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COMMENTARY

# Receptors Mediating Adenovirus Attachment and Internalization

*Jeffrey M. Bergelson**

Division of Immunologic and Infectious Diseases, Children's Hospital of Philadelphia, Philadelphia, PA 19104, U.S.A.

**ABSTRACT.** Adenovirus infection requires that the virus attach to cells and be internalized. Interaction between the viral fiber protein and specific cell surface receptors, such as the 46-kDa coxsackievirus and adenovirus receptor (CAR), is responsible for attachment; a second interaction between the viral penton base and cell surface integrins facilitates virus internalization. Expression of receptors may determine whether tissues are susceptible to adenovirus infection and adenovirus-mediated gene delivery. BIOCHEM PHARMACOL **57**;9: 975–979, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** adenoviruses; receptors, virus; gene transfer; genetic vectors

The use of viral vectors for gene therapy has led to an increased interest in factors that govern virus tropism—the propensity of viruses to infect particular tissues. Attachment to a susceptible cell is the first step in infection, and expression of specific cell surface receptors for virus attachment is one important determinant of tropism. Of course, although attachment is important, the capacity of a cell to support nucleic acid replication, synthesis of viral proteins, or other events in the virus life cycle may also determine whether infection proceeds. Once attachment has occurred, contact with the receptor may facilitate subsequent events in infection, such as virus entry, delivery to an appropriate intracellular compartment, or removal of the viral capsid and release of the viral nucleic acid into the cell.

## ADENOVIRUSES AS VECTORS FOR GENE THERAPY

Adenoviruses cause human respiratory and gastrointestinal disease. Forty-seven serologically distinguishable human adenoviruses have been divided into six subgroups (A–F) on the basis of hemagglutination properties, DNA base composition, sequence homology, and oncogenic potential in rodents [1]. The molecular biology of the group C Ads† 2 and 5 has been studied most extensively. These viruses have been adapted as vectors for gene therapy [2]: genes essential for viral replication have been deleted, to produce vectors with limited capacity to cause disease and to permit the introduction of relatively large segments of foreign DNA. The potential advantages of adenovirus vectors for gene therapy include their relative safety (even replication-competent viruses generally cause only mild respiratory infections), the ease with which they can be produced, purified, and concentrated to high titer, and the fact that they can deliver genes to quiescent as well as to dividing cells. In practice, however, their use may be limited by inability to target efficient gene delivery to particular tissues of interest, and by anti-viral inflammatory responses, which have interfered with persistent expression of the delivered gene [3].

## ADENOVIRUS STRUCTURE

The adenovirus protein capsid, which encases the viral DNA, is an icosahedral structure, with 20 triangular surfaces; an elongated fiber projects from each of the 12 icosahedral 5-fold vertices. At its proximal end, the fiber is attached to a pentameric structure called the penton base. At its distal end, the fiber bulges out to form a globular "knob" domain. Purified fibers [4–6] and isolated recombinant knob domains [7, 8] bind to cells with high affinity, and block virus attachment, demonstrating that the fiber—specifically the knob domain—mediates virus attachment to the cellular receptor. The crystal structure of the knob domain has been determined: it is shaped like a 3-bladed propeller, with a deep central depression at the 3-fold symmetry axis [9]. Based on analysis of fiber sequences from viruses that do or do not bind the same receptor, amino acid residues within the depression have been proposed to interact with the cellular receptor.

* Correspondence: Jeffrey M. Bergelson, M.D., Abramson 1202E, Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, Philadelphia, PA 19104. Tel. (215) 590-3771; FAX (215) 590-2025; E-mail: bergelson@email.chop.edu

† *Abbreviations*: Ad(s), adenovirus(es); CAR, coxsackievirus and adenovirus receptor; MHC-I, major histocompatibility complex class I; PI3K, phosphoinositide-3-OH kinase; and MAP, mitogen-activated protein.

## ADENOVIRUS FIBER RECEPTORS

Cross-competition experiments demonstrate that the fibers of Ads 2 and 5 (belonging to group C) bind to the same receptor [4], whereas those of Ad 3 (belonging to group B) bind to a different receptor [10]. A variety of biochemical approaches were used to demonstrate proteins that could bind fibers—either immobilized on columns or in ligand blotting assays [5, 11, 12]—but none of these proteins was identified or shown to mediate virus attachment to cells.

### CAR

About 20 years ago, it was observed that Ad 2 competed for a cell surface attachment site with a structurally and genetically distinct virus, coxsackievirus B3, and it was proposed that these two unrelated viruses shared a common receptor [13]. Recently, using antibodies that recognized a putative coxsackievirus receptor, two groups independently cloned a 46-kDa cell surface protein and demonstrated that it is a receptor for coxsackieviruses and for adenoviruses as well [14, 15].

CAR is a transmembrane protein with two immunoglobulin-like extracellular domains and a long cytoplasmic domain; its cellular function, other than as a virus receptor, remains unknown. Cells transfected with CAR gain the capacity to bind adenovirus and purified adenovirus fibers and knob domains. In addition, expression of CAR on relatively resistant hamster [14] and murine cells [15] greatly facilitates adenovirus-mediated gene transduction, indicating that attachment to CAR is followed by virus internalization. The CAR locus resides on chromosome 21, consistent with the observation that introduction of chromosome 21 DNA into murine or hamster cells results in expression of a high-affinity adenovirus fiber receptor [16]. The murine CAR homolog (mCAR) is more than 90% identical to human CAR [15, 17]. Two forms of mCAR have been identified, which differ only in the cytoplasmic domain, and both function in adenovirus attachment and adenovirus-mediated gene delivery.

Ads 2 and 5, and their purified knob domains, bind to purified recombinant CAR, confirming that CAR is directly responsible for virus attachment [18]. In addition to these group C viruses, adenoviruses belonging to groups A, D, E, and F—but not group B adenoviruses such as Ad 3, which had been shown previously to bind to a different receptor [5, 10]—bind directly to purified CAR and to CAR-transfected cells [18]. It thus appears that CAR is a receptor for a variety of adenoviruses, although some of these may also bind to other unidentified receptors. No receptor for the subgroup B viruses has been identified thus far.

### *MHC Class I*

Other investigators have reported that the heavy chain of the human MHC-I molecule is also a receptor for Ad 5. MHC-I is composed of a 44-kDa heavy chain, expressed in association with a smaller (12 kDa) subunit, β2-microglobulin. Hong *et al.* [19] screened a phage-displayed peptide library with Ad 5 knob domains, and derived a consensus peptide sequence with homology to the α2 domain of the MHC-I heavy chain. A synthetic consensus peptide was shown to bind Ads 2 and 5. In addition, Daudi B cells, which are deficient in β2-microglobulin expression and thus deficient in MHC class I surface expression, bind little or no virus, but transfection with cDNA encoding β2-microglobulin restores both MHC-I expression and the capacity to bind virus.

These results strongly suggest that an MHC-I molecule functions as a receptor for Ad 5, but several questions still remain. MHC-I molecules are highly polymorphic, and the products of multiple MHC-I loci are likely to be expressed on a given cell, all in association with β2-microglobulin. It is not known which β2-microglobulin-associated molecule is responsible for adenovirus attachment to Daudi cells, and it is not yet clear whether all MHC-I molecules function in adenovirus attachment, or only a subset. The latter possibility seems more likely, given that some cell lines that lack fiber receptors—such as murine fibroblasts [20] and human fibroblasts [21] and monocytes/macrophages [22]—are likely to express at least some MHC-I.

## ROLE OF THE PENTON BASE IN ADENOVIRUS INTERNALIZATION

Once adenovirus has attached to the cell surface, it moves rapidly to clathrin-coated pits, and is internalized in endosomal vesicles [23]. Within the endosome, the viral structure is disassembled in a step-wise fashion, which results in the release of viral nucleic acid into the cytoplasm and its eventual transport to the nucleus where replication occurs [24]. Adenovirus is not surrounded by a lipid envelope, and its penetration into the cytoplasm does not depend on membrane fusion events. Instead, disruption of the endosome is believed to permit entry. Although the evidence is indirect, acid-induced conformational changes in the penton base protein, and a dramatic increase in its hydrophobicity, may be important in this process [23].

The penton base protein of many adenovirus serotypes contains the sequence RGD (Arg-Gly-Asp), which interacts with vitronectin receptors (the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$) on the cell surface [6]. This interaction is of relatively low affinity ($K_d$ 55 nM, compared with 1.7 nM for the fiber–receptor interaction), and is not responsible for virus attachment to the cell; however, although purified fiber remains fixed to the cell surface, purified penton base protein, once bound, is internalized rapidly. Although virus binds equally well to $\alpha_v$-negative and $\alpha_v$-positive cells, the latter are significantly more susceptible to infection. It thus appears that virus internalization is facilitated by interactions between the penton base and $\alpha_v$ integrins on the cell surface.

Whether these interactions are essential is not clear at this time. Cell lines that lack $\alpha_v$ integrins can still be

infected [6], although infection is less efficient, and it has been suggested that other integrins may also function in virus entry. A virus mutant that lacks the penton base RGD is still infectious, although virus entry is delayed [25]. Internalization of this mutant is more rapid in cells that have high levels of fiber receptor, suggesting that recruitment of additional fiber receptors into a complex with the multivalent viral capsid may also facilitate virus entry [26].

Penton base interaction with $\alpha_v$ integrins leads to activation of focal adhesion kinase and PI3K [27]. Agents, such as wortmannin, that inhibit PI3K also inhibit adenovirus internalization and adenovirus-mediated gene delivery, indicating that activation of PI3K by adenovirus is important for virus entry. Engagement of $\alpha_v$ integrins by extracellular matrix ligands also leads to activation of MAP kinases, and in one study, adenovirus infection was found to stimulate the Raf/MAP kinase signaling pathway [28]. However, other investigators found that MAP kinase inhibitors that blocked other integrin-dependent functions, such as cell migration, had no effect on adenovirus entry [27]. Thus, adenovirus interaction with $\alpha_v$ integrins appears to trigger specific signaling events that are involved in virus entry. It is possible that activation of PI3K serves to modulate a reorganization of the actin cytoskeleton that may be involved in virus internalization. Adenovirus internalization has also been shown to require dynamin, a cytosolic GTPase that regulates endocytosis in clathrin-coated vesicles [29].

## ROLE OF RECEPTORS IN TISSUE TROPISM

*In vitro*, susceptibility to adenovirus infection and adenovirus-mediated gene delivery has been associated with expression of both fiber receptors and $\alpha_v$ integrins. We do not yet have sufficient information about the tissue expression of the identified receptors (CAR, MHC-I, and $\alpha_v$ integrins), or the susceptibility of individual tissues to adenovirus infection, to know how—or whether—the receptors determine adenovirus tropism *in vivo*.

Clinically, Ads 2 and 5 are commonly associated with upper respiratory tract infections in humans, although infections of the lung, brain, heart, and pancreas also occur, and virus is often recovered from the intestinal tract. There has been no systematic study of CAR protein expression in different tissues, but CAR mRNA is most abundant in the human heart, brain, pancreas, and small intestine, with low levels expressed in the lung and liver, and little or none detected in leukocytes, skeletal muscle, or spleen [15, 17]. In mice, however, mCAR RNA is abundant in the lung and liver [15, 17]. Different patterns of receptor expression in different species may be an important consideration in extrapolating the results of gene therapy experiments performed in animal models.

High levels of CAR expression in the murine liver may explain the observation that when adenovirus vectors are administered systemically to rodents, gene expression is most prominent in the liver [30]; however, it is also possible that virus uptake by the liver occurs by other mechanisms. Very little CAR mRNA is expressed in murine skeletal muscle; nonetheless, gene delivery can be accomplished by direct injection of adenovirus vectors into muscle tissue [31]. It is not known whether this involves other receptors, or whether the very high local concentrations of virus achieved can result in entry by receptor-independent mechanisms.

MHC-I is expressed on virtually all nucleated cells, yet a number of cultured cells (including fibroblasts, endothelial cells, and monocytes/macrophages) appear to lack functional fiber receptors [21, 22]. As discussed above, this may indicate that only a subset of MHC-I alleles function in virus attachment. It is not yet known whether murine MHC-I also functions in virus attachment. β2-Microglobulin-knockout mice are deficient in MHC-I expression, yet gene transduction to the liver of these animals appears to be normal, suggesting that murine MHC-I is not essential for virus entry [3].

Expression of fiber receptors has been shown to be an important limiting factor in susceptibility to adenovirus-mediated gene delivery. Cells that lack fiber receptors are inefficiently transduced [21], and susceptibility to gene transfer by adenovirus vectors is greatly increased by introduction of fiber receptors [14, 15, 17], or by modifications of the virus that permit attachment to alternative receptors [21, 32].

However, at least *in vitro*, gene transduction can also occur by fiber-independent pathways. CHO cells express few detectable fiber receptors, and virus entry is greatly facilitated by expression of human CAR, yet, in the presence of high virus concentrations, inefficient entry and gene transduction do occur [14, 33]. Similarly, some hematopoietic cells do not express fiber receptors, yet inefficient attachment to these cells can occur in the absence of fiber, by direct interaction between the penton base RGD and RGD-binding leukocyte integrins, distinct from the $\alpha_v$ integrins that mediate virus internalization [22]. In an attempt to redirect virus to different target cells, vectors with modified fibers have been produced: for example, introduction of polylysine permits fiber attachment to heparan-sulfate, and facilitates gene delivery to cells that do not express fiber receptors [21].

## ABSENCE OF RECEPTORS—A POSSIBLE BARRIER TO ADENOVIRUS-MEDIATED THERAPY FOR CYSTIC FIBROSIS

The pulmonary disease in cystic fibrosis results from a defect in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel that is normally expressed in the superficial epithelium of the small airways. Because viral transmission occurs by the respiratory route, Ad vectors were considered attractive for delivery of CFTR to the airway, and initial studies showed that *in vitro* cultures of airway epithelium were easy to transduce with

adenovirus vectors. However, gene delivery has so far proved inefficient *in vivo* [34].

Human airway epithelium is composed of ciliated columnar cells that develop from an underlying layer of poorly differentiated basal cells. *In vitro*, primary cultures of respiratory epithelium, which are easily transduced, consist predominantly of undifferentiated cells; however, mature columnar epithelium is resistant to adenovirus infection or gene transduction [35, 36]. It has been suggested that, *in vivo*, Ad-mediated gene delivery may occur largely where the columnar epithelium is damaged, and basal cells are exposed to the airway lumen [36]. The resistance of columnar cells to Ad-mediated gene delivery has been related to the absence of fiber receptors from the apical membrane [37], and although CAR is expressed on well-differentiated columnar epithelium *in vitro*, it is restricted to the basolateral membrane and is inaccessible to viral vectors [38]. It is possible that other receptors involved in adenovirus entry are also absent from the luminal surface of the airway epithelium [38].

Although the *in vitro* data are suggestive, we do not yet know where fiber receptors are present in the human airway *in vivo*, or whether an absence of receptors poses an insurmountable barrier to cystic fibrosis gene therapy with adenovirus vectors. Modifications of the viral fiber may permit attachment to molecules specifically expressed on target cells, and development of new vectors, based on Ad serotypes that bind receptors other than CAR, may facilitate gene delivery to tissues that now appear refractory. A better understanding of the distribution of CAR and other fiber receptors in human tissues may help identify those sites where adenovirus-mediated gene delivery is likely to succeed, as well as those where other viral or non-viral delivery systems might better be employed.

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