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Efficient Transfer of Genes into Senescent Cells by Adenovirus Vectors via Highly Expressed $\alpha v\beta 5$ Integrin

Yoshihiro Hashimoto,*,† Kenjiro Kohri,* Hidetoshi Akita,*,† Kohnosuke Mitani,‡ Kyoji Ikeda,† and Makoto Nakanishi†

**Department of Urology, Nagoya City University, Mizuho-ku, Nagoya 467, Japan; †Department of Geriatric Research, National Institute for Longevity Sciences, Obu, Aichi 474, Japan; and ‡Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90024*

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Although various methods for transferring genes into mammalian cells have been established, none have been successful with senescent cells. In this report, we present evidence of the efficient transfer of a gene into human senescent fibroblasts using an adenoviral vector. By employing a recombinant adenovirus vector harboring the β-galactosidase gene (AdCAGβNR), we observed a good correlation between the proportion of β-galactosidase positive cells and population doubling of the infected cells. In addition, 1.5- to 6.0-fold greater β-galactosidase activity was observed in senescent fibroblasts (population doubling [PD] = 58) than in young cells (PD=15). Western blotting analysis revealed that, compared with young fibroblasts, senescent fibroblasts expressed larger amounts of $\alpha v\beta 5$ and $\alpha v\beta 3$ integrins which were thought to form part of the adenovirus receptor. These results suggest that higher expression of $\alpha v\beta 5$ and $\alpha v\beta 3$ integrins in senescent cells renders them more sensitive to adenovirus infection than young cells. Thus, adenovirus vectors may prove to be useful in gene therapy strategies directed against senescence-related disorders.

Key Words: **adenovirus; αv integrin; senescence; gene transfer.**

Normal human fibroblasts exhibit a finite proliferative lifespan in vitro and have been utilized as a model for studying cellular aging (1–3). A prominent feature of senescent human fibroblasts is their failure to proliferate in response to serum mitogens, with growth arrest at the late G1 phase (2,3). Although various methods for transferring genes into mammalian cells have been reported, involving the use of calcium-phosphate, dextran sulfate, electroporation, and cationic lipids (4–7), none have been successful with senescent cells because of their loss of replicative capacity. However, the development of methods for effective gene transfer into these cells is critical, both for better understanding of the aging process and for creating therapeutic strategies for age-related diseases.

Replication-defective forms of human adenoviruses have been used to deliver foreign genes into various types of cells (8–11). The successes achieved using adenovirus as a gene delivery vector are in part due to its highly efficient mode of cell entry and the fact that cell replication is not an essential part of the process. These advantages suggest that it might be suitable for gene delivery into senescent cells.

Entry of human adenoviruses into cells involves their interaction with two separate receptors on the cell surface. The initial binding of adenovirus to an as yet unidentified receptor is mediated by the fiber capsid protein (12). Subsequent cell entry occurs via the internalization of the virus into clathrin-coated vesicles (13) and is mediated by the binding of penton base protein to αv integrins (14,15).

In this study, we demonstrated that senescent cells express $\alpha v\beta 5$ integrin much more strongly than do young cells and that adenoviral vectors can deliver a gene into senescent cells more effectively than into young cells.

MATERIALS AND METHODS

Cell lines and recombinant adenovirus. High (PD=58) passaged human fibroblasts were used as senescent fibroblasts. Cells were grown in DMEM containing 10% FBS, 10 units/ml penicillin, and 10 μg/ml streptomycin, as described previously (16), and maintained with routine medium changes. Cultures were passaged by trypsinization with 0.05% trypsin. All chemicals for cell culture were obtained from GIBCO-BRL.

The recombinant adenovirus vector harboring the β-galactosidase gene (AdCAGβNR) contains the modified chicken β-actin promoter with the CMV-1E enhancer (CAG) and the SV40 polyadenylation

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signal in a minigene cassette inserted into the E1 deletion region of modified Ad5. Viral stocks were expanded in a transformed kidney cell line (HEK293) containing E1A. Cells were harvested 48h after infection and disrupted by four cycles of freezing and thawing. Cell debris were removed by two cesium chloride gradient ultracentrifugations. The virus titer was determined by plaque-forming activity in 293 cells. Concentrated virus was dialyzed with PBS containing 10% glycerol, aliquoted, and stored at −80 °C.

Detection of adenovirus-mediated gene delivery. Cells were plated in triplicate at a density of 8.0×10^3 cells/cm^2 in 6-well tissue culture plates, infected with various multiplicities (0 to 3×10^2 virus particles per cell) of AdCAGβNR and incubated for 0.5h at 37 °C. Uninfected viruses were then removed by washing with PBS, and cells were incubated for an additional 48h at 37 °C in a 5% CO_2-humidified incubator. The cells were washed once in PBS, fixed, and stained for β-galactosidase activity. Adenovirus-infected cells were detected by counting the β-galactosidase-positive cells.

β-galactosidase activity was determined as follows; infected cells were washed once in cold PBS, harvested in 200 μl of reporter lysis buffer (Promega) and the activity was measured using an assay kit (Promega).

Senescence-associated β-galactosidase. Senescence-associated (SA) -β-gal activity was detected as previously described (17). Cells were washed once with PBS, pH 7.2, fixed with 0.5% glutaraldehyde in PBS and washed in PBS supplemented with 1mM $MgCl_2$. Cells were stained in X-gal solution (1 mg/ml X-gal, 0.12 mM $K_3Fe(CN)_6$, 0.12 mM $K_4Fe(CN)_6$, 1mM $MgCl_2$ in PBS at pH 6.0) overnight at 37 °C.

Western blot analysis. Cells were plated at a density of 1.0×10^4 cells/cm^2 in 10-cm culture dishes. The cells were washed once in cold PBS and lysed in 350 μl of extraction buffer (0.5% NP40, 20 mM Tris, pH 7.4, 0.25 M NaCl, 1 mM EDTA, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml of aprotinin, and 100 μg/ml of leupeptin). Protein samples (30 μg each) were separated by SDS-PAGE (12.5% gels), transferred to nitrocellulose membranes, and detected with monoclonal antibodies against human αvβ3 and αvβ5 integrins (Chemicon), and polyclonal antibodies against human p16, p21 and p27 (Santa Cruz Biotechnology). Detection was enhanced by chemiluminescence (ECL, Amersham).

RESULTS AND DISCUSSION

Since replication-deficient adenoviral vectors are highly infectious and are capable of transferring genes into non-dividing cells, we first determined whether senescent fibroblasts are susceptible to adenovirus-mediated gene delivery. We used a recombinant adenovirus vector, AdCAGβNR, that constitutively expresses β-galactosidase which targets the nuclei of infected cells. Senescent (PD=58) and young (PD=25) human fibroblasts were infected with AdCAGβNR at multiplicities of infection (MOI) of 3 or 100. Infected cells were then detected by staining for β-galactosidase activity after 48h. Approximately 8% of young cells and 30% of senescent cells expressed β-galactosidase activity when the cells were infected at an MOI of 3, whereas infection of both young and senescent cells was almost complete at an MOI of 100 (Fig.1). To more precisely assess the sensitivities of senescent and young cells to adenovirus vectors, virus infection of four cell types with distinct PDs was performed at several MOIs. As shown in Fig. 2, at lower MOIs (below 30) there was a remarkable correlation between PDs of infected cells and the population of β-galactosidase-positive cells. At an MOI of 300, however, almost 100% of cells of all types were positive, indicating that all were saturated with adenovirus.

To quantitate the differences in infection rates between young and senescent cells, we directly assayed β-galactosidase activity in the cell lysates. Again, there was a good correlation between β-galactosidase activities in the lysates and PDs of the cells (Fig. 3). Lysates of senescent fibroblasts exhibited 1.5- to 6.0-fold greater activity than did those of young fibroblasts (Fig.3).

Senescent cells adopt a flattened enlarged shape (18). The proportion of morphologically senescent cells was found to increase among cells with higher PD (data not shown). To further confirm the relationship between PD and the degree of cellular senescence, we examined senescence-associated β-galactosidase activities, at an optimum pH of 6.0, as a senescence-associated marker (17). It should be noted that no senescence-associated β-galactosidase activities whatsoever were detected in cells at a pH of 7.4 which was used for measuring these activities following AdCAGβNR infection. As shown in Fig. 4, the proportion of β-galactosidase-positive cells correlated well with PD, demonstrating that higher β-galactosidase activity was present in cells with higher PD. Taken together with the above results, we conclude that senescent fibroblasts are more susceptible to adenovirus infection than are young fibroblasts and that the higher β-galactosidase activity in senescent cells reflects their higher susceptibility to adenoviruses but not a higher level of transcription of the β-galactosidase gene.

Cell surface αv integrin is thought to form part of the receptor for adenoviruses (15). Cells deficient in αv integrin are significantly less susceptible to adenovirus internalization and infection. It has previously been reported that certain integrins are overexpressed in senescent cells, compared with young cells (19−21). We therefore considered whether human senescent fibroblasts overexpress αv integrins which act as receptors for adenoviruses. Protein levels of human αvβ3 and αvβ5 integrins were examined in senescent and young human fibroblasts by Western blotting using specific antibodies against human αvβ3 and αvβ5 integrins. As shown in Fig. 5, αvβ5 and αvβ3 integrins were expressed at a higher level in senescent human fibroblasts than in young human fibroblasts. As controls, p21 and p16 were also expressed at higher levels in senescent cells than in young cells, but expression of p27 was the same in both. These results suggest that the higher susceptibility of senescent cells to adenoviruses may be explained at least in part by the overexpression of αvβ5 and αvβ3 integrins. In this regard,

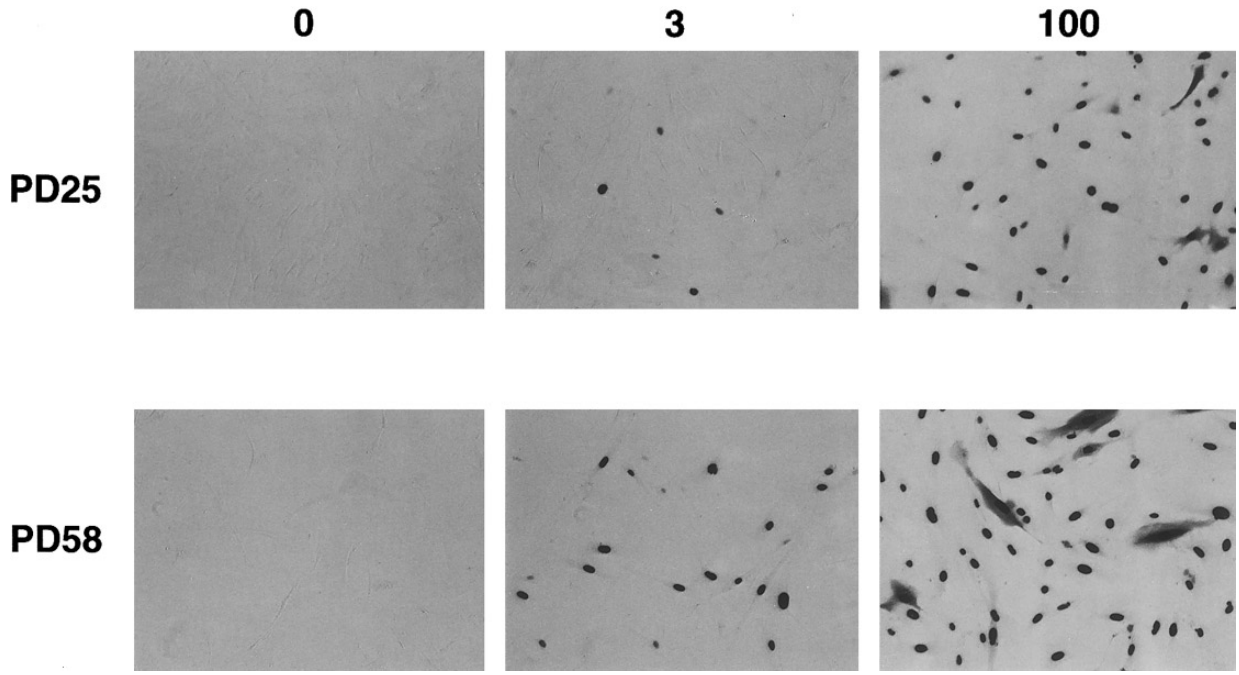

FIG. 1. Photomicroscopy of β-galactosidase-positive young (PD=25) and senescent (PD=58) human fibroblasts infected with AdCAGβNR. Cells were infected at MOIs of 3 or 100. Two days after infection, the cells were fixed and stained for β-galactosidase activity. An MOI of O represents control cells without infection. Magnification was $\times$200.

the intriguing results of a recent study showed that integrin αvβ5 plays important roles in adenovirus entry following internalization (22).

In the present study, we demonstrated that senescent cells are more susceptible to adenovirus gene transfer than are young cells. We have also shown that αvβ5 and αvβ3 integrins are overexpressed in senescent cells, which might account for the high susceptibil-

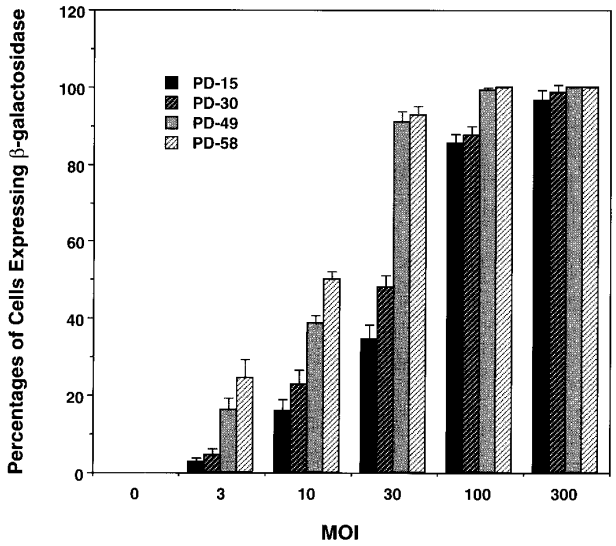

FIG. 2. Cells with higher PD were more susceptible to the adenovirus vector than those with lower PD. Cells were infected with various multiplicities of AdCAGβNR for 48h. The numbers of β-galactosidase-positive cells shown are the averages of three independent experiments. Standard deviations are indicated in the figures.

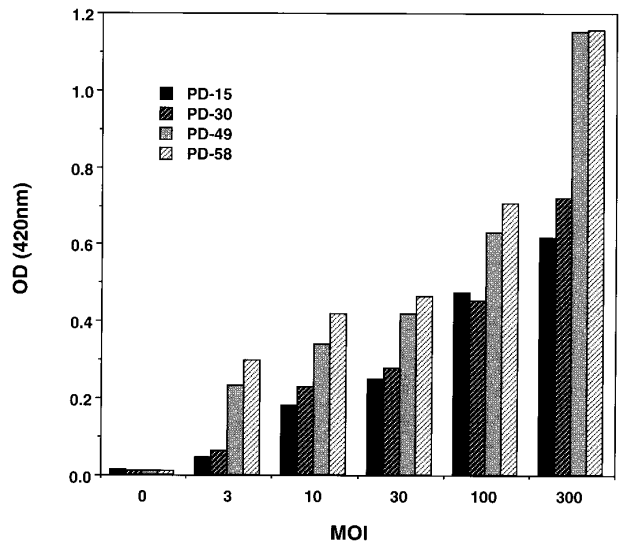

FIG. 3. Quantitation of β-galactosidase activity after adenovirus gene transfer. Cells were infected with various multiplicities of AdCAGβNR for 48h and harvested in reporter lysis buffer as described in Materials and Methods. Cell lysates were then used for the determination of β-galactosidase activity.

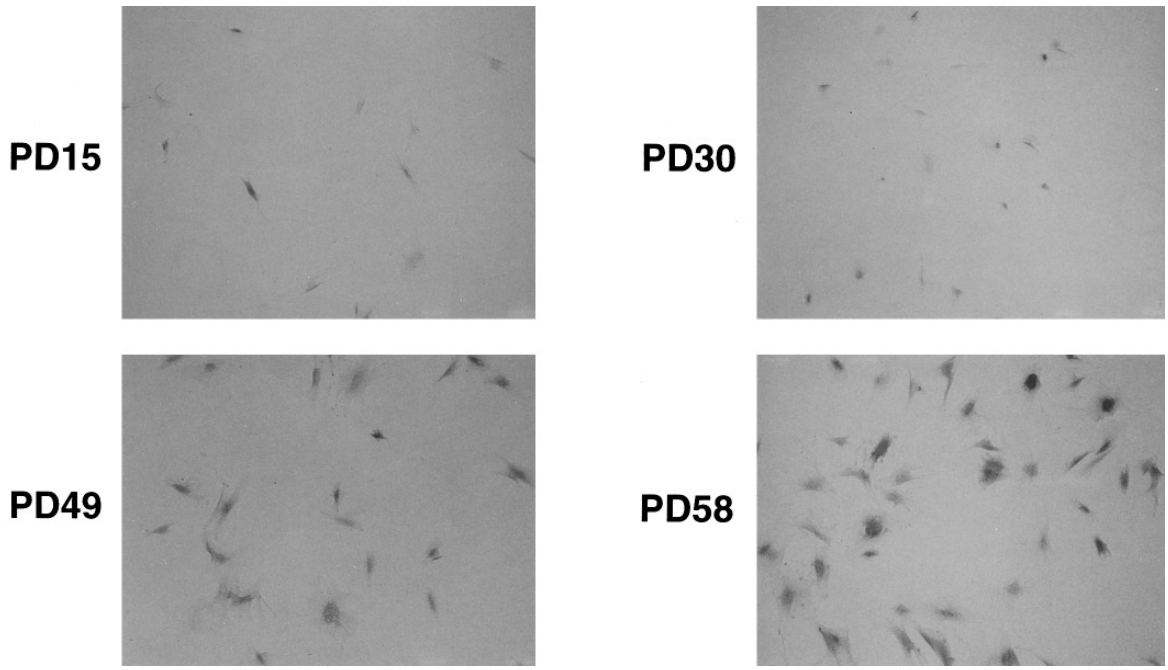

FIG. 4. Photographs of cells with the indicated PDs stained for β-galactosidase activity at a pH of 6.0. Photographs are at the same magnification ($\times$200).

ity of these cells to adenoviruses. Several disorders, such as impaired wound healing, anemia, and decreased immunity against various pathogens, have been thought to be caused by loss of proliferative capacity in senescent cells. Therefore, it is suggested that the adenovirus vector may prove to be a useful tool for efficient gene transfer into senescent cells as well as for the development of new strategies for combating age-related disorders.

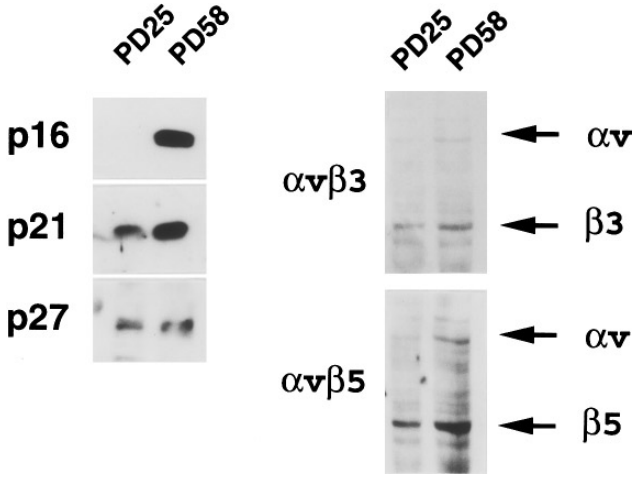

FIG. 5. Expression of human $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins and p16, p21, and p27 in young vs. senescent cells. Senescent (PD=58) and young (PD=25) human cells were lysed. Proteins (30 μg/lane) were separated by SDS-PAGE (12.5% gel) and transferred to a nitrocellulose membrane. They were then detected by Western blotting using antibodies against human $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, p16, p21, and p27.

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