Vascular Cell Adhesion Molecule-1 Augments Adenovirus-Mediated Gene Transfer

Yi Chu, Donald D. Heistad, Myron I. Cybulsky, Beverly L. Davidson

Abstract—We have reported that adenovirus-mediated gene transfer is augmented in the endothelium of atherosclerotic blood vessels. We observed that vascular cell adhesion molecule-1 (VCAM-1) shares some homology with the coxsackievirus and adenovirus receptor. Because VCAM-1 is upregulated on atherosclerotic endothelial cells, we hypothesized that VCAM-1 may act as an auxiliary receptor to augment adenovirus-mediated gene transfer. To test this hypothesis, stable NIH 3T3 cell lines that constitutively express VCAM-1 on the cell surface were generated. Recombinant adenovirus 5 (Ad5), which contains the reporter β-galactosidase gene, was used to compare Ad5 infection in VCAM-1 \(^\text{+}\) and parental NIH 3T3 cells. Total β-galactosidase activity and the number of transgene-positive cells were 6- to 10-fold and 5-fold higher, respectively, in VCAM-1 \(^\text{+}\) than in VCAM-1 \(^\text{−}\) cells. Ad5 binding to VCAM-1 \(^\text{+}\) cells was increased by 3-fold over VCAM-1 \(^\text{−}\) cells. Soluble VCAM-1 protein, present during infection or viral binding, reduced β-galactosidase activity in VCAM-1 \(^\text{+}\) cells in a dose-dependent manner. Taken together, we conclude that VCAM-1 can mediate adenovirus binding and infection. This may explain, in part, the previous finding that adenovirus-mediated gene transfer is augmented in atherosclerotic arteries. (Arterioscler Thromb Vasc Biol. 2001;21:238-242.)

Key Words: vascular cell adhesion molecule-1 ■ coxsackie and adenovirus receptor ■ adenovirus-mediated gene transfer ■ atherosclerosis

Endothelium in atherosclerotic vessels is an important target for gene transfer, both for the investigation of the role of gene products in atherogenesis and for the development of gene therapy.\(^1,2\) Adenovirus-based vectors have been used extensively in gene transfer to atherosclerotic animals.\(^3,4\) We and others have previously reported that adenovirus-mediated gene transfer to the endothelium was increased in atherosclerotic vessels compared with normal vessels.\(^5,6\)

Vascular endothelium is altered by atherosclerosis, with changes in a variety of intracellular and cell surface molecules.\(^7,8\) Because increased viral binding often translates into improved transduction efficiency,\(^9,10\) we considered candidate molecules on the surface of endothelium that could behave as surrogate receptors, allowing increased binding of adenovirus to the surface.

The receptor for adenoviruses types 2 and 5 has been identified as the coxsackie-adenovirus receptor (CAR).\(^11-14\) The receptor is expressed most abundantly on human heart and mouse liver and to varying degrees in other tissues.\(^11-13\) In addition to the high-affinity receptor, the adenovirus may\(^15\) or may not\(^16\) require the \(\alpha\)-integrins as coreceptors.

Vascular cell adhesion molecule-1 (VCAM-1), which is not expressed on normal endothelium, is expressed on the endothelium of atherosclerotic vessels.\(^17-19\) VCAM-1 functions as an adhesion receptor for leukocytes bearing very late antigen \(\text{4}^{20,21}\) and may contribute to leukocyte recruitment at the early stages of atherogenesis. Proatherogenic factors, including hypercholesterolemia, cytokines (eg, interleukin-1 \(\alpha\)), and advanced glycation end products, induce the expression of VCAM-1.\(^17,18,22\)

Like CAR, VCAM-1 belongs to the immunoglobulin gene superfamily, and we observed that there is homology between VCAM-1 and CAR. Therefore, we speculated that augmented adenovirus-mediated gene transfer to the endothelium of atherosclerotic blood vessels\(^5\) may result in part from the surface expression of VCAM-1. In the present study, we tested the hypothesis that VCAM-1 could serve as an auxiliary receptor for adenovirus by comparing adenovirus infection and binding in VCAM-1 \(^\text{+}\) and VCAM-1 \(^\text{−}\) cells.

Methods

Stable Cell Lines Expressing VCAM-1 on the Cell Surface

The plasmid VCAM-1 (pVCAM-1) contains VCAM-1 cDNA (rabbit 7D form, kindly provided by Dr Anthony Rosenzweig, Massachusetts General Hospital, Boston) under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter, as

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Adenovirus Infection of VCAM-1+ and Parental NIH 3T3 Cells

VCAM-1+ and parental NIH 3T3 cells were plated at 15,000 to 20,000 cells per well on a 96-well plate (Costar), which was pretreated with 2% gelatin (Sigma) to enhance cell adherence. At 90% confluence (24 to 48 hours after plating), triplicate wells were infected with recombinant adenovirus 5 (Ad5) containing the reporter β-galactosidase gene (Ad5βgal) at a multiplicity of infection of 0, 5, 25, and 125 infectious units per cell, in a volume of 40 μL per well. Two hours after incubation in a 37°C incubator, unbound virus was removed and replaced with 100 μL of fresh DMEM containing 10% FBS and antibiotics penicillin and streptomycin supplemented with G418 at 0.5 mg/mL. (All reagents were purchased from Life Technologies.) The cell proliferation curve is indistinguishable between VCAM-1+ and parental NIH cells (data not shown).

Histochemistry for β-Galactosidase

VCAM-1+ and parental NIH 3T3 cells were infected with Ad5βgal at a multiplicity of infection of 0, 5, 25, and 125 infectious units per cell. Twenty-four hours after infection, cells were assayed for β-galactosidase expression by using a chemiluminescent assay kit (Tropix), with total activity normalized to protein content. Protein content was measured by using a modified Lowry method with BSA as a standard (DC protein assay kit, Bio-Rad).

Adenovirus Binding Detected by Southern Blotting

VCAM-1+ and parental NIH 3T3 cells were plated on 60-mm plates. At confluence, the cells were equilibrated to 4°C for 1 hour. At this low temperature, virus binds cells through receptors but cannot enter low temperature, virus binds cells through receptors but cannot enter... 239

Competition of Viral Infection or Binding to VCAM-1+ Cells by Soluble VCAM-1

Soluble VCAM-1 protein (sVCAM-1, human 7D form with deleted transmembrane and cytoplasmic domains) was kindly provided by Dr Roy Lobb (Biogen Inc, Cambridge, Mass).29 sVCAM-1 was incubated at a final concentration of 0.1, 1, or 10 μg/mL with Ad5βgal at a multiplicity of infection of 125 infectious units per cell at 37°C for 15 minutes before infection (37°C for 2 hours) or binding (on ice for 30 minutes) to VCAM-1+ and parental NIH 3T3 cells. Cells were washed twice with DMEM/2% FBS after infection or binding to remove unbound virus. After 24 hours of incubation at 37°C, cell lysate was prepared and assayed for β-galactosidase activity as described in the previous section. Three independent experiments were performed with duplicates used for each sample.

Results

CAR is a cell membrane protein with 365 amino acid residues (aa) composed of a short leader, a 222-aa extracellular portion of 2 immunoglobulin domains, and a 107-aa intracellular portion.31 It has been reported that the first immunoglobulin domain is sufficient for the adenovirus-binding activity of intact CAR.30,31 A homology search using the BLAST program22 for the amino acid sequence of the 2 Ig domains revealed an overall 43% homology between the first Ig domain of CAR and the seventh Ig domain of VCAM-1 in an 86-aa-long segment (Figure 1). In addition, there is 57% homology between aa 100–141 of CAR and aa 271–312 of VCAM-1, 50% homology between aa 100–154 of CAR and aa 559–613 of VCAM-1, and 56% homology between aa 90–212 of CAR and 361–383 of VCAM-1.

NIH 3T3 cells, which lack a detectable receptor for adenovirus fiber,13,33,34 were cotransfected with pVCAM-1 and pMC1neoPolyA. pVCAM-1 expresses VCAM-1 on the cell surface under control of the RSV LTR promoter. pMC1neoPolyA expresses the neomycin-resistance product, conferring G418 resistance in transfected cells. FACS analysis indicated that stable transfecants constitutively expressed VCAM-1 on the cell surface (Figure 2). Mean fluorescence in VCAM-1+ cells was ~20-fold higher than

Figure 1. Homology between VCAM-1 and CAR. A, Overall structures of human and rabbit VCAM-1 (hVCAM-1 and rbVCAM-1, respectively) and human CAR (hCAR). Circles represent extracellular immunoglobulin domains. The longest region of homology is indicated as a bold line. B, Amino acid sequence of the homologous region, with bold letters indicating identity or similarity. Amino acid numbers are indicated and correspond to the full-length preprocessed forms.
that found in parental NIH 3T3 cells. The VCAM-1 cells, like parental NIH 3T3 cells, lack CAR expression, as detected by reverse transcription–polymerase chain reaction (data not shown).

Replication-deficient Ad5 containing the Escherichia coli lacZ gene driven by the RSV LTR promoter (Ad5\textsuperscript{gal}) was used for comparison of adenovirus infection in VCAM-1 and VCAM-1 NIH 3T3 cells. Cells were infected with Ad5\textsuperscript{gal} at 5, 25, or 125 infectious units per cell for 2 hours at 37°C, followed by removal of unbound virus. Twenty-four hours after infection, cell lysates were assayed for \(\beta\)-galactosidase activity, with total activity normalized to protein content. Total \(\beta\)-galactosidase activity was 6- to 10-fold higher in VCAM-1 than in parental NIH 3T3 cells (0.21 \(\pm\) 0.02 versus 0.019 \(\pm\) 0.003 at 5 IU per cell, 0.99 \(\pm\) 0.10 versus 0.17 \(\pm\) 0.08 at 25 IU per cell, and 7.38 \(\pm\) 1.12 versus 0.81 \(\pm\) 0.11 at 125 IU per cell; Figure 3). Three independent VCAM-1 cell lines were tested and showed similar results. This finding indicates augmented adenovirus infection in VCAM-1 cells.

Histochemistry for \(\beta\)-galactosidase activity was performed to determine whether increased levels of gene transfer as measured by enzyme activity were the result of the transduction of more cells. When VCAM-1 and parental NIH 3T3 cells were infected with Ad5\textsuperscript{gal} and then stained for \(\beta\)-galactosidase expression by 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside histochemistry, \(\approx\)5-fold more cells expressed \(\beta\)-galactosidase in VCAM-1 than in parental NIH 3T3 cells (Figure 4).

The results above are best explained by increased binding of virus to the cells when VCAM-1 is present on the cell surface. To test this possibility, cells were equilibrated at 4°C for 2 hours, unbound virus was removed, and bound viral DNA was isolated.\textsuperscript{26} The amount of DNA bound was quantified by Southern blotting.\textsuperscript{27} There was \(\approx\)3-fold more adenovirus DNA bound to VCAM-1 cells (2.7 \(\pm\) 0.8, \(n=3\)) than to parental NIH 3T3 cells (1 \(\pm\) 0, \(n=3\); Figure 5).

If VCAM-1 acts as an auxiliary receptor for adenovirus, then soluble VCAM-1 protein should compete with adenovirus to bind to VCAM-1–expressing cells. sVCAM-1 was incubated with Ad5\textsuperscript{gal}, and the mixture was added to VCAM-1 and parental NIH 3T3 cells. Cells were incubated either on ice (for binding) or at 37°C (for infection) and were washed to remove unbound virus. After 24 hours of incubation at 37°C, sVCAM-1 reduced \(\beta\)-galactosidase activity in the lysate of VCAM-1 cells (Figure 6). In control experiments using BSA, instead of sVCAM-1, BSA did not inhibit or augment \(\beta\)-galactosidase activity (data not shown). This finding supports the conclusion that VCAM-1 binds Ad5 in VCAM-1 cells.

**Discussion**

In the present study, we tested the hypothesis that VCAM-1 can mediate adenovirus binding and infection. This hypothesis was based (1) on the observation of the augmented adenovirus-mediated gene transfer efficiency to the endothe-
CAR, the high-affinity receptor for adenovirus,\textsuperscript{11–13} when introduced to CAR-lacking cells such as Chinese hamster ovary cells and NIH 3T3 cells, increased adenovirus-mediated gene transfer by 2 to 3 orders of magnitude.\textsuperscript{11} The first immunoglobulin domain is sufficient for the adenovirus-binding activity of intact CAR.\textsuperscript{30,31} In comparison, we found that augmentation in VCAM-1\textsuperscript{+} cells is \(\approx 3\)-fold for viral binding and 6- to 10-fold for transgene expression. The modest increase can best be explained by the fact that VCAM-1 is only moderately homologous to CAR. We have been unable to demonstrate direct binding of Ad5 to pure sVCAM-1 protein using ELISA-based assays. As such, binding affinities of Ad5 fiber to VCAM-1 could be substantially lower than for CAR.

Like VCAM-1, other immunoglobulin superfamily members also share some homology with CAR. We studied VCAM-1 because VCAM-1 is expressed on the surface of endothelium of atherosclerotic vessels. It is possible that other molecules may also mediate weak binding of adenovirus. Whether a molecule mediates virus binding depends on the context of the molecule. The major histocompatibility complex class I \(\alpha_2\) domain is a high-affinity receptor for Ad2 and Ad5.\textsuperscript{35–36} When expressed in hamster cells, however, it is not a high-affinity receptor for Ad5.\textsuperscript{37}

Factors other than VCAM-1 also may contribute to the augmentation of adenovirus-mediated gene transfer in the endothelium of atherosclerotic vessels. The present study, which demonstrated the effect of VCAM-1 on adenovirus-mediated gene transfer to NIH 3T3 cells, provides an example of a cell surface molecule that is moderately homologous to CAR and that can mediate the binding of adenovirus. Our results suggest that auxiliary low-affinity receptors may be used to direct or improve adenovirus-mediated gene transfer to CAR-negative cells.

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