

Gene Transfer to Human Pancreatic Endocrine Cells Using Viral Vectors

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We have studied the factors that influence the efficiency of infection of human fetal and adult pancreatic endocrine cells with adenovirus, murine retrovirus, and lentivirus vectors all expressing the green fluorescent protein (Ad-GFP, MLV-GFP, and Lenti-GFP, respectively). Adenoviral but not retroviral vectors efficiently infected intact pancreatic islets and fetal islet-like cell clusters (ICCs) in suspension. When islets and ICCs were plated in monolayer culture, infection efficiency with all three viral vectors increased. Ad-GFP infected 90–95% of the cells, whereas infection with MLV-GFP and Lenti-GFP increased only slightly. Both exposure to hepatocyte growth factor/scatter factor (HGF/SF) and dispersion of the cells by removal from the culture dish and replating had substantial positive effects on the efficiency of infection with retroviral vectors. Studies of virus entry and cell replication revealed that cell dispersion and stimulation by HGF/SF may be acting through both mechanisms to increase the efficiency of retrovirus-mediated gene transfer. Although HGF/SF and cell dispersion increased the efficiency of infection with MLV-GFP, only rare cells with weak staining for insulin were infected, whereas ~25% of β -cells were infected with Lenti-GFP. We conclude that adenovirus is the most potent vector for ex vivo overexpression of foreign genes in adult endocrine pancreatic cells and is the best vector for applications where high-level but transient expression is desired. Under the optimal conditions of cell dispersion plus HGF/SF, infection with MLV and lentiviral vectors is reasonably efficient and stable, but only lentiviral vectors efficiently infect pancreatic β -cells. *Diabetes* 48:745–753, 1999

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Ad-GFP, adenovirus expressing GFP; BrdU, bromodeoxyuridine; CK, cytokeratin; GFP, green fluorescent protein; HGF/SF, hepatocyte growth factor/scatter factor; ICC, islet-like cell cluster; Lenti-GFP, lentivirus expressing GFP; MLV-GFP, murine retrovirus expressing GFP; MOI, multiplicity of infection; PBS, phosphate-buffered saline.

Ex vivo gene transfer to human pancreatic endocrine cells is an attractive approach to gene therapy for diabetes. However, most efforts to transfer genes into primary pancreatic endocrine cells have had limited success because of insufficient gene transfer and transient gene expression (1,2). The development of methods for efficient and stable gene transfer to primary β -cells is necessary if genetic modification to improve the prospects for β -cell transplantation as a therapy for diabetes is to become feasible.

The available gene transfer vectors differ greatly in their ability to infect and stably express genes in different cell types. Therefore, it is important to characterize their ability to infect human pancreatic endocrine cells under several conditions. Nonviral techniques, including lipofection, electroporation, and biolistic particles, have been used to transfer genes into primary pancreatic islets (1), leading to transient gene expression. Viral vectors are attractive gene transfer vehicles because they often mediate highly efficient gene transfer. The most commonly used viral vectors are those derived from human adenoviruses and murine retroviruses. Successful adenovirus-mediated gene transfer to rodent islets has been described (3–5). Drawbacks of adenoviral vectors are the lack of integration into the host cell genome and the potent immune response directed against adenoviral structural proteins, leading to transient expression of the transgene (6). Murine retroviral vectors integrate stably into the host cell genome and do not induce the expression of any viral proteins, but they require cell division for integration, making them unsuitable for the infection of nondividing cells (7). A more recently developed class of vectors derived from lentiviruses, such as those belonging to the HIV family, can infect both dividing and nondividing cells, including pancreatic β -cells (8–11).

Previously, we have shown that cells from both fetal islet-like cell clusters (ICCs) and adult islets can be induced to divide in monolayer cultures when grown on HTB9 extracellular matrix in the presence of hepatocyte growth factor/scatter factor (HGF/SF) (12). Human fetal pancreatic endocrine cells expanded in this way were transplanted under the kidney capsule of nude mice, where they differentiated successfully into fully functional β -cells capable of reversing chemically induced diabetes in rodents (13,14). These results raise the possibility that expanded populations of human pancreatic endocrine and/or endocrine precursor cells can be used for transplantation in diabetes. However, genetic modification of expanded cells will be desirable before transplantation to avoid apoptosis, graft rejection, and recurrent autoimmunity.

The studies described here were directed at defining an optimal set of conditions for transferring genes into human pancreatic endocrine and endocrine precursor cells using viral vectors. The vectors used were derived from Moloney murine leukemia virus (MLV), HIV-1, and adenovirus type 5, each expressing the green fluorescent protein (GFP) gene (MLV-GFP, Lenti-GFP, and Ad-GFP). The experiments have been performed in intact adult islets and fetal ICCs and in cells derived from adult islets and fetal ICCs grown as monolayer cultures.

RESEARCH DESIGN AND METHODS

Plasmids and viruses. The MLV-based vector pLGFPRL (MLV-GFP) expresses the GFP gene under the control of the retroviral LTR promoter. The GFP gene was cloned into the *Bam*H1 site of the vector pLRNL (15). The HIV-based vector pHR'CGFP (Lenti-GFP) expresses the GFP gene under the control of an internal cytomegalovirus (CMV) promoter (10). Infectious retrovirus from both of these plasmids was produced as VSV-G pseudotypes as previously described (16). The adenovirus expressing GFP under the control of chicken β -actin promoter (Ad-GFP) was a gift from Dr. Y. Kanegae. Titters for all three viruses were determined on HeLa cells by serially diluting the virus and counting the number of foci of green cells. MLV titers were also measured by infecting rat 208F cells and counting the number of G418-resistant colonies. Retroviral infections were performed in the presence of 8 μ g/ml polybrene. Adenovirus titers were checked by plaque formation assay on 293 cells. Replication-competent lentivirus was assayed as previously described and was absent in all cases (8).

Tissues and tissue culture. Human fetal pancreases at 20–24 weeks of gestation were obtained from the Anatomic Gift Foundation (Laurel, MD) and Advanced Bioscience Resources (Oakland, CA). Informed consent for tissue donation was obtained by the procurement centers. Approval for the use of human fetal and adult tissue was obtained from the University of California, San Diego institutional review board. Fetal ICCs were obtained after digestion of minced fetal pancreases with Collagenase P (Boehringer Mannheim, Indianapolis, IN) as previously described (17). Human adult islets were provided by Dr. C. Ricordi (Diabetes Research Institute, University of Florida, Miami, FL) and Dr. B.J. Hering (University of Minnesota, MN). They were isolated with an automated method as described (18). For each experiment, translucent fetal ICCs with a high epithelial cell content (19) were hand-picked under direct visualization with a dissecting microscope. Dithizone-positive adult islets were similarly purified. Purified ICCs and islets were then cultured for 2–3 days in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, and 1 μ g/ml amphotericin B. Glucose concentrations were 11 and 5.5 mmol/l glucose in the fetal and adult cultures, respectively. Medium and HGF/SF were refreshed every other day. Suspension cultures were performed by incubating 100 fetal ICCs or

adult islets in 1 ml medium. Monolayer cultures were performed by plating ICCs or islets on tissue culture dishes coated with HTB9 extracellular matrix in the presence of 10 ng/ml HGF/SF as described (12). To study the effect of HGF/SF on infection efficiency, HGF/SF treatment was discontinued 48 h before infection. To study the effect of cell dispersion on infection efficiency, single-cell suspensions were made by incubating the monolayer culture in nonenzymatic dissociation medium (Sigma, St. Louis, MO) to detach them from the matrix. These cells were replated on a fresh matrix-coated plate to form dispersed cultures and grown to 30–40% confluence before infection.

Flow cytometric analysis. Quantitative analysis of the infected cells was done by flow cytometry (FACStar Plus; Becton Dickinson, Mansfield, MA) on at least 5,000 cells per sample. Forty-eight hours after infection, the cultured cells were dissociated into a single-cell suspension, washed in phosphate-buffered saline (PBS) containing 3% fetal calf serum, and fixed in 4% paraformaldehyde in PBS. The percentage of GFP⁺ cells and the mean fluorescence intensity of the positive cells was determined after compensating for autofluorescence using uninfected cells as a negative control. In all cases, flow cytometric analysis of the cells demonstrated a low degree of cell death (<2%), as assessed by forward scatter and autofluorescence. To confirm that the infected cells were epithelial rather than mesenchymal, two-color flow cytometry with double-labeling for the human epithelial antigen Ber-EP4 in addition to GFP was performed. Forty-eight hours after infection, the cells were incubated with a monoclonal anti-Ber-Ep4 antibody (1:100 dilution) (Dako, Carpinteria, CA). This antibody reacts with glycoproteins present on the surface of all human epithelial cells, including those in the fetal and adult pancreas (17). The surface-labeled cells were then counterstained with phycoerythrin-labeled goat F(ab')₂ anti-mouse immunoglobulin G (Tago Immunologicals, Burlingame, CA).

Virus binding and internalization. Virus binding and internalization were assayed by incubating a single-cell suspension of 10⁶ cultured fetal cells or a monolayer culture with retroviral particles (multiplicity of infection [MOI] 50–100) for 2 h at 4°C or 37°C. Cells were then washed with Hanks' balanced salt solution (HBSS) four times. Cells in suspension culture were incubated with a mouse anti-VSV-G IgG2a monoclonal antibody (Sigma), counterstained with phycoerythrin-labeled anti-mouse IgG, and analyzed by flow cytometry. Cells in monolayer culture were fixed in 4% paraformaldehyde and stained for VSV-G using the immunoperoxidase technique (20). For quantification of virus entry to cells on the outside compared with cells in the center of the monolayer culture, we defined an area within 50 μ m from the edge of the monolayer culture as periphery, whereas the other cells were considered in the center of the monolayer culture. Enough fields were examined to score 500 cells each in the periphery and in the center of the culture.

Immunohistochemistry and confocal microscopy. Immunohistochemistry for GFP was performed using the immunoalkaline phosphatase technique (21) with a rabbit polyclonal anti-GFP antibody (diluted 1:1,000) (Clontech) as the primary antibody. Simultaneous analysis of GFP and pancreatic hormone or cytokeratin

TABLE 1
Infectivity of adult islets and fetal ICCs with adenovirus, MLV, and lentiviral vectors

Tissue and culture condition	% GFP ⁺ cells		
	Adenovirus	MLV	Lentivirus
Adult islets			
Suspension	53.4 ± 15.5	<1	2.6 ± 1.2
Nondispersed monolayer	90.7 ± 1.1	6.5 ± 0.4	17.2 ± 1.0
Nondispersed monolayer + HGF/SF	ND	7.8 ± 0.8	28.1 ± 5.2
Dispersed monolayer	ND	12.0 ± 0.15*	39.2 ± 5.9*
Dispersed monolayer + HGF/SF	ND	26.1 ± 4.2*†	65.4 ± 7.2*†
Fetal ICCs			
Suspension	32.2 ± 12.7	<1	<1
Nondispersed monolayer	97.7 ± 0.5	1.9 ± 1.1	24.5 ± 3.3
Nondispersed monolayer + HGF/SF	ND	3.45 ± 0.7	39.0 ± 4.6†
Dispersed monolayer	ND	15.2 ± 6.3*	68.9 ± 10.1*
Dispersed monolayer + HGF/SF	ND	33.1 ± 7.2*†	84.9 ± 4.5*

Data are means ± SE. *Significant difference between dispersed and nondispersed cultures; †significant differences between HGF/SF versus no HGF/SF treatment. Lenti-GFP infection was significantly higher than that of MLV-GFP under all conditions (*P* < 0.05 for nondispersed cells and *P* < 0.01 for dispersed cells). In suspension, adenovirus and retrovirus infection was performed at an MOI of 10⁶ colony-forming units (CFUs)/islet and 10⁴ CFU/islet, respectively. In monolayer culture, cells were infected at MOI 20–50. Infection of cultured pancreatic cells with Ad-GFP was very high and in preliminary experiments was not affected by HGF/SF; therefore, the effect of cell dispersion and HGF/SF on infection efficiency was not studied further. ND, not done.

(CK)-19 expression was done by growing adult islets or fetal ICCs on glass coverslips coated with HTB9 matrix. Detachment and reattachment of the endocrine pancreatic cells to the glass coverslips resulted in poor adherence and growth; therefore, confocal microscopy was performed on nondispersed monolayer cultures. The cells were fixed 48 h after infection, permeabilized in 0.1% Triton-X100 in PBS for 10 min, and blocked by 50 mmol/l glycine in PBS, 2% donkey serum, and 2% bovine serum albumin. After washing, the cells were incubated with sheep polyclonal anti-human insulin antibody (diluted 1:600) (The Binding Site, San Diego, CA), mouse monoclonal anti-glucagon antibody (diluted 1:1,000) (Sigma), and mouse monoclonal anti-CK-19 antibody (diluted 1:20) (Sigma). After a 1-h incubation, the cells were washed several times and incubated with lissamine rhodamine sulfochloride (LRSC)-conjugated donkey anti-sheep and indo-dicarbocyanine (CY5)-conjugated donkey anti-mouse antibodies, respectively (diluted 1:100) (Jackson ImmunoResearch Labs, West Grove, PA). Analysis was done with a microscope (model Nikon Diaphot 200) equipped with a laser scanning confocal attachment (Bio-rad MRC 1024 and Lasersharp software). Color composite images were generated using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA). Bromodeoxyuridine (BrdU) uptake was performed by incubation for 16 h with 0.1 mmol/l BrdU, fixation in 4% paraformaldehyde, and incubation with a 1:20-diluted mouse anti-BrdU monoclonal antibody (Dako) as the primary antibody. Cell nuclei that had incorporated BrdU were detected with immunoperoxidase staining followed by light hematoxylin counterstaining.

Statistical analysis. Experiments on infection efficiency in suspension and monolayer culture were carried out on at least three different preparations of adult and fetal islets/ICCs. Data on infection efficiency are presented as mean \pm SE. Comparisons were performed by the unpaired Student's *t* test. $P < 0.05$ was considered significant.

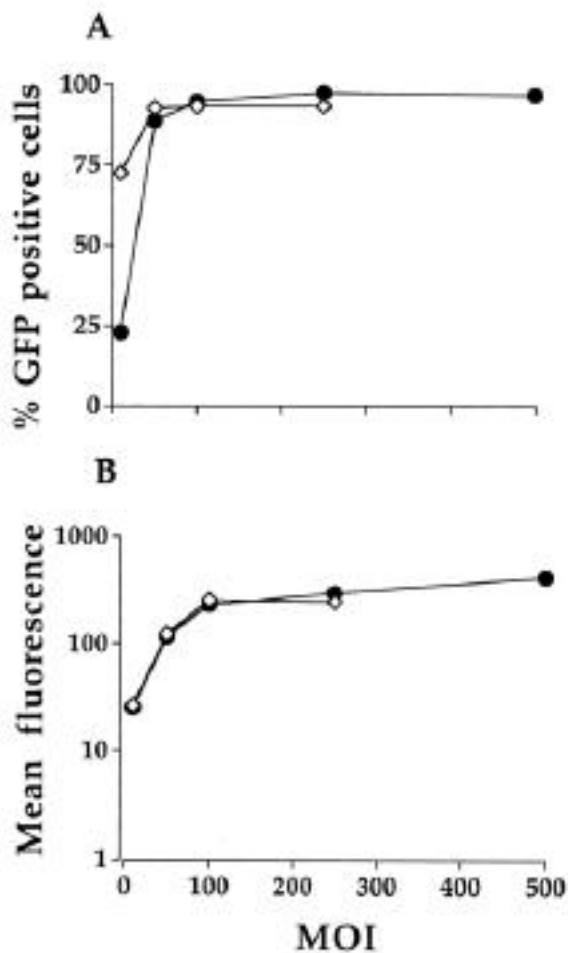


FIG. 1. Dose response curve of adenovirus-mediated gene transfer to adult islets (\diamond) and fetal ICCs (\bullet). The percentage of infected cells (A); the change in mean fluorescence of GFP-positive cells at different MOIs (B). Fluorescence values are arbitrary and are presented on a logarithmic scale.

RESULTS

Adenoviral but not retroviral vectors are able to infect a substantial fraction of fetal and adult pancreatic cells kept in suspension culture. When infected in suspension culture with Ad-GFP, 53.4% of cells from adult islets and 32.2% of cells from fetal ICCs expressed GFP by flow cytometric analysis (Table 1). Although Ad-GFP infection of adult islets seemed to be more efficient than that of fetal ICCs, the difference was not statistically significant ($P = 0.2$). In contrast, the infectivity of adult islets and fetal ICCs in suspension with MLV-GFP or Lenti-GFP at the maximal achievable MOI was extremely poor (Table 1). Thus, we consistently observed that MLV-GFP infected $<1\%$ of the cells in both adult islets and fetal ICCs (Table 1). Similarly, Lenti-GFP infected only 2.6% of adult islet cells and $<1\%$ of the cells contained in fetal ICCs (Table 1).

The efficiency of infection of adenoviral and retroviral vectors is increased by growing the cells in monolayer culture. The relatively low infectivity of intact ICCs and islets, particularly with the retroviral vectors, could have been due to physical constraints on the accessibility of virus particles to the cells on the interior of the ICCs and islets. Therefore, we tested whether growing cells from adult islets and fetal ICCs in monolayer culture, where all of the cells would be exposed to virus, would result in an increased infection efficiency.

Culture in monolayer before infection resulted in a marked increase in the infection efficiency with all three viral vectors

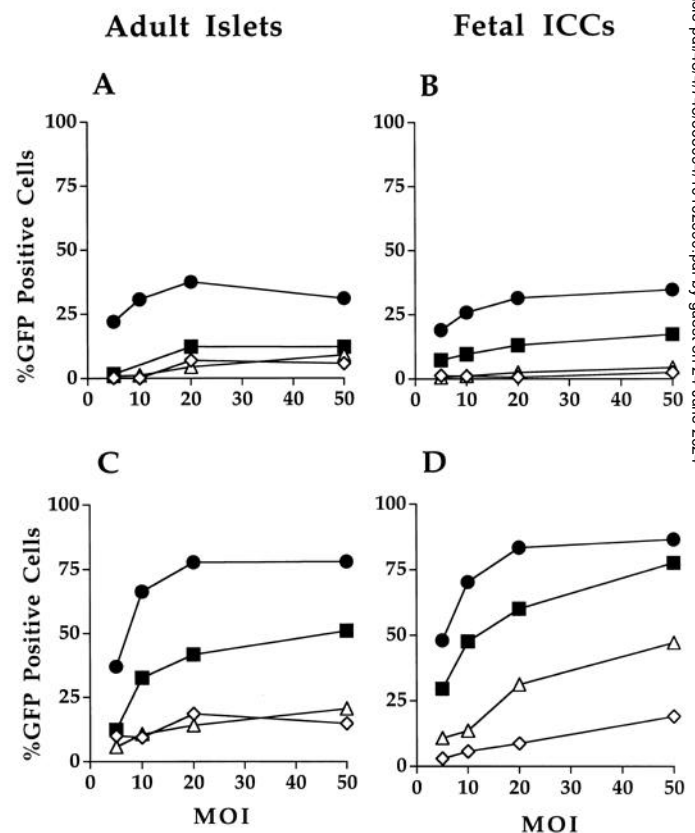


FIG. 2. Dose-response curve of infection with MLV-GFP (A, B) and Lenti-GFP (C, D) of adult islets and fetal ICCs in monolayer culture. \triangle , \diamond , nonspread cells with and without HGF/SF treatment; \bullet , \blacksquare , spread cells with and without HGF/SF treatment.

(Table 1). Ninety to ninety-five percent of the cells were infected by Ad-GFP at an MOI of 50. In addition, adding more virus resulted in an increase in the fluorescence per cell, most likely reflecting the entry of multiple adenovirus copies into the cells (Fig. 1). There was a direct correlation between

the MOI used for infection and cell death, which was most prominent at a high MOI of 500–1,000.

The efficiency of infection with MLV-GFP was also improved by growing the cells in monolayer culture, 2 and 6.5% for cells from ICCs and adult islets, respectively.

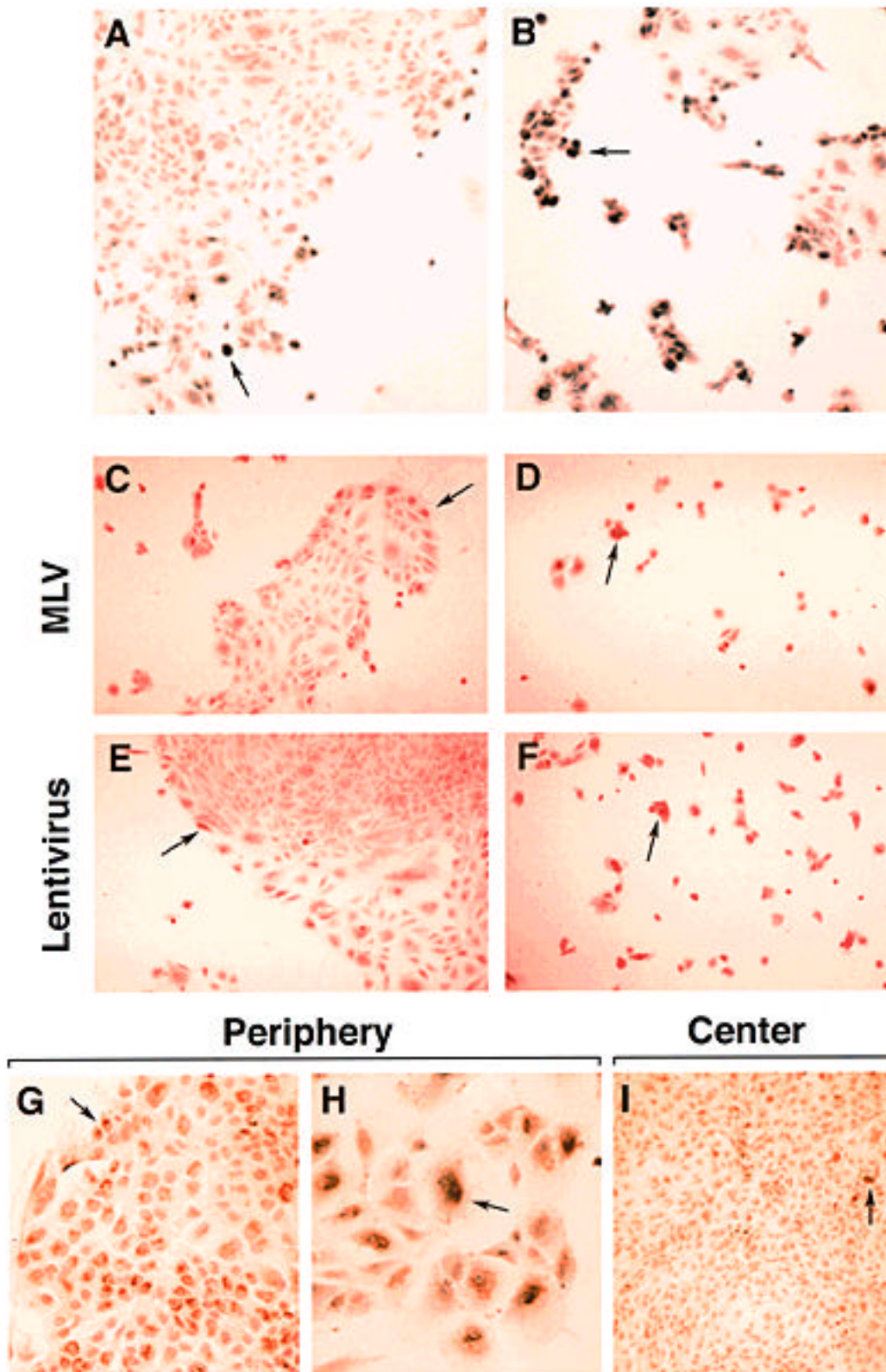


FIG. 3. *A, B:* BrdU uptake in fetal ICCs cells grown in a monolayer culture. Cultures were incubated for 16 h with 0.1 mmol/l BrdU and stained using the mouse monoclonal anti-BrdU as the primary antibody. Cell nuclei that incorporated BrdU were detected with the immunoperoxidase staining followed by light hematoxylin counterstaining. Note the preferential BrdU uptake into nuclei of cells located in the periphery of the cultures (*A*). BrdU labeling index of dispersed cells is higher and the distribution of the positive cells is homogenous (*B*). Arrowheads indicate BrdU-positive cells. *C–F:* The distribution of fetal pancreatic cells infected with retroviral vectors. Infected cells were identified by immunalkaline phosphatase staining. Rabbit polyclonal anti-GFP antibody was used as the first antibody. Note preferential infection of the periphery of the culture with MLV and lentiviral vectors (*C* and *E*), whereas infection is homogenous in dispersed cells (*D* and *F*). *G–I:* The distribution of internalization of viral particles into cultured pancreatic cells. Cells were incubated with VSV-G-pseudotyped retroviral particles (MOI 50–100) for 2 h at 37°C. The cells were stained for VSV-G using the immunoperoxidase technique. Note the preferential internalization of VSV-G particles into cells in the periphery of the culture (*G*). The VSV-G particles were concentrated in the perinuclear region (*G, H*). Only a few cells in the center of the monolayer stained positively for VSV-G (*I*). Original magnifications $\times 87$ (*A–F*), $\times 64$ (*G*), $\times 128.5$ (*H*), and $\times 26$ (*I*).

Lenti-GFP infectivity was higher than that of MLV-GFP, 24.5 and 17.2% for cells from ICCs and adult islets, respectively. Dose-response curves (Fig. 2) show that transduction efficiency was optimal at an MOI of around 20 for both MLV-GFP and Lenti-GFP. Higher MOI resulted in increased cell death with no effect on the percentage of GFP⁺ cells.

The peak value of mean fluorescence and the percentage of GFP⁺ cells after Ad-GFP infection was obtained on day 2, after which there was a gradual decline. In contrast, mean fluorescence increased after infection with MLV-GFP and Lenti-GFP (data not shown).

Mitotic activity is an important determinant of infection efficiency for retroviral vectors, but not for adenoviral vectors. Because lentiviral vectors can infect nondividing cells, the increased ability of lentivirus over murine retrovirus to infect cells grown in monolayer culture could have been due to a low mitotic index of the cells. In particular, cells on the interior of a monolayer culture may be mitotically inactive because of close proximity to one another and consequent contact inhibition. To test this hypothesis, immunohistochemical analysis of BrdU uptake was performed on monolayer cultures. As predicted, cells on the periphery of the monolayers displayed a higher frequency of BrdU incorporation when compared with cells located in the middle of monolayers (Fig. 3A and B). To increase the mitotic rate of all the cells in the culture, we tested the effect of dispersing the cells from a monolayer culture followed by replating the single-cell suspension onto fresh matrix. After dispersion of the cells, the mitotic index increased from 12.7 ± 2.5 to $23.7 \pm 2.8\%$ ($P < 0.05$).

The second intervention tested was the use of HGF/SF, which we have previously shown to increase the mitotic index of human pancreatic cells (22,23). In monolayer cultures, HGF/SF had no effect on the efficiency of infection with Ad-GFP or MLV-GFP. There was a trend toward HGF/SF having a positive effect on the efficiency of infection of adult islets and fetal ICCs with Lenti-GFP, but it did not

reach statistical significance (Table 1, Fig. 2). In sharp contrast, HGF/SF did increase the percentage of BrdU labeling when the cells were dispersed, up to $36.4 \pm 0.9\%$ ($P < 0.05$). Moreover, HGF/SF had a substantial effect on infection efficiency with both MLV-GFP and Lenti-GFP. When dispersed adult and fetal pancreatic cells were cultured in the presence of HGF/SF, there was a 1.5- to 2.2-fold increase in the infection efficiency with MLV-GFP and Lenti-GFP. This was surprising, given that infection of cells with the lentivirus vector should be less sensitive to the mitogenic effects of HGF/SF than the MLV vector. To investigate this further, the location of infected cells within the monolayer culture was determined by immunohistochemistry. Immunoalkaline phosphatase staining of nondispersed fetal pancreatic cells showed that Lenti-GFP and MLV-GFP preferentially infected cells on the periphery of the monolayer culture (Fig. 3C-F) (see below).

Cells on the periphery of a monolayer culture preferentially bind and internalize VSV-G-pseudotyped virus. While differential mitotic activity could explain the increased infectivity of cells on the periphery with MLV-GFP, the superior infectivity of Lenti-GFP remained puzzling. One possible explanation was that there could be increased binding and/or internalization of virus in cells on the periphery of nondispersed monolayer cultures. Since the VSV-G envelope protein is on the surface of both the MLV and lentivirus vectors, this protein was used as a marker for binding and internalization.

Immunostaining for VSV-G protein after incubation of monolayer cultures with retroviral particles showed that VSV-G particles were internalized into the cells and localized in the perinuclear region (Fig. 3G-I). VSV-G⁺ cells were more abundant in the periphery of the monolayer culture, $36.2 \pm 2.7\%$ on the outside vs. $9.2 \pm 3.1\%$ in the center of the culture ($P < 0.01$). Thus, the preferential infectability of the cells on the periphery could be due to preferential binding and internalization as well as to a higher mitotic index. However, the percentage of VSV-G⁺ cells did not increase after treatment with HGF/SF, suggesting that the major effect of HGF/SF was on the mitotic index rather than on virus binding.

We were interested to determine whether the cells on the interior of the monolayer culture were fundamentally different in their ability to bind and internalize virus from those on the periphery, or whether the differences were due to location within the monolayer culture. Therefore, we performed a binding and internalization analysis on single-cell suspensions (Fig. 4). Flow cytometric analysis using an anti-VSV-G antibody showed that the virus bound to ~95% of the cells, demonstrating that the vast majority were able to bind and internalize virus. The change in mean fluorescence of the infected pancreatic cells was similar to that of the 293 cell line, which can be infected efficiently with retroviral vectors. Therefore, the local environment within the interior of the monolayer culture must have negative effects on binding and/or internalization.

Dispersion of cells from islets and ICCs increases infection efficiency with retroviral vectors that is further enhanced by HGF/SF. Because both the BrdU uptake and binding/internalization studies suggested that nondispersed monolayer culture was not the optimal environment for infection with MLV or lentiviral vectors, the effect of dispersing the cells throughout the tissue culture dish was studied. Dispersion of the cultured cells was the

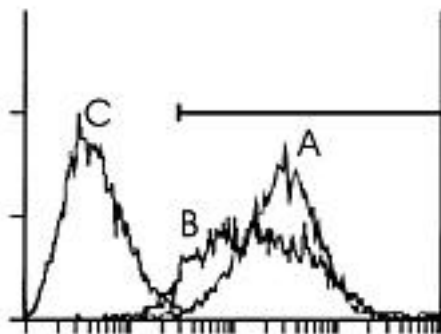


FIG. 4. Flow cytometric analysis of cells from cultured ICCs. Virus binding and internalization were assayed by incubating a single-cell suspension of 10^6 cultured fetal cells (MOI 50–100) for 2 h at 4°C (A) or 37°C (B). Cells in suspension culture were incubated with a mouse anti-VSV-G IgG2a monoclonal antibody and counterstained with phycoerythrin-labeled anti-mouse IgG. The percentage of cells containing VSV-G particles was determined after compensating for autofluorescence using uninfected cells as a negative control (C).

most important factor in increasing the infectivity of pancreatic endocrine cells with MLV-GFP and Lenti-GFP (Table 1, Fig. 2). HGF/SF had a further positive effect on infection efficiency of dispersed cells. When dispersed cells were cultured in the presence of HGF/SF, ~33% of fetal and adult pancreatic cells were infected with MLV-GFP and 85 and 74% of cultured cells from ICCs and adult islets were infected with Lenti-GFP.

Human β -cells can be infected with high efficiency by lentiviral but not MLV vectors. A critical issue for both gene therapy and basic science applications of gene transfer technology is the ability of the various vectors to infect β -cells. While Lenti-GFP infected 25% of adult β -cells, MLV-GFP did not infect any of the cells with strong positive staining for insulin (Table 2, Fig. 5) but did infect rare cells with weak insulin staining. In fetal ICCs, only rare cells were double-labeled for insulin and GFP after infection with Lenti-GFP. No insulin-positive cells expressing GFP were found after infection with MLV-GFP. However, the low percentage of insulin-positive cells in fetal ICC preparations infected with MLV- and Lenti-GFP (< 5%) precludes meaningful quantification.

Adenoviral vectors efficiently infect β -cells from adult islets but not fetal ICCs and are inefficient at infecting α -cells. Ad-GFP exhibited marked differences in the efficiency with which it infected different cell types in the fetal and adult pancreas. While confocal microscopy demonstrated that most fetal and adult pancreatic cells were infected with the virus, including 75% of the insulin-positive cells from adult islets, α -cells were relatively resistant to infection with Ad-GFP (~5%) (Table 2, Fig. 5). Insulin-positive cells from the fetal pancreas were much more resistant to infection than adult β -cells (infection efficiency of 20%). Flow cytometric analysis showed that ~90% of the cells grown in monolayer culture are epithelial. The high infectivity of pancreatic epithelial cells, together with the inefficient infection of endocrine cells in the fetal pancreas, suggest that the majority of infected fetal cells are hormone-negative putative endocrine precursors, which are the predominant cells contained in ICCs (17).

DISCUSSION

The studies described here demonstrate that both adenoviral and lentiviral vectors are capable of transferring genes efficiently into insulin-expressing cells. MLV vectors, while ineffi-

cient at transferring genes directly into insulin-expressing cells, infect a substantial percentage of cells from both the fetal and adult pancreas. A few cells that were infected with MLV showed weak staining for insulin. These cells may be β -cells that dedifferentiate as a result of proliferation (24). Studies on highly expanded populations of human islet cells strongly suggest that this is the case (24a). However, the precise origin of the MLV-infected cells that stained weakly positive for insulin was not addressed in this study, and therefore they were not counted as β -cells in the analysis.

The percentage of GFP⁺ cells after infection with lentivirus was higher compared with MLV. This may be in part because of differences in promoter activity. Also, transgene expression from different viral vectors may vary. To minimize underestimation of infection efficiency due to weak GFP expression, we used flow cytometric analysis, a highly sensitive technique to detect fluorescent cells. The striking difference in the efficiency of infection of insulin-positive cells with MLV and lentivirus is explained by the low proliferation rate of the hormone-positive cells and the fact that lentivirus, but not MLV, can infect nondividing cells.

Gene transfer to intact adult islets and fetal ICCs in culture is attractive because it minimizes the manipulation of cells, therefore preserving their viability. Infection of fetal ICCs and adult islets by retroviral vectors in suspension was poor, however, <3% of the cells being infected. Ad-GFP was the only viral vector that efficiently infected human islets in suspension, but infection of adult islet cells was only 50% and even less for fetal ICCs. Previous studies using adenoviral vectors to infect rodent islets showed that only cells in the periphery of the islets are transduced (5). Moreover, β -cells are more abundant in the center of adult islets, so only rare β -cells are transduced after adenoviral vector infection of intact islets (5). It is likely that the geometry of the islet does not allow cells in the inner core of the islet/ICC to be exposed to viral particles. In line with this notion is the finding that human islets in suspension were more easily infected by Ad-GFP than were fetal ICCs, although the infectivity in monolayer culture was similar. It is our impression that fetal ICCs have a more compact structure, which may interfere with virus exposure. In addition, large ICCs (>150 microns) were more difficult to infect compared with smaller ICCs (data not shown).

TABLE 2
Infectivity of adult and fetal pancreatic β -cells

Culture condition	% GFP ⁺ -insulin ⁺ cells*		
	Adenovirus	MLV	Lentivirus
Adult islets			
Suspension	NA	<1	~3
Monolayer	74.3 ± 5.4	<1	25.7 ± 7.6
Fetal ICCs			
Suspension	NA	<1	<1
Monolayer	20.0 ± 6.7	<1	NA

Data are means ± SE. Monolayer analysis was performed on nondispersed monolayer cultures. The percentage of GFP⁺ β -cells was similar with and without HGF/SF. Islet preparation from two independent donors, ages 18 and 48 years, were used for the analysis of infection of adult islets. Three fetal pancreases were used for the infection analysis of fetal ICCs. *Percentages of insulin-positive cells; only cells with bright red staining for insulin were considered positive. NA, not applicable (the number of positive cells was too low for meaningful quantification).

Although retroviral gene transfer to human adult islets and fetal ICCs in suspension is low, it may be sufficient for some applications in which local expression of a gene product in a small percentage of cells is sufficient to achieve a biological effect. Examples would be the expression of secreted molecules such as immunomodulatory cytokines. However, the low infectivity of intact adult islets and fetal ICCs precludes the use of retroviral vectors for applications requiring transfer to most or all cells to induce expression of intracellular proteins. For these applications, the data presented

here indicate that infection in dispersed monolayer culture is required to obtain a sufficiently high infection efficiency.

Both MLV-GFP and Lenti-GFP preferentially infected cells on the periphery of monolayer cultures. Infection with MLV-GFP and Lenti-GFP was markedly increased by dispersion of the cells and HGF/SF treatment. Growth factors, including HGF/SF, have been shown previously to enhance the efficiency of infection of MLV and ecotropic retroviral vectors (25,26). This enhancement was thought to be due to effects of the growth factors on the mitotic rate of the target cells.

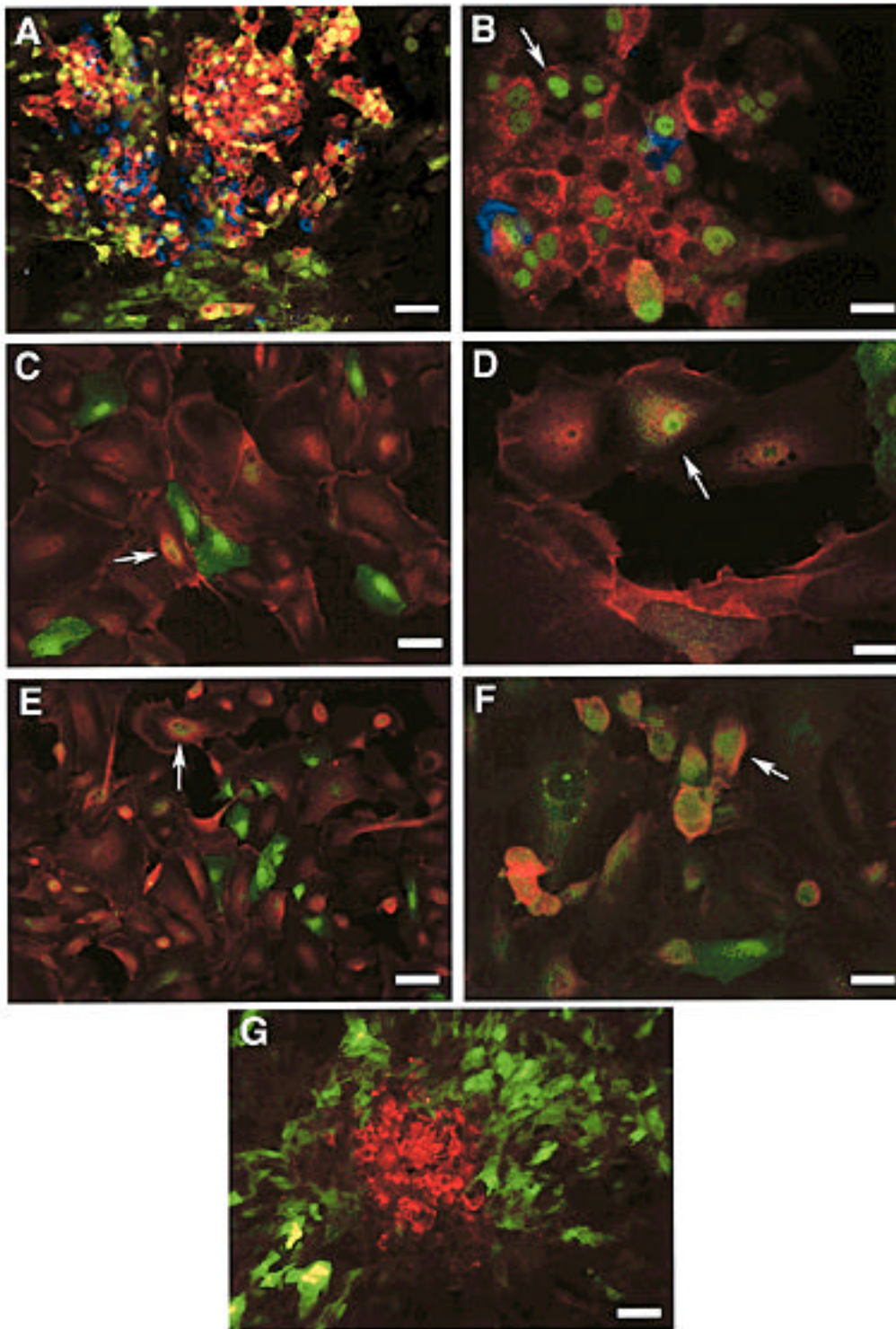


FIG. 5. Confocal microscopic analysis of cultured adult and fetal endocrine pancreatic cells infected with Ad-, MLV- and Lenti-GFP (green). Each sample is double-immunostained for insulin (red) and glucagon (blue). Arrowheads in *B-F* indicate cells with colocalization of GFP and insulin. Infection of cultured adult islets with Ad-GFP is shown in *A* and *B*, MLV-GFP in *C* and *D*, and Lenti-GFP in *E* and *F*. Infection of fetal ICCs with Ad-GFP is shown in *G*. Bars are 50 μm (*A*, *E*, *G*), 25 μm (*B*, *C*, *F*), and 12.5 μm (*D*).

Therefore, the effect of HGF/SF on the efficiency of infection with lentivirus vectors, which are able to infect nondividing cells, was unexpected. One possibility is that lentiviral vectors may exhibit a preference for dividing cells, at least in some cell types. Another is that HGF/SF may have effects on virus binding and internalization that were not detected using the methodology employed in this study. This would not be entirely unexpected, since HGF/SF has dramatic effects on many cellular processes in addition to the cell cycle, such as its scatter effect. The receptor for VSV-G protein is thought to be a ubiquitously present phospholipid, but we have demonstrated previously that the binding of VSV-G–pseudotyped retrovirus to human hematopoietic cells can be increased by soluble growth factors (27). Since both MLV and lentivirus vectors are pseudotyped with the VSV-G envelope protein, restriction of VSV-G binding/internalization could explain the preferential transduction of dispersed cells and of cells in the periphery of the monolayer culture observed for both viruses.

Ad-GFP was the most efficient vector for gene transfer to human pancreatic cells. However, the loss of transgene expression after Ad-GFP infection of proliferating cells grown in monolayer cultures, in contrast to the stable expression after MLV-GFP and Lenti-GFP infection, demonstrates the importance of integrating viral vectors in settings where the cells actively proliferate. During cell division, the DNA of replication-defective adenoviral vector is rapidly lost. Therefore, the percentage of transgene-expressing cells decreases over time.

The preferential infection of some cell types in the fetal and adult pancreas with Ad-GFP was striking. Adult β -cells were efficiently infected, while fetal β -cells were not. Glucagon-positive cells were hardly infected at all in either the fetal or adult pancreas. The explanation for this preferential infection is likely to reside in differences in the expression of adenovirus receptors on the cell surface. The receptor for adenovirus was recently cloned and was shown to bind adenovirus and coxsackie B virus in a fiber-dependent manner (28). Other receptors may also be important in adenovirus binding (29). Integrins, which are molecules involved in cell adhesion, are also important for adenovirus entry into different cells. Both $\alpha\beta3$ and $\alpha6\beta1$ integrins have been implicated in adenovirus internalization (30). The differential infectivity of cell types within the pancreas could be due to the expression of the adenovirus receptor and different integrins in the cell membrane. The striking difference in the efficiency of infection of adult α - and β -cells within the same tissue preparation demonstrates that the ability of different viral vectors to infect cells should be determined separately for each cell type.

The present study provides new information on the properties of gene transfer to human endocrine pancreatic cells by viral vectors. Our studies demonstrate that efficient gene transfer to human endocrine pancreatic cells with different viral vectors is feasible, but its efficiency will depend on culture conditions and on the cell type to be infected. The establishment of an efficient method for viral vector-mediated gene transfer to endocrine pancreatic cells by dispersion of cultured cells and HGF/SF treatment, and the use of new viral vectors such as those based on lentiviruses, will advance the basic science and therapeutic application of gene transfer to diabetes research.

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