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Simvastatin Protects against Amyloid β and HIV-1 Tat-Induced Promoter Activities of Inflammatory Genes in Brain Endothelial Cells

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ABSTRACT

Increased deposition of amyloid β (Aβ) is characteristic for normal aging and human immunodeficiency virus-1 (HIV-1)-associated alterations of the central nervous system. In addition, both Aβ and HIV-1 are known to induce cellular oxidative stress and disruption of the blood-brain barrier (BBB). Therefore, we hypothesize that Aβ and HIV-1 protein Tat can potentiate their proinflammatory effects at the brain endothelium level. To address this hypothesis, we studied promoter activity of three proinflammatory genes in an in vitro BBB model of human brain microvascular endothelial cells (HBMEC) cocultured with a human astrocyte cell line producing Tat (SVGA-Tat cells) and exposed to Aβ. Treatment of HBMEC with Aβ(1-40) in the presence of SVGA-Tat cells resulted in a significant up-regulation of E-selectin, CC chemokine ligand-2, and interleukin-6 promoter activities and protein levels compared with the individual effects of Aβ or Tat. In addition, Aβ markedly amplified E-selectin promoter activity in HBMEC cocultured with HIV-1-infected Jurkat T cells. Simvastatin, the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, effectively blocked proinflammatory reactions induced by Aβ in cocultures with SVGA-Tat cells or with HIV-1-infected Jurkat cells. The present study indicates that a combined exposure to Aβ and Tat or HIV-1 can synergistically potentiate the expression of inflammatory genes in brain endothelial cells. In addition, simvastatin may provide a beneficial influence by reducing these effects at the BBB level.

A new challenge in the HIV/AIDS epidemic in Western countries is an increase in infection rates among the older population. It is estimated that approximately 15 to 20% of HIV-1-infected people in the United States are 50 years of age or older (Shah and Mildvan, 2006). This is partly caused by increased survival rates as a result of more effective antiretroviral therapy. In addition, the rates of new HIV-1 infections are rapidly growing in older people. Because of a variety of sociological, biological, and medical implications, this trend may further increase in the near future. These facts are of significant concern because elderly patients develop AIDS more rapidly and have higher morbidity and mortality rates. In addition, older patients are more likely to develop HIV-associated dementia. This phenomenon seems not to be related to the length of HIV-1 infection (Valcour and Paul, 2006), suggesting that aging can independently aggravate HIV-1-associated central nervous system pathology.

Among a variety of mechanisms that can be affected by HIV infection and aging, alterations of the blood-brain barrier (BBB) seem to be especially important. Even normal aging can lead to the BBB disturbances, including disrupted microvascular integrity, higher permeability to albumin, and accumulation and depositions of amyloid β (Aβ) that precede neuronal degeneration and dementia (Price and Morris, 1999). In addition, disturbances of the BBB are associated with HIV-1 infection and HIV-1 trafficking into the brain (Persidsky and Poluektova, 2006).

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ABBREVIATIONS: HIV, human immunodeficiency virus; Aβ, amyloid β; AD, Alzheimer's disease; AIDS, acquired immune deficiency syndrome; BBB, blood-brain barrier; CCL-2, CC chemokine ligand-2, chemoattractant protein-1; EGFP, enhanced green fluorescent protein; HBMEC, human brain microvascular endothelial cell; IL, interleukin; NF-κB, nuclear factor-κB; LTR, long terminal repeats; SVGA, a human astrocytic subclone of SVG cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

It has been hypothesized that the BBB pathology in HIV-1 infection is mediated by soluble factors released from infected cells. One such mediator may be the HIV-1 protein Tat, which normally serves as a transcriptional transactivator critical for HIV-1 replication (Nath and Geiger, 1998). In support of its pathogenic role, Tat was detected in the perivascular cells of AIDS patients with HIV-associated encephalitis (Gartner, 2000). Tat can easily cross cell membranes and can affect brain endothelial cells, including stimulation of inflammatory responses and alterations of tight junction protein expression (Toborek et al., 2005). It is interesting that Tat can elevate Aβ levels in the central nervous system by inhibiting the Aβ-degrading enzyme, neprilysin (Rempel and Pulliam, 2005). This phenomenon may have important implications for older HIV-infected patients. Indeed, HIV-1 infection and prolonged antiretroviral therapy were demonstrated to contribute to an increase in Aβ deposition in older HIV-infected individuals (Green et al., 2005). In addition, Aβ levels in the cerebrospinal fluid were shown to correlate with AIDS dementia complex (Brew et al., 2005).

Aβ is the major constituent of senile plaques and cerebrovascular deposits. It is deposited in the brain parenchyma and cerebral blood vessels in normal aging and Alzheimer's disease (AD). It was demonstrated that the BBB can regulate Aβ transport into the brain via two main receptors: the low-density lipoprotein receptor-related protein 1 and the receptor for advanced glycation end products (Deane and Zlokovic, 2007). The main forms of Aβ are the 1 to 40 and 1 to 42 fragments originating from amyloid precursor protein by physiological proteolytic processes. Soluble Aβ(1-40) is present mainly in the circulation and in cerebral vascular amyloid. In contrast, Aβ(1-42) is mainly localized to the senile plaques (Seubert et al., 1992). Exposure to Aβ can affect the metabolism of endothelial cells by several mechanisms, including the induction of inflammatory responses (Paris et al., 2002; Toborek et al., 2005). Aβ can also stimulate the infection of target CD4-positive cells by HIV-1 (Wojtowicz et al., 2002).

Based on these reports, we hypothesize that Aβ and HIV-1 Tat can potentiate their toxic effects. Because of their role in HIV and aging pathology, we focused on the mechanisms of microvascular endothelial injury and the BBB dysfunction. The present study indicates that Aβ(1-40) and Tat can cross-amplify their proinflammatory effects in an in vitro model of the BBB.

Materials and Methods

Cell Cultures

Human Brain Microvascular Endothelial Cells. Brain endothelial cells used in the present study represent the first stable, fully characterized, and well-differentiated human brain endothelial cell line (Weksler et al., 2005). Human brain microvascular endothelial cells (HBMECs) were cultured in EBM-2 medium (Clonetics, East Rutherford, NJ) supplemented with vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, basic fibroblast growth factor, hydrocortisone, ascorbate, gentamicin, and 2.5% fetal bovine serum (FBS) as described originally (Weksler et al., 2005). Collagen type I (BD Biosciences Pharmingen, San Jose, CA) was used for coating the cell culture dishes.

Tat-Expressing Astrocytes. SVGA cells, which represent a human astrocyte cell line, were stably transfected with Tat expression construct (SVGA-Tat cells) as described and characterized previously (Chauhan et al., 2007). Control SVGA cells were transfected with an empty vector. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2.

Jurkat T Cells. Jurkat cells (a human peripheral blood leukemia T cell line) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2.

Coculture Models. HBMEC were plated onto polycarbonate membranes of the upper chamber of the Transwell system (pore size, 0.4 μm; Corning Costar, Corning, NY). SVGA, SVGA-Tat, or Jurkat cells were cultured in the lower chamber of the system.

Generation of HIV-1 Stock and Infection of Jurkat Cells

HIV-1 stock was generated using 293T cells (American Type Culture Collection, Manassas, VA). The cells were grown in DMEM containing 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). When the cultures reached ~70% confluence, the cells were transfected with the NL4-3 plasmid containing full-length proviral DNA using calcium phosphate. After transfection, cells were incubated for 24 h in RPMI 1640 medium with 10% FBS and penicillin plus streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin). Then culture supernatants were collected, filtered through 0.45 μm filters (Millipore, Bedford, MA), and frozen at −80°C. HIV-1 p24 levels in the supernatants were determined by enzyme-linked immunosorbent assay (ZeptoMetrix, Buffalo, NY) as the marker of HIV-1 infection.

HIV-1 stock was used to infect Jurkat T cells. In brief, 4×10^6 Jurkat cells were cultured in T-25 flasks (Corning Costar) in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were infected with viral isolate containing 65 ng of HIV-1 p24 and incubated at 37°C and 5% CO_2. The cultures were maintained for up to 18 days with fresh medium added every other day. Noninfected Jurkat cells were used as controls.

Treatment Factors

Aβ(1-40) (active peptide) and Aβ(40-1) (negative control with the reverse amino acid sequence) were purchased from Anaspec (San Jose, CA) and dissolved in sterile ultrapure water obtained from ELGA Purelab Classic (Lowell, MA). Freshly solubilized Aβ solutions without preaggregation were used in our experiments. Such form of Aβ was demonstrated to induce proinflammatory reactions in isolated rat brain microvessels (Paris et al., 2002).

The cells were treated with Aβ(1-40) at the concentrations of 0.1 to 1 μM for 20 h in EBM-2 medium. HIV-1 Tat strongly binds to serum proteins; therefore, all experiments in cocultures with SVGA-Tat cells were performed in EGM-2 medium without serum or growth factors. However, the experiments that involved treatment with Aβ in the presence of HIV-infected Jurkat cells were performed in cell culture media containing 10% FBS. Cells cultured on chamber slides were treated with 1 μM Aβ(1-40) and SVGA or SVGA-Tat conditioned medium. In selected experiments, coculture systems were pretreated for 15 min with 5 or 10 μM simvastatin followed by cotreatment with Aβ(1-40) for 20 h. Both simvastatin and Aβ were added to the upper and lower chambers of the Transwell systems. Simvastatin was left in cell culture media for the duration of Aβ treatment. Our preliminary studies (data not shown) indicated that simvastatin concentrations between 5 and 20 μM did not affect cell viability.

Promoter Constructs

Inflammatory gene promoter constructs (pGL3 E-selectin, pGL3 CCL-2, and pGL3 IL-6) were generated based on the pGL3 luciferase basic vector (Promega, Madison, WI). In brief, 5′-flanking regions of human E-selectin (−1014/+36), CCL-2 (−1699/+18), and IL-6

(−1026/+15) were amplified by polymerase chain reaction from human genomic DNA and individually cloned into the pGL3 basic vector by inserting fragments between MluI and NcoI site.

To generate pcDNA3 Tat86 expression construct, HIV Tat(1–86) sequence was amplified using Tat1 BamHI primer, 5′-AGA TCT GGA TCC ATG GAG CCA GTA GAT CCT-3′ and Tat86 XhoI primer, 5′-GAA TTC CTC GAG CTA TTC CTT CGG GCC TGT CGG GTC CCC TCG GGA TTG GGA GGT GGG TTG CTT TGA TAG AGA AGC TTG-3′ harboring exon II of Tat (underlined) and pNL4–3 template. The amplified fragment was digested with BamHI and XhoI and then cloned into the pcDNA3 vector.

To generate the pGL3 LTR construct, 5′-LTR region of HIV-1 was amplified by polymerase chain reaction from the pNL4–3 plasmid and cloned into the pGL3 basic vector. pEGFP LTR construct was a gift from Dr. Avindra Nath (Johns Hopkins University, Baltimore, MD). Promoterless pEGFP was generated from the pEGFP LTR construct by eliminating the 5′-LTR region. *Renilla reniformis* luciferase pRL-TK control vector was purchased from Promega.

Transient Transfections of HBMEC

HBMEC were cultured on the filter inserts in cocultures with SVGA, SVGA-Tat, or Jurkat cells and grown to 90% confluence. To prepare a transfection solution, lipofectin (4 μl/ml) was mixed with Opti-MEM medium (both from Invitrogen) for 30 min. Then, 2 μg/ml pGL3 E-selectin, pGL3 CCL-2, or pGL3 IL-6 promoter constructs was added, followed by incubation for an additional 10 min at room temperature to allow DNA-lipofectin complexes to form.

Filter inserts with HBMEC were removed from the coculture systems, placed in empty 12-well plates, and washed twice with Opti-MEM to remove serum residues. Then, 250 μl of the transfection solution containing individual promoter gene constructs was added to each filter insert, and the cells were incubated for 5 h at 37°C. Transfection solutions were aspirated, and cultures were returned into the Transwell coculture systems. Cultures were allowed to recover in a normal growth medium for 24 h. Then, the growth medium was replaced with serum free EGM-2 basal medium (for endothelial cells) and DMEM (for astrocytes), and cocultures were treated with Aβ added into both the upper and lower chambers.

Transient Transfections of Astrocytes

SVGA cells were transfected using Lipofectamine 2000 or lipofectin (both from Invitrogen). In brief, SVGA cells were seeded on the 6- or 24-well plates and grown to ~90% confluence. Before transfections, Opti-MEM medium was mixed with Lipofectamine 2000 (10 μl/well for the 6-well plates) or lipofectin (1 μl/well for the 24-well plates) for 30 min. Then, pEGFP LTR, pEGFP (both 500 ng/well for the 6-well plates), pcDNA3, pcDNA3 Tat86 (2 μg/well for the 6-well plates or 400 ng/well for the 24-well plates), and pRL-TK (10 ng/well for the 24-well plates) were added to prepare individual transfection solutions. The cells were washed with Opti-MEM, the specific transfection mixtures were added to each well, and transfections were performed for 4 h at 37°C. Then, cultures were washed and allowed to recover in growth medium for 20 h. Astrocytes transfected with the EGFP constructs were imaged using an inverted fluorescence microscope. In cultures transfected with other constructs, luciferase activity was determined as described below.

Luciferase Activity Assay

Firefly and *R. reniformis* luciferase activities were measured using the Dual Luciferase Assay System (Promega). In brief, cells were washed with phosphate-buffered saline and incubated on a shaker for 30 min at 24°C with passive lysis buffer. Cell lysates were transferred to the Eppendorf tubes and centrifuged to remove cell debris. Then, 10 μl of the cell extracts was mixed with 100 μl of luciferase assay reagent containing luciferin plus ATP and luminescence was measured in a luminometer with dual automatic injector (Turner Designs, Sunnyvale, CA). The samples were then mixed with the Stop and Glo reagent, and *R. reniformis* luciferase activity was determined as an internal control.

All transfection results from astrocytes were expressed as the ratio of the firefly to *R. reniformis* luciferase activity. On the other hand, Aβ used for treatment of HBMEC interfered with the *R.*

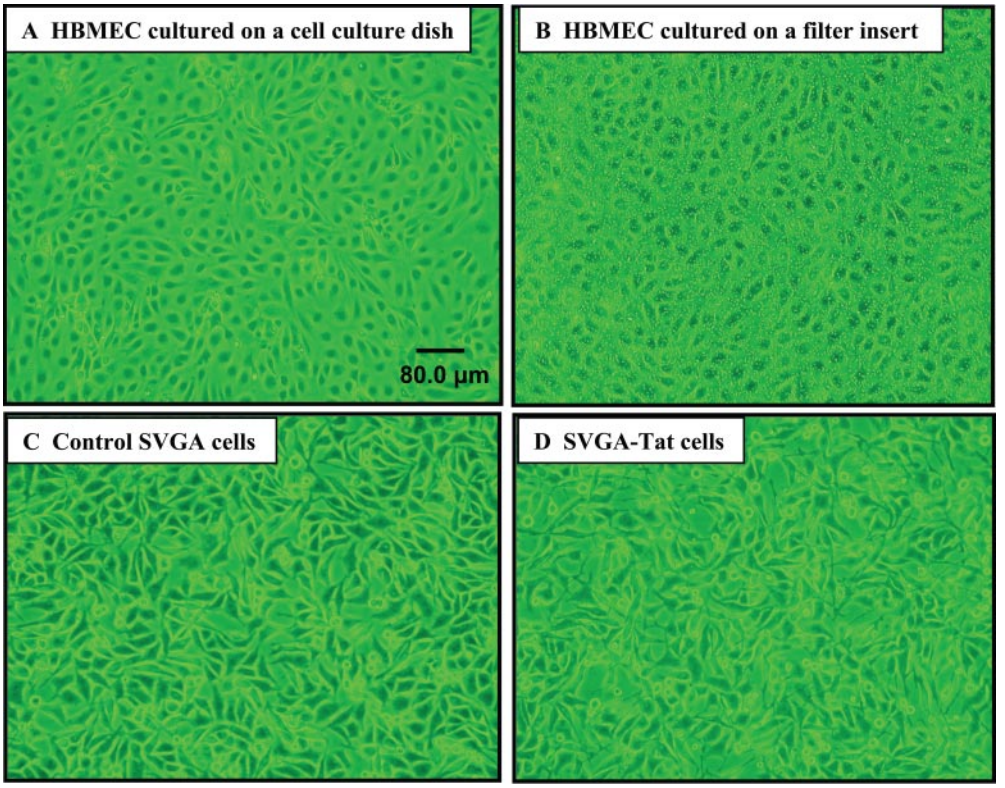

Fig. 1. Morphological characterization of immortalized HBMECs, control astrocytic cell line (SVGA), and SVGA overexpressing Tat. Top, phase-contrast micrographs of a confluent HBMEC monolayer with the typical morphology of elongated, fusiform shape of endothelial cells cultured on a cell culture dish (A) and on a filter insert (B). Endothelial cells cultured on filter inserts have no morphological changes compared with those grown on cell culture dishes. Bottom, phase-contrast micrograph of confluent control SVGA astrocytes (C) and SVGA-Tat astrocytes (D). Control SVGA and SVGA-Tat cells exhibit similar morphology characteristic for astrocyte cultures, such as multipolar shape and overlapping morphology.

reniformis luciferase assay. Therefore, firefly luciferase activity in HBMECs was normalized to total protein levels, and the transfection results were expressed as relative light units per microgram of protein.

Immunofluorescence Microscopy

HBMECs cultured on Permanox chambered plastic slides were fixed with ethanol for 30 min at 4°C. After washing with PBS and blocking with 3% bovine serum albumin in PBS for 30 min, samples were incubated overnight at 4°C with rabbit polyclonal anti-E-selectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The excess of primary antibody was removed, slides were washed with PBS and incubated with Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology) for 2 h at room temperature. After washing with PBS, slides were mounted using ProLong Gold Antifade reagent containing 4′,6-diamidino-2-phenylindole (Invitrogen) to visualize the nuclei. Specimens were covered with coverslips and evaluated under an epifluorescence Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan). The images were captured using a Spot charge-coupled device camera system.

Enzyme-Linked Immunosorbent Assay

Concentrations of CCL-2 and IL-6 were quantified using commercially available human CCL-2 and IL-6 Immunoassay kits (R&D Systems, Minneapolis, MN). Assays were performed using aliquots of cell culture media collected from both the luminal and abluminal chambers of the Transwell coculture systems.

Statistical Analysis

Data were analyzed using SigmaStat 2.0 (SPSS Inc., Chicago, IL). One- or two-way analysis of variance was used to compare responses among treatments. Treatment means were compared using Bonferroni's least significant procedure and $p < 0.05$ was considered significant.

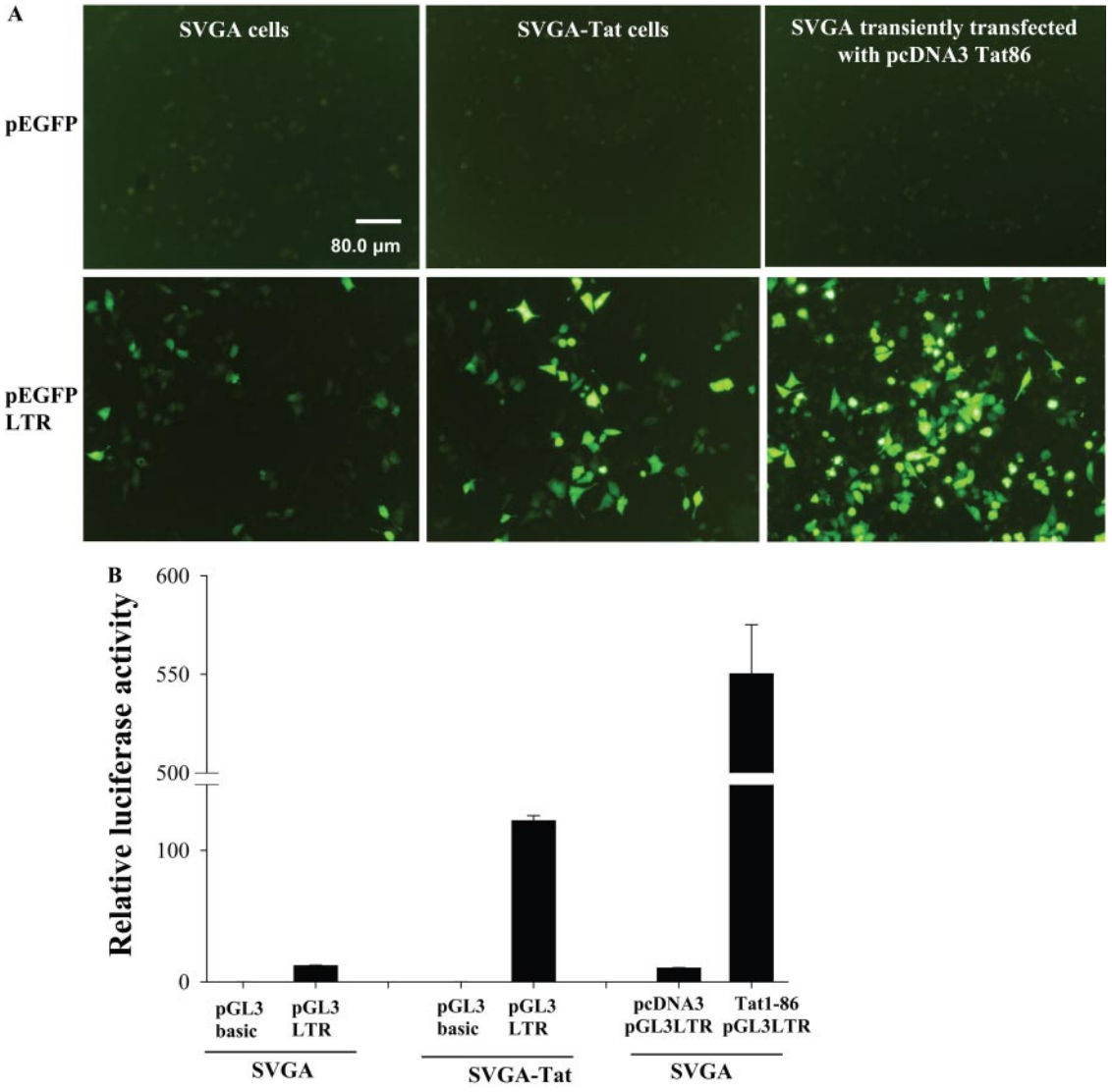

Fig. 2. Tat expression by SVGA-Tat astrocytes. A, control SVGA and SVGA-Tat astrocytes were transfected with the pEGFP LTR (bottom) or promoterless pEGFP (negative control, top) constructs. To confirm the specificity of Tat detection, normal SVGA astrocytes were transiently transfected with the pcDNA3 Tat86 expression vector and cotransfected with the pEGFP LTR or the promoterless pEGFP constructs. EGPF expression was detected by immunofluorescence microscopy. B, to quantify Tat transactivation, control SVGA and SVGA-Tat astrocytes were transfected with pGL3 LTR or pGL3 basic vector (negative control). As a positive control, SVGA astrocytes were transiently transfected with pcDNA3 Tat86 expression vector (or pcDNA3 empty vector) and pGL3 LTR. To normalize transfection rates, all cells were cotransfected with the *R. reniformis* luciferase reporter plasmid pRL-TK. Luciferase activity was analyzed by dual luciferase assay 20 h after transfections and normalized according to *R. reniformis* luciferase activity. Values are mean ± S.E.M. ($n = 4–6$).

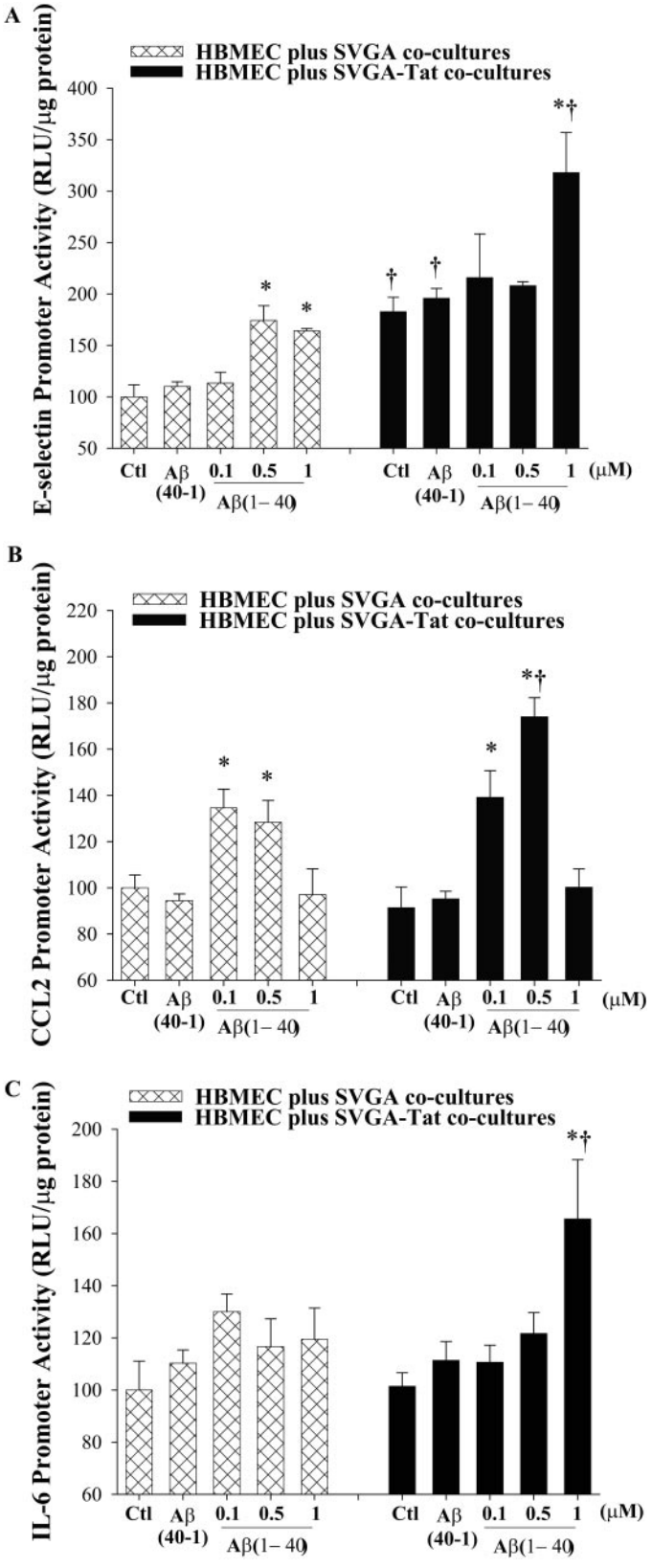

Fig. 3. Aβ amplifies Tat-induced promoter activities of proinflammatory genes in brain endothelial cells. HBMECs cocultured with control or Tat-expressing astrocytes (SVGA and SVGA-Tat cells, respectively) were transfected with the firefly luciferase reporter constructs containing the human E-selectin (A), CCL-2 (B), and IL-6 (C) promoter sequences. Cells in the coculture system were treated with the indicated concentrations of Aβ(1-40) 24 h after transfections. Aβ(40-1) at the concentration of 1 μM was used as a negative control. Luciferase activity was analyzed in HBMECs after a 20-h exposure to Aβ. Values are mean ± S.E.M., $n = 4$. *, statistically different compared with the corresponding controls within the HBMECs plus SVGA or HBMECs plus SVGA-Tat cocultures. †, data in cocultures of HBMECs with SVGA-Tat are significantly different compared with cocultures of HBMEC with control SVGA and exposed to the corresponding concentration of Aβ.

Results

Morphological Characterization of HBMECs and SVGA-Tat Cells. HIV-1 can infect astrocytes, which then produce viral proteins such as Tat. Astrocytes constitute the most abundant cell type in the brain, and viral proteins produced by infected astrocytes are likely to have significant neuropathological effects. Therefore, the present study was based on coculture systems of HBMEC with astrocytes expressing HIV Tat protein (SVGA-Tat cells) or normal SVGA cells used as controls.

The phase contrast micrographs in Fig. 1 depict confluent HBMECs and SVGA cells. HBMECs cultured on cell culture dishes exhibited typical monolayer morphology of elongated, fusiform shape of endothelial cells. The experimental design of the present study required HBMEC cultures on filter inserts. Therefore, we also determined that HBMECs cultured on microporous filters had normal morphology. In addition, confluent SVGA-Tat and SVGA control cells exhibited typical morphology characteristic of astrocyte cultures.

Tat Expression by SVGA-Tat Astrocytes. To confirm the expression and activity of Tat protein in our culture systems, SVGA-Tat and control SVGA astrocytes were transfected with pEGFP LTR (Fig. 2A) or pGL3 LTR (Fig. 2B) promoter constructs. SVGA-Tat cells transfected with pEGFP LTR (Fig. 2A, middle) exhibited markedly stronger EGFP fluorescence compared with control SVGA cells (Fig. 2A, left). In contrast, transfections with the promoterless pEGFP construct revealed only background fluorescence both in SVGA-Tat and control SVGA cells. To further confirm the specificity of these responses, control SVGA cells were transiently transfected with pcDNA3 Tat86 and pEGFP LTR constructs. As illustrated (Fig. 2A, right), this procedure resulted in efficient and specific LTR transactivation, similar to that in SVGA-Tat astrocytes.

To further characterize SVGA-Tat cells, Tat transactivation was quantified by transfection with the pGL3 LTR construct, followed by measuring of luciferase activity. Transfections with the pGL3 basic vector were performed as negative controls. A strong induction of luciferase activity was observed in SVGA-Tat cells but not in control SVGA cells (Fig. 2B). Strong transactivation of LTR promoter was also observed in control SVGA cells transiently transfected with the pcDNA3 Tat86 and pGL3 LTR constructs (positive control).

Tat and Aβ Selectively Amplified Promoter Activities and Protein Levels of Proinflammatory Mediators in HBMECs. Coculture systems of HBMECs with SVGA-Tat astrocytes were used to investigate the combined effects of Aβ and Tat on promoter activity of selected proinflammatory genes. HBMECs cocultured with SVGA-Tat or control SVGA cells were transfected with the firefly luciferase reporter constructs containing human E-selectin, CCL-2, or IL-6 promoter sequence. Then, both HBMEC and astrocytes were exposed to 0.1, 0.5, or 1 μM Aβ(1-40) for 20 h. In cocultures of HBMECs with control SVGA cells, Aβ induced a marked increase in the promoter activity of E-selectin (Fig. 3A) and

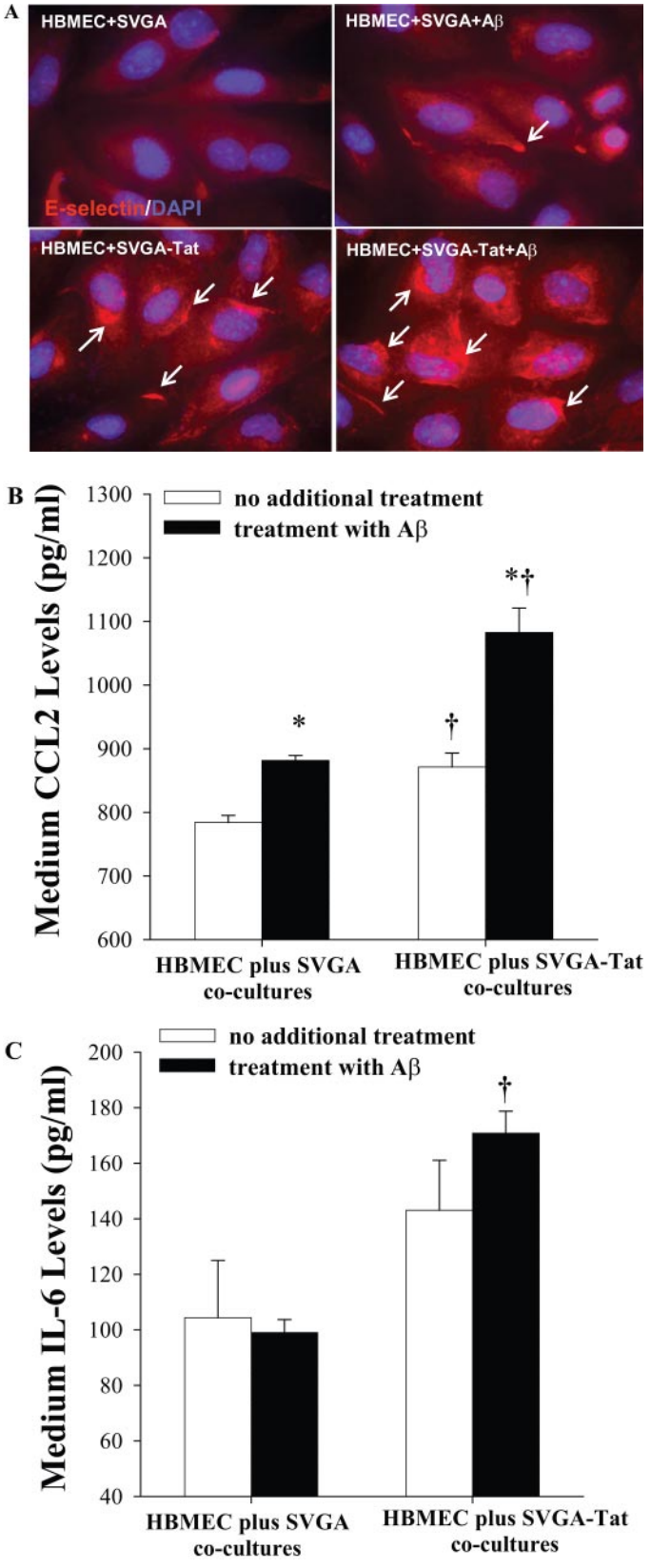

Fig. 4. Aβ and Tat synergistically amplify protein expression of proinflammatory mediators in brain endothelial cells. A, HBMECs cultured on chambered slides were exposed to 1 μM Aβ(1-40) and/or conditioned media from SVGA or SVGA-Tat cells for 6 h. E-selectin immunoreactivity was determined by immunofluorescence microscopy. Red color reflects E-selectin-positive immunoreactivity, and blue color represents 4′,6-diamidino-2-phenylindole staining for DNA that visualizes the nuclei. Arrows indicate intensification of E-selectin immunoreactivity by Aβ and/or SVGA-Tat conditioned media. B and C, HBMECs were cocultured with SVGA-Tat or control SVGA cells and treated with Aβ(1-40) at 0.5 μM (B) or 1 μM (C). CCL-2 and IL-6 protein levels were determined in the culture media by enzyme-linked immunosorbent assay. Values are mean ± S.E.M. (n = 3–8). *, statistically different compared with the corresponding controls within the HBMECs plus SVGA or HBMECs plus SVGA-Tat cocultures. †, data in cocultures of HBMECs with SVGA-Tat are significantly different compared with cocultures of HBMECs with control SVGA and exposed to the corresponding concentration of Aβ.

CCL-2 (Fig. 3B); however, it did not affect promoter activity of IL-6 (Fig. 3C). On the other hand, exposure to Aβ in cocultures with SVGA-Tat cells markedly amplified promoter activities of all studied proinflammatory genes (Figs. 3, A–C). These effects were achieved by 1 μM Aβ for E-selectin and IL-6 promoters and by 0.5 μM Aβ for CCL-2 promoter.

To verify the specificity of the Aβ(1-40) effects, cocultures were also exposed to the reverse-sequence Aβ peptide (Aβ40-1) at the concentration of 1 μM. As illustrated in Fig. 3, A to C, Aβ(40-1) did not affect transactivation of the inflammatory gene promoters.

In addition to promoter activities, we determined the effects of Aβ and/or SVGA-Tat on protein levels of E-selectin, CCL-2, and IL-6 (Fig. 4, A–C). Based on the results of the dose-response experiments presented in Fig. 3, A to C, Aβ was used at the concentration of 1 μM to stimulate E-selectin and IL-6 protein expression and at 0.5 μM to induce CCL-2 protein production. As illustrated in Fig. 4A (top right), treatment with Aβ alone slightly elevated E-selectin immunoreactivity. Similar effects were also apparent in HBMECs exposed to conditioned medium from SVGA-Tat cultured (bottom left). However, the most marked increase in E-selectin immunoreactivity was observed in HBMECs treated with Aβ in the presence of conditioned medium from SVGA-Tat cells (bottom right). Potentiation of proinflammatory effects of Aβ and SVGA-Tat were also observed in experiments in which levels of secretary CCL-2 and IL-6 proteins were determined in cell culture media (Fig. 4, B and C, respectively).

Simvastatin Protected against Tat and Aβ-Induced Stimulation of Promoter Activities of Proinflammatory Genes in HBMECs. Statins have been demonstrated to have pleiotropic effects, including anti-inflammatory and anti-HIV properties. Therefore, we hypothesized that they can protect against Tat and Aβ-induced proinflammatory effects. To address this hypothesis, HBMECs were cocultured with SVGA-Tat or control SVGA cells and transfected with the pGL3 E-selectin, pGL3 CCL-2, or pGL3 IL-6 construct. Then, the cocultures were pretreated for 15 min with 10 μM (Fig. 5, A and B) or 5 μM (Fig. 5C) simvastatin, followed by exposure to Aβ(1-40) for 20 h. Aβ was used at the concentration of 1 μM to stimulate E-selectin and IL-6 promoters and at 0.5 μM to induce CCL-2 promoter. Consistent with data presented in Figs. 3 and 4, Aβ in the presence of SVGA-Tat induced a marked increase in E-selectin (Fig. 5A), CCL-2 (Fig. 5B), and IL-6 (Fig. 5C) promoter activities. Treatment with simvastatin up-regulated the IL-6 promoter activity in control (i.e., not exposed to Aβ) cocultures of HBMECs with SVGA-Tat cells. Nevertheless, simvastatin effectively protected against up-regulation of E-selectin and IL-6 promoters in cocultures of HBMECs with SVGA-Tat and exposed to Aβ. Inhibition of IL-6 promoter activity was achieved by 5 μM simvastatin; however, 10 μM simvastatin was required to block E-selectin promoter activity.

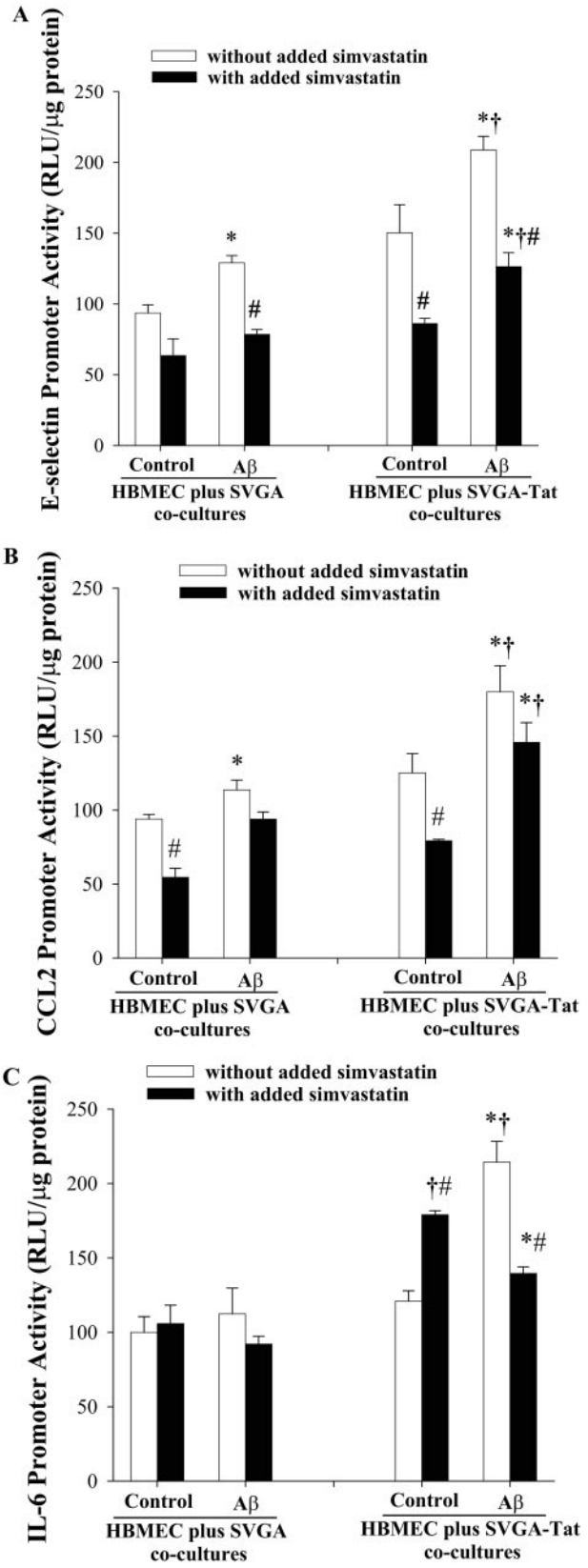

Fig. 5. Simvastatin protects against Aβ and Tat-induced promoter activities of proinflammatory genes in brain endothelial cells. Transfections and luciferase activity assays were performed as described in the legend to Fig. 3. In addition, selected cultures were pretreated for 15 min with 10 μM (A and B) or 5 μM (C) simvastatin before coexposure to 1 μM (A and C) or 0.5 μM (B) Aβ(1-40) for 20 h. Values are mean ± S.E.M. (n = 4–6). *, statistically different compared with the corresponding controls within the HBMECs plus SVGA or HBMECs plus SVGA-Tat cocultures. †, Data in cocultures of HBMEC with SVGA-Tat are significantly different compared with cocultures of HBMECs with control SVGA and exposed to the corresponding concentration of Aβ. #, data in cocultures exposed to simvastatin are significantly different compared with the corresponding cocultures without added simvastatin.

Aβ Amplified HIV-1-Stimulated Transactivation of E-Selectin Promoter in HBMECs. In the final series of experiments, we investigated whether exposure to Aβ can stimulate E-selectin promoter activity in the presence of HIV-1. HBMECs were transfected with the pGL3 E-selectin construct and cocultured with HIV-1-infected or control Jurkat cells for 24 h. Cultures were then pretreated for 15 min with 10 μM simvastatin or vehicle control and exposed to 1 μM Aβ(1-40) for 20 h. The experiments were performed 7, 14, and 18 days after infection of Jurkat cells. The results were similar; therefore, individual data from these separate experiments were combined in a single figure (Fig. 6). Stimulation of HBMECs by HIV-1-infected Jurkat cells resulted in a sufficient increase in E-selectin promoter activity. In addition, treatment with Aβ markedly elevated these effects. Most importantly, pretreatment with simvastatin effectively protected against both HIV-1 and HIV-1 plus Aβ-induced transactivation of the E-selectin promoter construct.

Discussion

The mechanisms of HIV entry into the brain are still unclear; however, they may occur through the so-called Trojan horse mechanism, via HIV-infected monocytes crossing the BBB (Gartner, 2000). Thus, induction of inflammatory reactions that mediate monocyte adhesion to the brain endothelium and transendothelial migration are of particular significance. Therefore, in the present studies, we focused on the expression of inflammatory mediators, such as E-selectin (an adhesion molecule), CCL-2 (a chemokine), and IL-6 (an inflammatory cytokine) in brain endothelial cells. Up-regulation of these inflammatory mediators can induce alterations of the BBB integrity by facilitating adhesion and migration of leukocytes into the brain (Buckner et al., 2006). The present study was based on coculture systems in which HBMECs were exposed to Tat produced by transfected astrocytes. Such cocultures are clinically relevant, because HIV can infect astrocytes, which then are able to produce viral proteins, such as Tat. Astrocytes along with HBMECs are the structural and functional part of the BBB. In addition, astrocytes are susceptible to Aβ treatment and can influence HBMEC inflammatory responses. Indeed, we observed that Aβ stimulated E-selectin promoter activity in HBMECs cocultured with SVGA astrocytes (Fig. 3A) but not with Jurkat cells (Fig. 6).

The facts that 1) deposits of Aβ increase in normal aging (Price and Morris, 1999), 2) Aβ can induce vascular alterations (Deane and Zlokovic, 2007), and 3) Aβ may be involved in HIV-associated pathology (Wojtowicz et al., 2002) prompted us to study the hypothesis that Aβ and Tat may cross-potentiate their toxic effects and alter the functions of the BBB. Indeed, the results of the present study indicated that coexposure to Aβ(1-40) and Tat or HIV-1-infected cells can synergistically elevate promoter activity of E-selectin, CCL-2, and IL-6. These effects were also confirmed at the protein levels (Fig. 4). Although the proinflammatory influence of Tat and Aβ in

endothelial cells has been recognized (Toborek et al., 2005), the evidence that these factors can exert synergistic proinflammatory effects is the new finding of the present study.

As illustrated in Fig. 3, exposure to Aβ resulted in a dose-dependent increase in promoter activities of E-selectin and IL-6. On the other hand, CCL-2 promoter was activated by Aβ at the concentration of 0.1 and 0.5 μM but not at 1 μM. Although Aβ is generally considered to be a pro-oxidative factor, evidence indicates that Aβ-mediated regulation of cellular oxidation may depend on the ratio of Aβ to other pro-oxidative factors. Indeed, pro-oxidative/antioxidative effects of Aβ can be regulated by the molar ratio of Aβ to Cu^{2+}. When Cu^{2+} is bound to two molar equivalents of Aβ, lipid peroxidation is inhibited and Aβ exerts antioxidant effects (Hayashi et al., 2007). Thus, it is possible that these finely tuned regulations of cellular redox balance might be responsible for the lack of CCL-2 stimulation by 1 μM Aβ.

The transfection experiments with the promoter constructs performed in the present study indicate that induction of inflammatory genes by Aβ, Tat, or HIV-1 occurs primarily at the transcriptional levels. In agreement with these results, it has been shown that Tat-induced expression of tumor necrosis factor-α is regulated by an NF-κB-dependent pathway in macrophages and astrocytes (Chen et al., 1997). The NF-κB-dependent pathway is also involved in Tat-induced up-regulation of IL-6 and IL-8 expression in human breast cancer cells (Lee et al., 2005). In addition, treatment with Tat can induce DNA binding activities of other transcription factors, such as cAMP response element-binding protein and activator protein-1, which are known to regulate inflammatory responses (Kumar et al., 1998; Toborek et al., 2005).

Aβ also seems to induce the activity of several transcription factors involved in the regulation of inflammatory gene expression. For example, Aβ treatment resulted in a poly-(ADP-ribose) polymerase-1-facilitated DNA binding of NF-κB (Chiarugi and Moskowitz, 2003) in microglial cells. Exposure to Aβ stimulated activation of early growth response-1 transcription factor, which is involved in the expression of cytokines (tumor necrosis factor-α and IL-1β) and chemokines (macrophage inflammatory protein-1β, CCL-2, and IL-8) in monocytes (Giri et al., 2004). Transcription factor CCAAT/enhancer-binding protein-δ was also shown to be involved in Aβ-mediated induction of IL-6 and CCL-2 (Hu et al., 2000). Thus, Tat and Aβ-mediated stimulation of a variety of transcription factors may be responsible for cross-potentiation of inflammatory responses as observed in our study.

Another important finding was that treatment with statins, such as simvastatin, protected against Aβ and Tat or HIV-1-induced inflammatory responses. Statins are drugs that effectively diminish cholesterol biosynthesis by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase. In HIV-infected patients, statins have been successfully introduced to improve disturbances in lipid profile (Dubé et al., 2003). On the other hand, statins such as simvastatin can also increase NF-κB activation and superoxide production via heme oxygenase-1-dependent mechanism (Hsieh et al., 2008). Such effects could be responsible for an increase in IL-6 promoter activity observed in simvastatin-treated HBMEC in the presence of SVGA-Tat cells.

Observed in the present study, the protective effects of simvastatin on Tat and Aβ-induced inflammatory responses are supported by literature reports. For example, it was shown that statins down-regulated vascular cell adhesion molecule-1, E-selectin, IL-6, IL-8, CCL-2 expression, and had immunomodulatory effects in endothelial cells (Greenwood et al., 2006). In monocytes, statins inhibited adhesion and migration (Pozo et al., 2006). Statins also decreased inflammatory responses in microglia and attenuated experimental autoimmune encephalomyelitis (Nath et al., 2004). Anti-inflammatory properties of statins seem to be related to decreased synthesis of several isoprenoid intermediates, which normally are involved in post-translational modification of proteins, including the γ-subunit of heterotrimeric G proteins and Ras and Rho proteins (Liao, 2005). Isoprenylation of Ras and Rho is required for covalent attachment, subcellular localization, and membrane trafficking of these molecules. The Ras and Rho signaling can stimulate inflammatory reactions; thus, a decrease in isoprenylation prevents activation of these kinases and may induce anti-inflammatory responses (Liao, 2005).

In the present study, simvastatin not only protected against Tat and Aβ-induced inflammatory reactions but also was highly effective against HIV-mediated stimulation of E-selectin promoter transactivation. The protective effects suggest that statins may be a valuable group of drugs for supplemental therapy in HIV infection. Indeed, a short-term statin treatment was demonstrated to decrease HIV-1 viral load in patients and inhibit HIV-1 infection through down-regulation of Rho activity (del Real et al., 2004). However, these effects were not con-

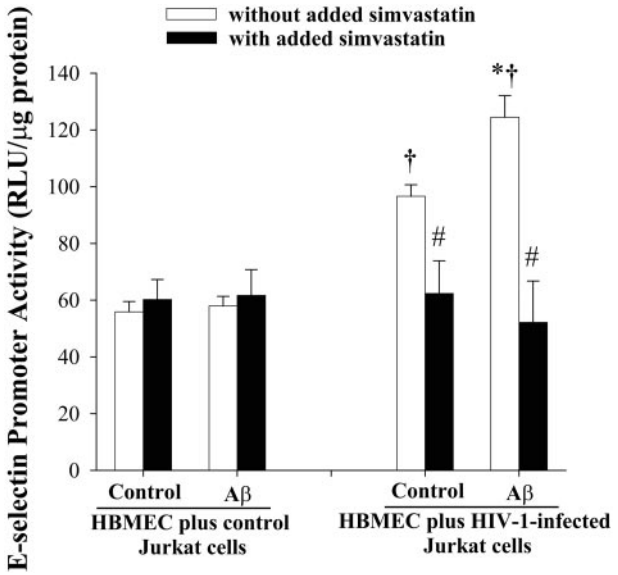

Fig. 6. Simvastatin protects against Aβ and HIV-1-induced promoter activity of E-selectin in brain endothelial cells. Transfections with the pGL3 E-selectin promoter construct were performed as described in the legend to Fig. 3. The next day, the transfected HBMECs were placed in cocultures with normal or HIV-1-infected Jurkat cells, followed by a treatment with 1 μM Aβ(1-40) for 20 h. Selected cultures were pretreated for 15 min with simvastatin (10 μM) before Aβ(1-40) coexposure. E-selectin promoter activity was analyzed by luciferase assay and normalized to cellular protein levels. Values are mean ± S.E.M. (n = 6–10). *, statistically different compared with the corresponding controls within the cocultures of HBMECs with normal or HIV-1-infected Jurkat cells. †, data in cocultures of HBMECs with HIV-1-infected Jurkat cells are significantly different compared with cocultures of HBMEC with normal Jurkat cells and exposed to the corresponding concentration of Aβ. #, data in cocultures exposed to simvastatin are significantly different compared with the corresponding controls without added simvastatin.

firmed in another study in which no anti-HIV activity of statins was observed in cell culture or in HIV-positive patients (Moncunill et al., 2005). It also should be noted that most statins are metabolized through the CYP3A4 pathway, which is inhibited by protease inhibitors widely used in antiretroviral therapy. Thus, interactions between statins and protease inhibitors may result in an increase in tissue statin concentrations, leading to drug toxicity. On the other hand, statins such as pravastatin and fluvastatin are the least influenced by the CYP3A4 pathway and, therefore, might be safer when administered with protease inhibitors (Dubé et al., 2003).

Evidence also indicates that statins may provide useful treatment in pathologies related to Aβ pathology, such as AD dementia. Retrospective epidemiological studies revealed that patients with high cholesterol levels and treated with statins less frequently developed AD (Jick et al., 2000). A relative safety of simvastatin was demonstrated in a 5-year clinical study (Pedersen et al., 1996), suggesting that statins might be safely used to prevent the development of AD. The beneficial effects of statins were also shown in patients with mild to moderate AD (Sparks et al., 2006). Finally, experimental studies revealed that statins can protect neurons against Aβ toxicity, increase learning capabilities in mice, and decrease Aβ levels both in cultured hippocampal neurons and in vivo in guinea pigs (Fassbender et al., 2001; Li et al., 2006). However, there are also reports that do not support these findings or even suggest that statins can induce cognitive impairment (Padala et al., 2006). Thus, to elucidate these controversies, at least two large multicenter trials have been initiated using simvastatin and atorvastatin (Miida et al., 2005) in an attempt to better understand the effects of statin therapy on dementia.

In summary, Aβ(1-40) and HIV-1 Tat protein cross-amplified promoter activities of three different proinflammatory genes in HBMECs. Similar synergistic effects were observed in HBMECs exposed to Aβ in the presence of HIV-1-infected Jurkat cells. It is most interesting that simvastatin effectively attenuated these effects. The present results indicate that Aβ and HIV Tat may synergistically induce inflammatory reactions in brain endothelial cells. In addition, statins may provide a beneficial influence by reducing these effects at the BBB level.

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