells were extracted for 30 min with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (Roche Diagnostics). Protein concentrations were measured by the BCA assay (Thermo Scientific Inc., Rockford, IL). Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore Corporation, Billerica, MA). The membranes were blocked in 5% or 10% non-fat milk in 0.1% Tris-buffered saline containing tween-20 (TBS-T) for 1 h. Blots were then incubated with the primary antibodies (1:1000) in 1% non-fat milk in TBS-T overnight at 4°C. Primary antibodies were phospho (p)-ERK (E-4 clone, sc-7383, Santa Cruz Biotechnology, Santa Cruz, CA), ANTXR1 (PAB6677, Abnova, Walnut, CA), and ANTXR2 (16723-1-AP, Protein Tech Group, Chicago, IL). Blots were then washed with TBS-T for 30 min and incubated with HRP conjugated goat anti-mouse, rabbit anti-goat, or goat anti-rabbit secondary antibodies (1:5000) for 1 h at room temperature. Blots were probed with antibody to GAPDH (FL-335 clone, sc-25778, Santa Cruz Biotechnology) as a loading control.

Intracellular cAMP enzyme-linked immunosorbent assay

The Biotrak cAMP enzyme-linked immunosorbent assay (ELISA) kit (GE Healthcare, Buckinghamshire, England) was used to measure intracellular cAMP concentrations, according to the manufacturer’s instructions. Standards (0–3200 fmol cAMP), and samples were added to the wells of a donkey anti-rabbit Ig-coated plate. cAMP concentration was measured by competition for binding to a specific rabbit anti-cAMP primary antibody in the presence of known amounts of a cAMP-peroxidase conjugate. Concentrations of cAMP in the experimental samples were quantified using a standard curve.

3 Results

Effects of LeTx and EdTx on J774A.1 cells and HAECs

We first determined whether LeTx and EdTx were cytotoxic for J774A.1 cells. To form the toxins, 500 ng/mL PA was added to the cultures, with varying concentrations of LF or EF. LeTx formed from PA and 1 ng/mL LF reduced J774A.1 cell viability at 96 h but not at 6 h (Figure 1(A)). However, LF concentrations greater than 10 ng/mL caused 100% cell death within 6 h. Moreover, the combination of PA (500 ng/mL) and LF concentrations of 10, 100, or 1000 ng/mL resulted in 100% cell death within 2 h (Figure 1(B)). In contrast to LeTx, EdTx failed to affect macrophage viability even at 500 ng/mL PA and EF concentrations as high as 1000 ng/mL (Figure 1(C)). A cell adhesion assay was performed to identify an anthrax toxin-mediated adhesion change. We observed no change in cell adhesion by J774A.1 cells in the presence of LeTx, except that adhesion was reduced at LeTx concentrations that induced cell death (1, 10, 100, and 1000 ng/mL; Figure 1(D)). However, high doses of EdTx (1–1000 ng/mL) caused a striking, dose-dependent loss of J774A.1 cell adhesion. Interestingly, low doses (0.1–0.01 ng/mL) of EdTx appeared to increase J774A.1 cell adhesion.
Figure 1  Effects of LeTx and EdTx on J774A.1 cell viability, adhesion, and morphology. The effect of different concentrations of the anthrax toxins on cell viability was assessed at 6 and 96 h incubation (A, C) or at varying times in the first 10 h of incubation (B). (D) Cells were incubated with the indicated concentrations of LF or EF in presence of PA (500 ng/mL). Cell adhesion, expressed as normalised CI, was measured with the xCELLigence system for 20 h prior to and 40 h after toxin challenge. (E) J774A.1 cells were treated with a combination of PA and LF or EF (1 ng/mL). Cell morphology was monitored in the same area of the wells for 12 h using real-time live cell imaging. The scale bar is 100 µm (see online version for colours).

To better understand the effects of LeTx and EdTx on J774A.1 cell morphology, we visualised the cells at 0, 2, and 6 h after toxin addition using real-time live cell imaging. In contrast to EdTx, LeTx caused significant cell death by 6 h (Figure 1(E)). Taken together, these findings demonstrated that macrophage cell death was maximal in the presence of LeTx formed from 1 ng/mL LF and 500 ng/mL PA.

We next examined the effects of anthrax toxins on the viability and adhesion of HAECs. The kinetics of the LeTx effect on HAEC viability was different from that observed for macrophages. LeTx (1 ng/mL LF and 500 ng/mL PA) reduced HAEC cell viability at much later incubation time (48 or 72 h; Figure 2(A)). In contrast, EdTx did not significantly affect HAEC viability at any incubation time (data not shown). Although LeTx induced HAEC death at concentrations between 1 ng/mL and 1000 ng/mL, changes in cell adhesion were observed with less than 0.1 ng/mL LF (Figure 2(B)). Similarly, EdTx slightly reduced cell adhesion (Figure 2(B)) and also increased the permeability of the HAEC layer to albumin (Figure 2(C)) at concentrations that had no effect on cell viability. Consistent with the toxins’ effects on cell viability,
Human aortic endothelial cells compare favourably with macrophages

cell debris was apparent in cultures containing LeTx, but not EdTx, when the cells were visualised by real-time live cell imaging for 24 h (Figure 2(D)).

**Figure 2** Effects of LeTx and EdTx on HAEC viability, adhesion, permeability, and morphology. (A) Cell viability was measured following incubation of HAECs with increasing concentrations of LeTx for up to 72 h. (B) Cell adhesion was measured with the xCELLigence system. Cell adhesion was determined at 15-s intervals for the first 4.5 h and then every 15 min up to 72 h. (C) The albumin permeability assay was performed after cells were treated with EdTx for 48 h. (D) HAECs were treated with a combination of PA (500 ng/mL) and LF or EF (10 ng/mL). Cell morphology was determined in the same area for 12 h by real-time live cell imaging. The scale bar is 100 µm (see online version for colours).

**Effects of LeTx and EdTx on cytokine expression by J774A.1 cells and HAECs**

Stimulation of macrophages with bacterial lipopolysaccharide (LPS) potently induces proinflammatory cytokine expression [23–25]. To examine the effects of anthrax toxins on cytokine expression, we assessed TNF-α, IL-1β, and IL-6 mRNA expression in J774A.1 cells incubated with LPS in the presence or absence of LeTx or EdTx. Addition of LeTx dose-dependently decreased the LPS-induced transcription of TNF-α, IL-1β, and IL-6 in these cells (Figure 3(A)–(C)), whereas co-treatment with EdTx dramatically inhibited LPS-induced cytokine mRNA expression, even at very low EdTx concentrations (Figure 3(D)–(F)).

In contrast, LeTx and EdTx had distinct effects on LPS-induced expression of IL-6 and IL-8 mRNA in HAECs (Figure 4). While LeTx slightly inhibited LPS-induced IL-6 mRNA levels, EdTx had a synergistic effect on LPS-induced transcription of IL-6 and IL-8 in HAECs.
Figure 3  LeTx- and EdTx-induced changes in cytokine expression in J774A.1 cells. J774A.1 cells were treated with LPS (100 ng/mL) in the presence or absence of the indicated concentrations of LeTx or EdTx for 6 h. Levels of TNF-α (A, D), IL-1β (B, E), and IL-6 (C, F) mRNA were measured by qRT-PCR and normalised to expression of the reference gene GAPDH.

Figure 4  LeTx- and EdTx-induced changes in cytokine expression in HAECs. HAECs were treated with LPS (100 ng/mL) in the presence or absence of the indicated concentrations of LeTx or EdTx for 6 h. Levels of IL-6 (A, C) and IL-8 (B, D) mRNA were measured by qRT-PCR and normalised to expression of the reference gene HPRT.
Expression of ANTXR1 and ANTXR2 anthrax receptors on J774A.1 cells and HAECs

The mechanism of anthrax toxin entry into cells is thought to be different according to the cell type or receptor usage. The receptor type has a profound influence on the pH required for pore formation and the translocation of anthrax toxin into the cell [26]. We measured the mRNA and protein levels of the anthrax receptors by qRT-PCR and immunoblotting, respectively, and found that both receptors were expressed at much higher levels in HAECs than in J774A.1 cells (Figure 5(A)). Previous work has suggested that the anthrax toxin enters endothelial cells via cell surface anthrax receptors, whereas phagocytosis is the main mechanism by which the toxins gain entry to macrophages [27,28]. Moreover, anthrax may exhibit different pathogenic mechanisms in HAECs localised to the interior of blood vessels compared with macrophages present in tissues [19]. Thus, the expression of high levels of the anthrax receptors by HAECs clearly suggests that these cells may be ideal for the study of the toxins’ pathogenic mechanisms of action in humans.

Figure 5  Expression of anthrax toxin receptors and authentication of the experimental toxin preparations in J774A.1 cells and HAECs. (A) Upper panel: Total RNA was isolated from J774A.1 cells and HAECs, and expression of ANTXR1 and ANTXR2 mRNA was determined by qRT-PCR. Expression levels were normalised against HPRT. Lower panel: Cell lysates from J774A.1 cells and HAECs were subjected to immunoblotting with antibodies specific for ANTXR1 and ANTXR2. GAPDH was detected as a loading control. (B) HAECs were treated with LeTx (500 ng/mL of PA and 1000 ng/mL of LF) for the indicated times. Cell lysates were subjected to immunoblotting with antibodies specific for phosphorylated ERK1/2. GAPDH was detected as a loading control. (C) HAECs were treated with EF alone or with or EdTx (500 ng/mL of PA plus 1000 ng/mL of EF). At the indicated times, cells were harvested and intracellular cAMP levels were measured by ELISA.
Inactivation of ERK by LeTx and induction of cAMP by EdTx in HAECs

Finally, we verified that the toxins used in our study with HAECs evoked similar intracellular effects as in macrophages. Phosphorylation of macrophages ERK1 and ERK2 has been reported to be inhibited by pretreatment with LeTx [29,30]. On the other hand, an earlier report demonstrated that a brief treatment with EdTx caused a robust increase in intracellular cAMP levels in murine macrophages [31]. Our findings here are consistent with these earlier observations. Treatment of HAECs with LeTx inhibited the phosphorylation of ERK1/2 in a time-dependent manner (Figure 5(B)). Similarly, treatment of HAECs with EF alone or with EdTx both increased intracellular cAMP concentrations in HAECs compared with untreated cells (Figure 5(C)). These results indicated that anthrax toxins have the same effects on HAECs and macrophages.

4 Discussion

The effects of anthrax toxin have been investigated in an array of cell types (Table 2). Identifying the events occurring during the early macrophage response to *B. anthracis* exposure is important to our understanding of the pathogenesis of the disease. Human alveolar macrophages play a key role in the innate immune response to *B. anthracis* spores. In contrast, we found that the murine macrophage cell line J774A.1 was very sensitive to LeTx toxicity and showed reduced viability within a few hours of treatment. Chakrabarty et al. reported that dendritic cells, derived from peripheral blood monocytes, exhibited weak activation of ERK1/2, strong activation of p38, but no activation of JNK after endocytosis of *B. anthracis* spores [32]. The alveolar macrophage, which plays a vital role in pulmonary defense, is the primary site of *B. anthracis* spore germination in murine models of inhalation infection [33]. In another study, LeTx triggered apoptosis in both human umbilical cord endothelial cells and dermal microvascular endothelial cells [34]. Neutrophils are also important in the defense against anthrax in humans; moreover, differentiated NB4 neutrophils are capable of killing *B. anthracis* in vitro [35]. LeTx shows a more subtle effect on motility and chemotaxis in human neutrophils than in macrophages [35].

Blood vessels are the key target tissue for anthrax pathogenesis. Anthrax toxins especially target the vascular endothelium, and symptoms of systemic anthrax infection are mostly closely related with the vascular system [18]. HAECs have advantages over macrophages as an in vitro model for anthrax studies, because they allow a more biologically relevant analysis of the interaction between anthrax toxins and the human host. Because there has been little investigation of HAECs as a target of anthrax toxins, we compared the effects of the toxins on HAECs and J774A.1 macrophages.

The results of our study indicated that HAECs were more resistant than J774A.1 cells to LeTx based on the cell viability experiments. On the other hand, cell adhesion by HAECs was reduced by LeTx at doses that did not induce cell death (0.01 and 0.1 ng/mL). Although the effect of EdTx on cell adhesion differed between the 2 cell types, high doses of EdTx can clearly reduce cell adhesion. Moreover, EdTx also affected cell permeability. We observed cell morphology using real-time live cell imaging, which showed that LeTx affected macrophage morphology within 6-h incubation but did not affect endothelial cell morphology until 24-h incubation. Our assessment of cytokine modulation showed that LPS-induced expression of TNF-α, IL-β, and IL-6 mRNA was
Human aortic endothelial cells compare favourably with macrophages

...dramatically decreased by LeTx and EdTx in J774A.1 cells. This data is consistent with a previous study that reported that proinflammatory cytokine production by macrophages is inhibited by anthrax toxins [36]. Because LeTx and EdTx had different effects on the expression of cytokines, especially IL-6, in macrophages and HAECs, anthrax toxins may modulate the immune response by having distinct effects on different host cells.

Table 2  Studies of anthrax toxins in different cell types

| Cell type                                   | Toxin   | Results                                                                 | Reference |
|---------------------------------------------|---------|-------------------------------------------------------------------------|-----------|
| Primary mouse macrophages                   | Spores  | Important for survival of *B. anthracis* in early stages of infection   | [33]      |
| Mouse J774A.1 macrophages                  | LeTx    | Apoptosis by LeTx through p38 MAPK inhibition                           | [29]      |
| Human umbilical cord endothelial cells and d | LeTx    | MAPK inhibition and anti-angiogenesis                                   | [19]      |
| dermal microvascular endothelial cells      |         |                                                                         |           |
| Mouse macrophages                           | Spores  | Inhibition of cytokine response                                          | [37]      |
| Human lung microvascular endothelial cells  | LeTx    | LeTx-induced endothelial barrier dysfunction                           | [38]      |
| Human lung microvascular endothelial cell   | LeTx    | Dysfunction of endothelial cell induced by LeTx was independent of MAPK | [39]      |
| Human alveolar macrophages                  | Spores  | Induction of cytokines and chemokines through MAPK pathway              | [32]      |
| Human umbilical cord endothelial cells and HL60 line | LeTx | Inhibition of MAP kinase signaling                                      | [34]      |
| Human alveolar macrophages                  | LeTx    | Inhibition of cytokine secretion                                        | [40]      |
| Human coronary artery endothelial cells     | LeTx    | No enhancement of TNF-α-induced VCAM-1 or E-selectin expression         | [41]      |
| Human brain microvascular endothelial cells | LeTx    | Inhibition of neutrophil signaling pathway                              | [42]      |
| Human differentiated NB4 neutrophils        | LeTx    | Suppression of chemokine production                                     | [35]      |
| Human coronary artery endothelial cells     | LeTx    | Vascular pathogenesis may result from a direct interaction of LeTx with endothelial cells | [43]      |
| Human umbilical cord endothelial cells      | LeTx    | Increase of endothelial permeability by LeTx cytotoxicity              | [44]      |

In summary, this is the first report showing that HAECs are suitable for anthrax toxin research. Both toxin receptors – ANTXR1 and ANTXR2 – are expressed at much higher levels in HAECs than in J774A.1 cells; thus, the receptors may allow the toxins to enter endothelial cells once released from macrophages. HAECs may thus be an excellent cell type for further studies on anthrax pathogenesis. Despite substantial progress in understanding anthrax pathogenesis, the ever-increasing threat that *B. anthracis* spores may be used as a biological weapon has increased the need for more detailed research on anthrax pathogenesis. The key finding of our study is that we have identified HAECs as a pathologic target of anthrax toxins. Our findings will therefore have immense value in the further development of protective measures against anthrax infection.
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