Vascular Biology

Effect of Vectors on Human Endothelial Cell Signal Transduction
Implications for Cardiovascular Gene Therapy

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Objective—Endothelium is an important target for gene therapy. We have investigated the effect of viral and nonviral vectors on the phenotype and function of endothelial cells (ECs) and developed methods to block any activation caused by these vectors.

Methods and Results—Transduction of ECs with viral vectors, including adenovirus, lentviruses, and Moloney murine leukemia virus, can induce a pro-inflammatory phenotype. This activation was reduced when nonviral vectors were used. We demonstrate that after transduction there is upregulation of dsRNA-triggered antiviral and PI3K/Akt signaling pathway. Blockade of the NFκB, PI3-K, or PKR signaling pathways all operated to inhibit partially virally induced activation, and inhibition of both PKR and PI3-K pathways totally blocked EC activation. Furthermore, inhibition of IFN-α/β in addition to PI3-K was effective at preventing EC activation.

Conclusions—Viral vectors, although efficient at transducing ECs, result in their activation. Blockade of the signaling pathways involved in viral activation may be used to prevent such activation. (Arterioscler Thromb Vasc Biol. 2006; 26:462-467.)

Key Words: endothelial cells ■ gene therapy ■ nonviral vectors ■ signal transduction ■ viral vectors

The endothelium is an attractive target for gene therapy because of its accessibility and importance in the pathophysiology of a wide range of conditions. Gene delivery to endothelial cells (ECs) can be accomplished using viral or nonviral vectors. In general, viral methods are effective at delivering genes to ECs; however, viruses can stimulate cytokines. This opens up novel therapeutic interventions aimed at modulating the effect of viral vectors on ECs.

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EC Isolation and Culture
Human saphenous veins ECs (HSVECs) and foreskin microvascular endothelial cells were prepared and cultured as described. For all experiments, the ECs were pooled from at least 5 patients as previously described.

Transfection and Transduction
The E1a-, partial E1b-, partial E3-adenovirus serotype 5 (Ad) vector, Ad-EGFP (Clontech, Palo Alto, Calif), Ad-0, RAd35, the HIV-EGFP/0 and EIAV-EGFP/0 (kind gifts from Oxford Biomedica, Oxford, UK), and the MMLV (pBullet-EGFP) were produced as described. For transduction, 10⁴ cells were incubated with virus in 100 μL optiMEM I for 2 to 3 hours, at which time the volume was increased to 0.5 mL by addition of culture medium. Immune complexes were used to transfect cells as described.

Flow Cytometry
The phenotype of ECs was assessed by flow cytometry 2 days after transfection with nonviral vectors or 3 days after transduction with viral vectors, because it was at these times that maximal gene expression was seen. Cell staining was performed using mouse antibodies (Ab) conjugated with allophycocyanin (activated protein C [APC]) or primary Ab, followed by goat anti-mouse-APC. When indicated, ECs were stimulated with 80 ng/mL tumor necrosis factor (TNF)-α, 80 ng/mL interleukin (IL)-1β, and 80 ng/mL IFN-γ, followed by flow cytometric analysis 12 hours after stimulation.

Cytokines
IFN-γ, IL-12 p70, IL-1β, IL-6, IL-8 and TNF-α were measured as described. Supernatants were obtained 4 days after transduction.
Reverse-Transcription Polymerase Chain Reaction Assay

After transduction or transfection, the ECs were isolated with magnetic beads coated with mAb against CD105 (Caltag, Silverstone, UK). Reverse-transcription polymerase chain reaction assays were performed using the paired primers for 2'-5' OA synthetase-1, IRF-1, IFN-α2, IFN-β1, and β-actin.8,16

SDS-PAGE and Western Blotting

Cell lysates were analyzed by Western blot as described.17,20 Antibodies used are listed in supplementary data.

Leukocyte Adhesion and Transmigration Assays

Adhesion and transmigration were performed as previously described.17

Blockade of Signaling Pathways

The following inhibitors were used to block specific pathways: NFκB pathway with PSI (10 μmol/L), MG132 (20 μmol/L), or SN50 (20 μmol/L) (all from Calbiochem); the PKR pathway with 2-amino-purine (Sigma) (10 μmol/L), MAPK p38 with SB23906 (10 μmol/L) (Tocris Cookson Ltd, Bristol, UK), JNK with SP600125 (10 μmol/L) (Calbiochem), STAT3 with STAT3 inhibitor peptide (3 mmol/L) (Calbiochem), PI3 K/Akt pathway with LY294002 (50 μmol/L) (Tocris Cookson Ltd, Bristol, UK), JNK with SP600125 (10 μmol/L) (Calbiochem), STAT3 with STAT3 inhibitor peptide (3 mmol/L) (Calbiochem), PI3 K/Akt pathway with LY294002 (50 μmol/L) (Sigma), or Wortmannin (100 nM) (Sigma). Trichodion (Alexis Platform, Nottingham, UK) was used to inhibit the NFκB, AP-1 and STAT-1 pathways (80 μmol/L), and Dexamethasone (Sigma) (5×10⁻⁷ M) was used to inhibit several pathways. In all cases, ECs were cultured in the inhibitor for 24 hours before addition of viral particles. To block cytokines, ECs were cultured in the presence of mAbs to IL-1α, IL-1β, IFN-α, IFN-β and/or TNF-α (20 μg/mL) (R&D systems).

Statistical Analysis

The Student t test was used for simple comparison between 2 means. For multiple comparisons, data were analyzed by ANOVA.

Results

Comparison of Efficiency of Viral and Nonviral Vectors at Transducing ECs

We first tested the ability of viral vectors to transduce primary HSVECs. We optimized transduction efficiency of all these viral vectors (Figure I, available online at http://atvb.ahajournals.org). As previously reported,2,7,22 adenoaviral and lentiviral vectors are efficient at transducing HSVECs. However, although MMLV vectors can transduce HUVECs,23 we found that MMLV resulted in poor transduction of HSVECs. Little toxicity (<20%) was seen with all viral vectors. Whereas nonviral vectors can be used to delivery genes to ECs,12,13 their efficiency is, in general, low. The best transfection efficiency was seen with immunoliposomases made with anti-CD71 Ab (~40%) (Figure I).

Effect of Transfection/Transduction on EC Phenotype

As previously reported,7 we found that exposure of HSVECs to Ad vectors (with a MOI of 500) (either Ad-EGFP (Figure I; Figure II, available online at http://atvb.ahajournals.org) or control (Ad0)8 (data not shown)) resulted in considerable upregulation of adhesion molecules and MHC class II molecules. After lentiviral transduction, we saw a similar upregulation of adhesion molecules CD54, CD62, and CD106 on HSVECs. Exposure to retroviral vectors also resulted in upregulation of adhesion molecule expression (though this was less in the case of CD106). However, transfection of HSVECs with nonviral vectors caused only slight upregulation of CD62 and CD106, equivalent to that seen in response to DNA alone (Figure I; Figure II). Incubation of HSVECs in medium/buffers used to prepare viruses did not affect the phenotype of the cells (data not shown).

To determine whether similar upregulation was seen in ECs from other sources, we repeated these experiments with microvascular ECs isolated from human foreshin. Similar upregulation of CD54 (Figure I), CD62, and CD106 (data not shown) was seen after viral transduction.

Viral Vectors Induce Production of Th1 Cytokines and Inflammatory Cytokines

We saw an upregulation of Th1 cytokines, IL-12 and IFN-γ, as well as enhanced secretion of TNF-α and IL-8, IL-1β, and IL-6 (Figure I; Figure III, available online at http://atvb.ahajournals.org).
Activation Pathways Induced by Viral Transduction

ECs produce type 1 IFNs in response to double-stranded RNA (dsRNA) by a pathway that is partially dependent on the cytosolic dsRNA-binding enzyme protein kinase R. Activation of this pathway has not been previously reported in ECs after viral vector transduction, although it was seen after the intracellular introduction of dsRNA into ECs. In addition, the NFκB pathway may be activated through the 2′-5′ OA synthetase/RNase L pathway. Therefore, we determined whether these pathways are activated in ECs after transduction. All 4 viral vectors triggered the production of type 1 IFNα/β at mRNA levels (Figure 2A) and IFN-α, β, and γ at the protein level (Figure 1; Figure III), together with upregulation of 2′-5′ OA synthetase and interferon (IFN) regulatory factor 1 (IFR-1) at the protein level (Figure 1; Figure III), together with upregulation of 2′-5′ OA synthetase/RNase L pathway (Figure 2A). We also saw phosphorylation of PKR and its downstream substrate eIF-2α, indicative of PKR pathway activation, and upregulation of PI3 kinase and its downstream substrate PKB/Akt (Figure 2B). Similar data were observed with microvascular endothelium (data not shown).

These data suggest that all 3 pathways may operate to activate (among others) the NFκB pathway (as shown by increases in phosphorylated IkB) (Figure 2B). This is one of the central pathways for EC activation. Viral preparation medium did not activate any of the signaling pathways (data not shown).

To address whether the differences in EC activation between viral and nonviral vectors is caused by the much higher EGFP expression after viral transduction, we transduced HSVECs at a lower multiplicity of infection (MOI) (Ad-EGFP, MOI 50; EIAV-EGFP, MOI 75, HIV-EGFP, MOI 500), resulting in an equivalent number of cells expressing EGFP to that seen after immunolipoplex transfection (40% to 50%). All viral vectors activated the IFN-α, β, PKR, and PI3K/Akt pathways (Figure 2C). We then transfected HSVECs with immunolipoplexes, dendriplexes, immunolipoplexes, or plasmid DNA alone, HSVECs were analyzed 2 days later (A) by reverse-transcription polymerase chain reaction to measure mRNA levels for PKR, 2′-5′ OAS, IRF-1, IFN-α, IFN-β and β-actin, (B) by Western blotting for expression of phosphorylated and total proteins for PKR, eIF-2α, IkBα, PKB/Akt, and PI3 kinase, as well as β-actin. The data shown are representative of 3 experiments. C, ECs were analyzed after transduction at lower MOI (Ad-EGFP MOI 50, EIAV-EGFP MOI 75, and HIV-EGFP MOI 500). Alternatively, immunolipoplex-transfected cells were drug-selected to ensure 100% transfection efficiency (nonviral selected). To exclude the effect of differential production of EGFP, immunolipoplex-transfected HSVECs were sorted to select cells (nonviral sorted) with an equivalent MFI to virally transduced cells (Ad-EGFP). Cells transfected with Ad encoding β-galactosidase (Ad-β-gal) or transfected with a plasmid encoding β-galactosidase (nonviral β-gal) were also analyzed. Cells were transfected with Ad-0, EIAV-0, and HIV-0 (viruses not encoding a transgene). All ECs were Western-blotted for phosphorylated PKR, IkBα, PKB/Akt, and β-actin.
in leukocyte adhesion and transmigration assays. When the ECs were transduced with Ad, lentivirus, and MMLV, the adherence and transmigration of leukocytes (in particular granulocytes and lymphocytes) was markedly increased to a comparable extent to that seen after cytokine activation (Figure IV, available online at http://atvb.ahajournals.org). There was no increase in adhesion or transmigration after transfection with the nonviral vectors (data not shown).

**Virally Activated HSVEC's Fail to Respond to Subsequent Activation by Proinflammatory Cytokines**

After transfection or transduction, HSVEC's were cultured for 5 days. They were then challenged with proinflammatory cytokines. Virally transduced HSVEC's were less responsive in term of adhesion molecule expression and TNF-α secretion but had higher (in the case of Ad-transduced HSVEC's) production of IL-8 (Figure 3A; Figure V, available online at http://atvb.ahajournals.org). Similar observations were seen when the subsequent activation was with oxidized LDL (data not shown).

To determine why HSVEC's previously transduced with virus had a reduced response to pro-inflammatory agents, we cocultured the HSVEC's in the presence of IL-1β and TNF-α for 5 days. HSVEC's that were chronically stimulated with these cytokines failed to upregulate adhesion molecules and MHC class expression on subsequent activation (Figure 3B). If HSVEC's were transduced with virus in the presence of neutralizing antibodies to IL-1 and TNF-α, they were able to respond to activation stimuli in a similar manner to untransduced HSVEC's (Figure 3B). These data indicate that IL-1 and TNF-α are necessary and sufficient for the effect seen.

**Inhibition of Signaling Pathways After Viral Transduction**

After viral transduction, there is activation of PI3-K and PKR pathways, which stimulate the NFκB pathway. We used 3 strategies to inhibit NFκB activation after viral transduction: (1) prevention of NFκB (p55 and p65) translocation (SN50); (2) prevention of IκB breakdown (proteasome inhibitors PSI and MG132); and (3) induction of IκBα synthesis25 and direct interference with NFκB-dependent transactivation26 (dexamethasone). Activation was determined by upregulation of adhesion molecules (Figure 4), cytokine secretion, and effects on the signaling molecules (Figures VI and VII, available online at http://atvb.ahajournals.org). Inhibition of the NFκB pathway partially prevented HSVEC activation after exposure to Ad. Trichodion, which inhibits AP-1 and STAT-1 in addition to
NFkB, partially prevents HSVEC activation. These data suggest that additional inhibition of AP-1 and STAT-1 has little effect in preventing viral activation of HSVECs over and above that seen with inhibition of NFkB alone.

Having shown that the downstream inhibition of virally activated signaling did not completely prevent EC activation, we investigated the upstream inhibition of PI3-K, PKR, MAPK p38, JNK, and STAT3 pathways. Inhibition of MAPK p38, JNK, and STAT3 pathways had a minimal effect on EC activation. However, inhibition of the PI3-K or PKR pathways resulted in a partial inhibition of activation.

**Effect of Blocking Cytokine Production on HSVEC Activation**

The PKR pathway induces IFN-α and IFN-β, whereas the PI3K pathway produces TNF-α and IL-1α and IL-1β. These cytokines are capable of autocrine signaling resulting in positive feedback loops that amplify activation of HSVECs. We therefore blocked these cytokine pathways, alone or in combination with inhibition of the signaling pathways. Addition of neutralizing antibodies to TNF-α and IL-1α and IL-1β only minimally inhibited the NFkB and PKR pathways after viral transduction, and had little effect on adhesion molecule expression. Addition of neutralizing antibodies to IFN-α or IFN-β (alone or in combination) had little effect on HSVEC activation, whereas blockade of all 4 cytokines did partially inhibit HSVEC activation.

**Effect of Inhibition of Both PKR and PI3K Pathways**

Inhibition of either PKR or PI3K on their own results in partial inhibition of HSVEC activation after transduction. However, addition of both LY294002 and aminopurine totally inhibits upregulation of HSVEC activation after viral transduction (Figure 4). We inhibited the cytokines stimulated by these pathways. Thus, inhibition of the PI3K pathway with LY294002 at the same time as blocking IFN-α and IFN-β results in total inhibition of HSVEC activation (Figure 4). However, inhibition of PKR with aminopurine together with anti-TNF-α and IL-1 antibodies only partially inhibited activation (data not shown). The use of all these inhibitors and all these neutralizing antibodies did not affect the transgene expression (data not shown).

**Effect of Inhibitors on Leukocyte Adherence and Transmigration**

NFkB, JNK, MAPK p38, and PKR inhibitors, Dexamethasone and Trichodion, which partially inhibited HSVEC activation, also partially blocked the increase in adhesion and transmigration of leukocytes after Ad transduction. However, treatment of HSVECs with a combination of PKR and PI3K inhibitors, or with PI3K inhibition in conjunction with IFN-α and IFN-β blockade, resulted in no augmentation of leukocyte adhesion or transmigration after Ad transduction (Figure VIII, available online at http://atvb.ahajournals.org).

**Discussion**

In this report we have characterized the effect of viral transduction on EC phenotype and function. Infection of A594 cells, vascular neointimal cells, or pulmonary tissues with Ad vectors induces upregulation of CD54, with E1-E4+ Ad vectors, but not E1-E4- vectors, inducing long-term expression of CD54 and CD106. We have observed not only an upregulation of adhesion molecule expression but also an increase in the secretion of Th1-type proinflammatory cytokines by ECs after transduction with Ad, lentivirus, and MMLV.

The NFkB pathway is primarily responsible for activation of ECs. However, there are several ways in which NFkB can be activated by viral transduction. In response to viral infection, ECs can be activated by dsRNA. This is partially dependent on the cytosolic dsRNA-binding protein kinase R (PKR). In dendritic cells (DCs), activation by dsRNA does not depend on the toll-like receptor (TLR) 3, a surface receptor for dsRNA, but requires endosomal recognition through TLR7 and MyD88.

In this report, we show that viral vectors activate the 2'-5' OA synthetase/RNase L and PKR pathway in ECs. The consequences of viral transduction can vary, with ECs having a reduced ability to upregulate adhesion molecules and cytokine production when activated 5 days after transduction. This effect could be produced by addition of TNF-α and IL-1β for 5 days, and was reversed by addition of neutralizing antibodies to these cytokines, indicating that this resulted from secretion of proinflammatory cytokines by transduced ECs.

Ad vectors can produce inflammatory responses at high doses after intravenous administration in vivo through production of cytokines and chemokines. The administration of transcription-defective Ad-GFP particles induced the early but not the late peak of chemokine and cytokine gene expression. These data indicate that Ad vector-induced inflammation is capsid-dependent in the early phase and transcription-dependent in the late phase. Ad-mediated activation of DCs was recently attributed to the high levels of TNF-α expression by these cells. Ad-induced TNF-α production was found to be necessary for DC maturation, in a manner dependent on signaling by PI3-K but not MyD88. Our observations are consistent with a similar pathway operating in human ECs, in as much as we saw the activation of PI3-K and high production of TNF-α after Ad transduction.

Having established pathways that were activated after viral transduction, we investigated the effect of inhibiting them. Inhibition of NFkB did not completely abolish virally induced adhesion molecule expression and pro-inflammatory cytokine production. However, inhibition of both PI3-K and PKR resulted in total inhibition of EC activation, suggesting that the PI3-K and PKR pathways not only activate NFkB but also other downstream effector pathways. Use of other inhibitors suggested that blockade of the MAPK p38, JNK, STAT-1, and STAT3 pathways has little effect on the activation of ECs by viral transduction. Both the PKR and the PI3-K pathways are associated with cytokine production (IFN-α/β for PKR and TNF-α/IL-1α/β for PI3-K), serving as positive feedback loops that may help “lock” the ECs into an activated phenotype. Inhibition of these pathways on their own has only a partial effect on EC activation; however, inhibition of the PI3-K pathway in combination with blockade of type 1 IFNs totally abolished EC activation. These data indicate strategies that might prevent nonspecific EC activation by viral vectors.

In this report, we have chosen HSVEC as our primary model because saphenous veins are used widely as conduit in many
bypass operations, and genetic approaches have been proposed to prevent graft restenosis. However, there are differences in EC biology, depending on their origin. Microvascular ECs are an important target for some applications, and we found similar activation of these cells after transduction.

In conclusion, we have shown that transduction with viral vectors has dramatic consequences for ECs in terms of their phenotype, the activation pathways induced, and their function. This can modulate the effect of gene therapy by inducing local inflammation, which may modulate the underlying disease process and also induce anti-vector or anti-transgene immune responses. Nonviral vectors, however, are relatively innocuous and fail to activate significantly ECs. However, they are less efficient at mediating gene expression! An understanding of the consequences of EC transduction and the pathways by which it is mediated is important in allowing the design of improved strategies involving the viral vector modification of ECs.

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11. Tan et al Effect of Gene Therapy on Human Endothelial Cells


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