Decreased Efficiency of Adenovirus-Mediated Gene Transfer in Aging Cardiomyocytes

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Background—Aging is an independent risk factor for the development of cardiovascular disease. Clinical application of myocardial gene transfer may be best suited in the elderly. In vivo gene transfer by adenovirus is less efficient in aging myocardium.

Methods and Results—When infected with adenovirus containing β-galactosidase (β-gal) and green fluorescent protein (GFP) driven by cytomegalovirus promoters in vitro, aging rat cardiac myocytes exhibit significantly lower infectivity and delayed transgene expression compared with adult controls. Abnormalities of viral internalization may be one mechanism accounting for this difference. To investigate this, we studied expression levels of the coxsackievirus and adenovirus receptor (CAR) as well as other potential integrins involved in the internalization of adenoviruses. CAR expression tended to be upregulated whereas among potential integrins, αβ1 was downregulated in aging cardiac myocytes. Blocking the β1 component of αβ1 further decreased infectivity, suggesting that the interaction between the penton base of the adenovirus and β1 maybe a crucial component of the viral entry mechanism.

Conclusions—These results suggest that it is integrin-stimulated internalization rather than the adenovirus-CAR interaction that plays a vital role in adenoviral entry. The downregulation of integrins observed in senescent cells may be a key mechanism accounting for the decrease in viral infectivity seen in these cells. These findings have implications for the gene therapy treatment of myocardial failure in the elderly. (Circulation. 2003;107:1170-1175.)

Key Words: aging □ gene therapy □ myocytes □ viruses □ receptors

Clinical application of myocardial gene transfer may be best suited in the elderly in whom heart failure is a problem of epidemic proportions. In the aging myocardial cell, a number of cellular abnormalities exist that are characterized by a decrease in specific gene expression.1,2 These pathways are potential targets for gene therapy. However, we have previously found that adenoviral gene transfer in vivo is less efficient in aging myocardium.3 Indeed, we showed that 2 days after in vivo gene transfer with adenovirus containing β-galactosidase, significantly fewer cardiac myocytes were infected in old rats than in adult controls.

The aging heart exhibits numerous alterations that could explain these results, such as increases in cardiomyocyte necrosis and apoptosis, decreased vascularization, more fibrosis, and differential remodeling of the extracellular matrix.4–6 It is not clear, however, whether the reduced infectivity in aging myocytes arises as a consequence of these alterations in cellular phenotype, reflects an inherent resistance of the cells per se to adenoviral transduction, or represents some combination of both factors.

The efficiency of adenoviral vectors can be limited by a deficiency of the appropriate binding/entry mechanism of the target cell that is mediated via two families of cell surface receptors. The knob of the adenovirus fiber first binds to the coxsackievirus and adenovirus receptor (CAR), which functions as a high-affinity receptor for both Coxsackie B viruses and subgroup 2 and 5 adenoviruses.7,8 Internalization then occurs via an interaction of an arginine-glycine-aspartate (RGD) sequence on the adenovirus penton base with members of the integrin family of cell surface heterodimers. These heterodimers, including αvβ3, αvβ5, αβ3, and α5β1, all recognize the RGD sequence and could be involved in adenovirus internalization.9–11

Adult rat cardiac myocytes express very low levels of CAR.12 β1 integrins are the prime isoform expressed in postnatal cardiac cells,13 and αv is not detected in adult rat cardiac myocytes.14 To further explore the role of these integrins and determine the mechanism of decreased infectivity seen in aging cardiac myocytes, we (1) tested the efficiency of adenoviruses at mediating gene transfer and expression in aging cardiac myocytes in vitro, (2) extended these observations to another rat model of aging, (3) compared the level of adenovirus receptors and integrin expression (both CAR-dependent and CAR-independent pathways) in...
aging versus control adult cardiomyocytes, and (4) studied the involvement of integrins in the mechanism of adenovirus infection in cardiomyocytes.

Methods

Construction of Recombinant Adenoviruses
To construct the adenovirus for this study, we used the method described by He et al.\(^\text{13}\) whereby the backbone vector, which contains most of the adenoviral genome (pAd.EASY1), is used, and the recombination is performed in *Escherichia coli*. \(\beta\)-Galactosidase cDNA was subcloned into the adenoviral shuttle vector (pAd.TRACK), which uses the cytomegalovirus long-terminal repeat as a promoter. The shuttle vector used also has a concomitant green fluorescent protein (GFP) under the control of a separate cytomegalovirus promoter. The adenoviruses were propagated in 293 cells. The titers of stocks used for these studies measured by plaque assays were \(3 \times 10^{11}\) pfu/mL for Ad.\(\beta\)gal.GFP with particle/pfu ratios of 8:1. These recombinant adenoviruses were tested for the absence of wild-type virus by polymerase chain reaction of the early transcriptional unit E1.

Preparation of Cardiac Myocytes
Calcium-tolerant rat ventricular myocytes were isolated from hearts of male Fisher 344 rats age 24 to 29 months (National Institute on Aging, Bethesda, MD) and 3 to 6 months (adult controls).\(^\text{14}\) These were resuspended in ACCt medium consisting of DMEM containing 2 mg/mL BSA; (in mmol/L) 2 L-carnitine, 5 creatine, and 5 taurine; 100 IU/mL penicillin; and 100 mg/mL streptomycin and plated at a density of 50 to 100 cells/mm\(^2\) into laminin-precoated dishes (1 \(\mu\)g/cm\(^2\), Invitrogen). After 1 hour, attached cells were infected with Ad.\(\beta\)gal.GFP at various multiplicities of infection (MOIs) for 2 hours and then washed and cultured for different time periods.

Infectivity Measurements
GFP-positive cells were examined by fluorescence microscopy at different time points and at various MOIs and were counted by using an imaging system that counts cells exhibiting the same fluorescence intensity (Image Pro Plus). A minimum of 200 cells were assessed for each sample, and cells were then fixed for X-Gal staining or lysed for measurement of GFP expression by Western blot analysis.

To study the involvement of integrins in infectivity, cardiomyocytes were preincubated with different concentrations of RGD peptides (1.5 mmol/L, Sigma), anti-\(\beta\) integrin blocking antibodies (2 \(\mu\)g/mL, Santa Cruz Biotechnologies or Chemicon International) diluted in TBST containing 5% nonfat dry milk. After washings with TBST, the membranes were incubated with a 1:5000 dilution of peroxidase-conjugated mouse anti-goat IgG. The immune complexes were detected using a chemiluminescence kit (NEL DuPont). The intensity of each band was then analyzed using laser densitometry. For immunoprecipitation, 400 \(\mu\)g of total protein from the lysates was immunoprecipitated with anti-\(\alpha\) or anti-\(\beta\) antibodies, and the immune complexes were collected on protein A/G-Sepharose beads, washed with the lysis buffer, and finally subjected to Western blot analysis and probed for the \(\beta\) partners as described above.

Statistics
All data are presented as mean±SEM. The Student \(t\) test was used to analyze differences in infectivity and compare changes in integrin and CAR expression. Differences among ages and differences among multiple conditions were determined by 1-way ANOVA with a post hoc Tukey test. Statistical significance was accepted at \(P<0.05\).

Results

Infectivity Measurements
Infecting the same number of aging and adult cells with Ad.\(\beta\)gal.GFP at MOIs of 10 and 100 for 48 hours, we found that the percentage of aging GFP\(^+\) cells was significantly lower compared with adult (% of total: MOI 10, aging 16±2 \(P<0.05\) versus adult 75±6; MOI 100, aging 37±4 \(P<0.05\) versus adult 90±5) (Figure 1A). Similarly, the expression of GFP as assessed by Western blot was also lower in aging cells compared with adult (Figure 1B). These results were corroborated by the decrease in percentage of X-Gal–positive aging cells. This decrease in infectivity persisted at 72 hours after infection. Adult myocytes were maximally infected at MOI 100 after 48 hours, whereas aging ones reached this maximum control value at MOI 1000 (Figure 1A and 1B).

We also examined infectivity as a function of time after transfection (12, 24, and 48 hours) at MOI 1000. The aged myocytes had delayed \(\beta\)-Gal and GFP expression and reached maximum control values at 48 hours (Figure 1C). When these experiments were carried out on cardiomyocytes isolated from aged F344/Brown Norway hybrid rats (26 to 30 months old, National Institute on Aging), there was a similar decrease in infectivity between aging and adult myocytes (Figure 2).

CAR and Integrin Expression
Plated cells were lysed at 1 hour to measure CAR and integrin expression either individually or in heterodimeric form (\(\alpha\) and \(\beta\), \(\alpha\) and \(\beta\), \(\alpha\) and \(\beta\)), by immunoprecipitation and/or Western blot analysis. Ventricular sarcolemmal membrane preparations from the whole heart were also analyzed to confirm the in vitro result and to bypass the possible enzymatic effect of cell isolation on integrin expression. Cell lysates from the cultured rat ventricular myocytes were prepared by scraping and homogenizing the cells in ice-cold lysis buffer (1% Triton X-100; [in mmol/L] 150 NaCl, 10 Tris [pH 7.4], 1 EDTA, 0.2 phenylmethylsulfonyl fluoride, and 0.2 sodium o-vanadate; and 0.5% Nonidet P-40). Sarcolemmal membranes were also prepared and extracted in the same buffer. The membranes or cell lysates were centrifuged, and total protein content was measured using the Bradford assay (Bio-Rad). Total protein (70 \(\mu\)g) was resolved by 7.5% SDS-PAGE, and proteins were transferred to nitrocellulose membranes. The membranes were stained with Pon-
Involvement of Integrins in Adenoviral Infection of Cardiomyocytes

Adult rat cardiomyocytes express mainly /H92521 integrins, which in heterodimeric form recognize the RGD sequence. As /H92521 integrin expression is decreased in aging cells, to further define the role of /H92521 integrins, we inhibited or activated integrin signaling in cardiomyocytes and studied the effects on infectivity. Because adenovirus possesses the RGD sequence, synthetic peptides with or without RGD sequences were incubated with cardiomyocyte controls for 30 minutes before Ad/H9252-/Gal/GFP infection (MOI 100 for 48 hours). Adenovirus infectivity in cardiomyocytes was significantly decreased (by 42%) by the RGD peptide at 1.5 mmol/L but not by the GRGESP-negative control peptide (Figure 4). Monoclonal antibodies directed against rat-specific /H92521 integrins (at 20 μg/mL) also partially decreased adenovirus infectivity by 44% (Figure 4). The IgM control had no effect on infectivity. Cardiomyocytes bind preferentially mainly to laminin via β integrin receptors, and laminin interacts with integrins by both RGD-dependent and -independent mechanisms. To further elucidate the role of integrins, adult and aging cardiomyocytes were incubated with the extracellular matrix protein laminin before adenovirus infection. When adult cells were incubated with laminin (50 μg/mL), there was an increase in infectivity at MOI 10 at 24 hours (Figure 5). At MOI 100 (maximum infectivity) there were no additional effects. Interestingly, laminin also induced an increase in infectivity in aging cells at MOI 10 (data not shown) and 100 at 24 hours (Figure 5).

Discussion

We have previously shown that adenoviral gene transfer in vivo by cross clamping is less efficient in the aging rat myocardium. These results could be related to observations made in animals and humans that indicate that the aging process of heart is characterized by alterations in coronary circulation. These alterations lead to a defect in vascular supply, associated with multiple foci of replacement fibrosis (and changes in extracellular matrix), local ischemia, and decreased metabolism, as well as necrosis and apoptosis of myocytes. The change in extracellular matrix surrounding mature skeletal muscle cells has especially been noted to impair adenoviral transduction efficiency. However, it is not known whether the decreased infectivity observed in vivo is due to a decrease in infectivity at the level of the aged cardiomyocyte.

Our results show that adenovirus gene transfer in vitro is indeed decreased in aging cardiomyocytes. Furthermore, aging myocytes exhibit delayed transgene expression. This observation is verified in a second model of aging rat. Transduction by adenoviral vectors can be limited by a number of factors including deficiency of virus binding to
CAR, impaired recognition of the RGD sequences of various integrins (for example, integrins $\alpha_3\beta_1$, $\alpha_3\beta_2$, $\alpha_5\beta_1$, and $\alpha_5\beta_1$) and hence decreased internalization of the virus by the myocyte. Previous studies have shown that CAR overexpression enhances adenoviral infection in neonatal rat cardiomyocytes, suggesting that CAR is a key determinant of cardiac susceptibility to adenovirus infection, with coreceptors $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrin not being limiting factors. CAR expression is low in adult rats and is upregulated during myocardial inflammation. Our results show low levels of CAR immunoreactivity in adult control myocytes and in ventricular sarcolemmal membrane preparations—observations consistent with those reported by Noutsias et al and Ito et al. They also suggest that CAR expression is not correlated with the decrease in infectivity in aging cardiomyocytes. This is supported by the observation that control cardiomyocytes, with much lower levels of CAR as compared with 293 cells, can still be highly transduced.

CAR has been found to be upregulated in hearts of patients with dilated cardiomyopathy. However, it is not clear whether the increase in CAR is causative for the left ventricular dysfunction. Recently, CAR has been found to be a transmembrane component of the tight junction and to act as a functional barrier to paracellular solute movement. Sequestration of CAR in tight junctions may limit virus infection across membrane surfaces. In dilated cardiomyopathy, tight junctions are disrupted; therefore, with increases in CAR, one can speculate that viruses in the bloodstream have access...
Relative CAR and Integrin Expression in Aging Cardiac Myocytes as Compared With Adult Cardiac Myocytes

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Adult (6 Months, n=6)</th>
<th>Aging (26 Months, n=6)</th>
<th>Adult (6 Months, n=3)</th>
<th>Aging (26 Months, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAR</td>
<td>1.00 ± 0.02</td>
<td>1.77 ± 0.41</td>
<td>1.00 ± 0.10</td>
<td>1.63 ± 0.31</td>
</tr>
<tr>
<td>αv</td>
<td>1.00 ± 0.16</td>
<td>0.90 ± 0.08</td>
<td>1.00 ± 0.15</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>β3</td>
<td>1.00 ± 0.07</td>
<td>0.89 ± 0.04</td>
<td>1.00 ± 0.12</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td>αvβ3</td>
<td>1.00 ± 0.10</td>
<td>1.03 ± 0.12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>β5</td>
<td>1.00 ± 0.04</td>
<td>1.05 ± 0.08</td>
<td>1.00 ± 0.08</td>
<td>0.90 ± 0.12</td>
</tr>
<tr>
<td>αvβ5</td>
<td>1.00 ± 0.09</td>
<td>0.95 ± 0.15</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>αv</td>
<td>1.00 ± 0.12</td>
<td>0.56 ± 0.02*</td>
<td>1.00 ± 0.15</td>
<td>0.59 ± 0.03*</td>
</tr>
<tr>
<td>β1</td>
<td>1.00 ± 0.03</td>
<td>0.82 ± 0.03*</td>
<td>1.00 ± 0.09</td>
<td>0.83 ± 0.05*</td>
</tr>
<tr>
<td>αvβ1</td>
<td>1.0 ± 0.05</td>
<td>0.53 ± 0.03*</td>
<td>1.0 ± 0.10</td>
<td>0.65 ± 0.04*</td>
</tr>
</tbody>
</table>

Levels of expression of integrins potentially involved in adenovirus entry in aging myocytes and sarcolemmal membranes expressed as fold change vs adult values. ND indicates not detected. *P<0.05.

Figure 4. Impairment of adult cardiomyocyte infectivity with RGD peptide and anti-β1 integrin antibody. Incubation with RGD peptide or anti-β1 integrin antibody for 30 minutes at 37°C significantly decreases subsequent transduction with adenovirus by 42% and 44%, respectively, compared with controls (n=3), whereas GRGESP peptide (1.5 mmol/L, n=2) has no effect on infectivity. CTL indicates adult control myocytes.

Figure 5. Western blot showing exposure to laminin for 30 minutes at 37°C before incubation with adenovirus enhances infectivity in aging (at MOI 100) and adult (at MOI 10) myocytes after 48 hours as assessed by expression of GFP.

more readily to the receptor causing direct injury to the cardiomyocyte. Furthermore, CAR has been proposed to be a “pathfinder” protein helping with cell-cell interaction in neonatal and embryonic hearts. In adult hearts, CAR expression is low because the structure of the heart is complete. However, with aging, fibrosis develops and there is disruption of the cardiomyocyte integrity. This may lead to the reactivation of the embryonic program leading to an increase in CAR.

The main integrins in adult cardiac myocytes are β1 integrins, but αv and αv may also be expressed. As these and other integrins have also been found to be involved in adenovirus transfer efficiency, we measured the level of expression of αv, β1, β3, β5, αv, and αv in aging cells.

Interestingly, we found that aging was associated with a decrease in β1 integrins that may have important global effects on cell signaling. This decrease in β1 integrin expression is also noted in aging mouse myocardium. We found that both RGD and blocking antibodies partially reduced infectivity, implicating the β1 integrin–RGD interaction as one of the mechanisms involved in the adenoviral infection process in cardiomyocytes in vitro. However, the partial inhibition observed suggests that this is not the only virus entry site in myocytes, and other binding and/or internalizing sites may coexist on the cell surface. Our results also show that myocytes have no detectable level of αv, a finding noted by Maitra et al. Laminin has been shown to bind to cardiomyocytes via primarily β3 integrins, and may subsequently initiate a cascade of intracellular signaling events that affect cell migration, proliferation, differentiation, and gene expression. In light of the differences observed in integrin expression between aging and adult cardiomyocytes, we examined the effect of laminin on transgene expression. Unexpectedly, we found that laminin enhanced infectivity in both adult and aged cardiomyocytes, even though β3 integrin expression is decreased in aging cells. It is intriguing to speculate that laminin may activate downstream signaling pathways in both adult and aging cells that facilitate transgene expression. The finding that adenovirus exploits specific integrins for entry into the cardiomyocytes has important implications in the design of an adenoviral vector with increased tropism toward this cell type. Our further understanding of these alterations could lead to the development of more efficient vectors to target abnormal signaling pathways in aging cardiomyocytes.
Acknowledgments
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