The Absence of Accessible Vitronectin Receptors in Differentiated Tissue Hinders Adenoviral-Mediated Gene Transfer to the Intestinal Epithelium *In Vitro*

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Purpose. Adenoviral (Ad) vectors have been used as efficient tools for gene therapy in various tissues, whereas in some differentiated epithelium transduction efficiency is almost abolished.
Methods. Caco-2 cell monolayers were chosen as an in vitro model for the differentiated intestinal epithelium. Fluorescence-labeled adenoviral particles were used for binding studies to cell surfaces. Internalization receptors for adenoviral uptake were decteted by a fluorescence-labeled vitronectin antibody. Gene expression was studied by using the β-galactosidase reporter gene. All experiments were done on undifferentiated and differentiated Caco-2 cells. Furthermore, adenoviral particles were allowed to bind to differentiated Caco-2 monolayers followed by a trypsinization step that disintegrates the monolayers and result in a cell suspension. Gene expression was tested after reseeding the cells into dishes.
Results. The results from adenoviral binding studies, vitronectin immunofluorescence detection and gene expression are in good agreement and indicate that virion binding as well as the expression of internalization receptors almost disappear in fully differentiated cells. Nonetheless, adenoviral binding to differentiated monolayers seems to be sufficient to cause up to 53% gene expression, but only if internalization of the vector can be induced by disintegrating the monolayers and releasing free vitronectin receptors.
Conclusions. These findings indicate that gene transfer to the intestinal epithelium utilizing adenoviral vectors is poor and ineffective, because of the lack of sufficient internalization receptors. If these receptors can be exposed in differentiated epithelium, transduction can be made more efficicient. Alternatively, a viral vector must be developed whose uptake mechanism is independent of integrin receptor expression like the enteral virus Ad40, or Ad5 could be conjugated to ligands that trigger viral internalization by receptor-mediated endocytosis.

KEY WORDS: adenoviral vector; Caco-2; gene transfer; integrin; intestinal epithelium; vitronectin receptor.

INTRODUCTION

Recently, gene therapy has gained prominence as a method for delivery of therapeutic peptides and proteins. Replication-deficient recombinant adenoviral vectors (Ad) have previously been shown to be efficient for transferring exogenous genes in a wide variety of cells in vitro and in vivo (1–4). Adenoviral vectors must cross the plasma membrane and enter the host cell to gain access to the cellular synthetic machinery in the nucleus and/or cytoplasm (5). Virus entry into the host cell has been divided into two events: binding to cell surface receptors and internalization via receptor-mediated endocytosis (5,6). A glycosylated protein with a molecular weight of 40,000 to 42,000 was isolated as the high affinity attachment site for adenoviral fibers on HeLa and KB cells (7). Vitronectin-binding α_V-integrins have been identified as secondary receptors that are distinct from the virus attachment receptor (8). They initiate Ad internalization through attachment to the penton base protein and enable viral endocytosis due to integrin-mediated signal transduction.

It has been recently reported that transduction efficiency in differentiated tissue is poor, probably because of a lack of accessible receptors responsible for internalization on the cell surface (9–12). Transduction efficiency may be dependent on the availability of the receptors and therefore, a sufficient density ("threshold level") of vitronectin receptors is required for efficient Ad-mediated gene transfer (8–11).

Gene transfer to the intestinal epithelium has been discussed as an alternative approach for oral peptide and protein delivery (12). The human intestinal cell line Caco-2 is commonly used as an in vitro model for the differentiated intestinal epithelium (13,14). Transduction efficiency in mature Caco-2 monolayers has been found to be very low when using an adenoviral vector for gene transfer (12). This raises the question whether in accordance to other epithelia, gene delivery to differentiated intestinal cells may be ineffective and difficult to achieve because of a lack of sufficient vitronectin receptor density. In the present study we characterized adenoviral-mediated gene transfer in differentiated Caco-2 cell monolayers with focus on the requirements for vitronectin receptors. Attachment of rhodamine-labeled adenovirus was compared with the occurrence of vitronectin receptors on the brush border membrane at different stages of Caco-2 differentiation. A striking agreement was found between integrin receptor expression and transduction efficiency in undifferentiated as well as mature Caco-2 cells.

MATERIALS AND METHODS

Materials

Tissue culture reagents were obtained from Gibco (Grand Island, NY) and tissue culture articles were from Corning (Corning, NY). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

Caco-2 cells (ATCC HTB37) passage numbers 58–71 were routinely maintained in Dulbecco's modified Eagle's Medium (DMEM) with 25 mM glucose, containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM Na-pyruvate, 1% L-glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml). Medium was changed every other day. 293 cells (ATCC

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ABBREVIATIONS: SOD Cu, Zn-superoxide dismutase; IL-2, interleukin-2; ILSOD cDNA, IL-2 signal peptide and human SOD fusion protein-coding cDNA; FR, rat skin fibroblast cell line (ATCC CRL 1213); X, xanthine; XO, xanthine oxidase; MDA, malondialdehyde; PGs, prostaglandins.

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CRL1573) were maintained in DMEM with 25 mM glucose, containing 10% FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml). All cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Preparation of Adenovirus

High titer recombinant adenovirus 5 containing the β-galactosidase reporter gene (Ad.RSVntlacZ) was prepared by amplification in the transformed human embryonic kidney cell line 293 using established methods (15,16). Virus was purified from cell lysate by cesium chloride centrifugation followed by desalting on a Sephadex G-50 column in phosphate buffered saline (PBS, Sigma). Transduction efficiency of the adenoviral vector was determined in 293 cells by detection of β-galactosidase activity and was expressed as lacZ forming units/ml (lfu/ml). Viral preparations show transduction efficiency of 1 lfu per 20 viral particles.

Rhodamine Labeling of Ad Virions

After a second CsCl gradient purification, virions were filtered through a Sephadex G50 column, equilibrated with 0.1 M $NaHCO_3$, pH 8.7. The concentration of the recovered virus was 6.7×10^{12} particles/ml. 15 μl of tetramethylrhodaminylisothiocyanate (TRITC, Sigma) in methanol at a concentration of 2.0 mg/ml was added to 0.6 ml of the virus suspension and incubated at 26°C for 4.5 hrs. The conjugated virions were separated from free TRITC on a Sephadex G50 column, equilibrated in PBS containing 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2$ and 0.9 g/l D-glucose. The number of TRITC molecules per virion was calculated by spectrophotometric measurements at 560 nm after subtraction of background absorption due to viral particles and revealed 620 TRITC molecules per virion. Calibration was performed with TRITC in PBS at four different concentrations and resulted in a linear slope with $r^2 = 0.9991$.

Attachment Studies with TRITC-Conjugated Ad (Ad-Rh)

Caco-2 cells were seeded on coverslips at a density of 10^4 cells/cm^2. After 3 and 7 days postseeding, Ad-Rh was added at an MOI of 10^6 particles/cell and adsorbed at 4°C for 60 min. After attachment, cells were washed with PBS and fixed in 3% paraformaldehyde in PBS for 30 min at room temperature. Samples were studied with a confocal laser scanning microscope at 586/610 nm (Bio-Rad MRC 600).

Gene Transfer

Gene transfer in Caco-2 cells was determined by detection of β-galactosidase activity. Caco-2 cells were seeded in six-well cell culture dishes at a density of 10^4 cells/cm^2 and grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. At different times after seeding, the complete medium was removed and cells were incubated with 0.25 ml Ad.RSVntlacZ (100 lfu/cell) in DMEM containing 2% FBS at 37°C. After 2 hrs, 2.5 ml of maintenance medium was added and cells were incubated for an additional 48 hrs. For the detection of β-galactosidase activity, cells were washed in PBS and fixed with 0.5% glutaraldehyde for 10 min at room temperature. Cells were then washed two times for 2–10 min with PBS containing 1 mM $MgCl_2$.

β-Galactosidase activity was detected by staining the cells for 4 hrs, using 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal, Gibco, Grand Island, NY) as a substrate (17,18). For microscopic analysis, PBS was added to the cells and a minimum of 200 cells was counted for each well.

Immunofluorescence Staining for $\alpha_v\beta_5$ Integrin

Caco-2 cells were seeded on glass coverslips at a density of $2 \cdot 10^4$ cells/cm^2. After 3, 5 and 15 days postseeding, cells were washed twice with PBS and fixed in 3% paraformaldehyde in PBS for 20 min at room temperature. Preparations were washed (3×) in 2% bovine serum albumin (BSA)-PBS and incubated with 100 μl primary monoclonal antibody against $\alpha_v\beta_5$ integrin (MAB 1961, Chemicon) for 20 min at room temperature. Cells were washed (3×) in PBS and then incubated with 100 μl FITC-labeled second antibody (IgG anti-mouse, Sigma) for 20 min at room temperature, washed with PBS (3×), and embedded in glycerol containing 10% PBS. Antibodies were diluted 1:20 in 2% BSA-PBS. Samples were studied using a fluorescence microscope with phase contrast optic (Zeiss). All photographs were taken on Kodak Ektachrome Elite 400 slide film. In all cases, no fluorescence was observed when the primary antibody was omitted.

The Effect of Trypsin on Ad.RSVntlacZ Transduction of Caco-2 Cells

Caco-2 cells were seeded on six-well culture plates (10^4 cells/cm^2) or on glass coverslips ($2 \cdot 10^4$ cells/cm^2). The initial cell growth is slower on glass coverslips compared to polystyrene and therefore, cells were seeded on glass in a higher density. For transduction, the complete medium was removed and replaced with 100 lfu Ad.RSVntlacZ suspended in 0.5 ml DMEM containing 25 mM Hepes. The samples were incubated for 1 hr at 4°C, followed by 30 min at 37°C, washed twice with HBSE (8 g/l NaCl, 6 g/l Hepes, 7.44 g/l EDTA, pH 7.4) and incubated with 2 mg/ml trypsin in HBSE for 10 min at 37°C. Cells were resuspended in Caco-2 medium, centrifuged to remove trypsin, seeded into six-well culture plates and incubated for 48 hr before assaying for β-galactosidase expression. Since not all cells attach again after trypsinizing, the cell number per well was determined by counting the cells in a hemacytometer.

RESULTS

Gene expression in Caco-2 cells using adenoviral vectors depends on the degree of cellular differentiation and is dramatically decreased in differentiated monolayers (Fig. 1). High levels of reporter gene expression (80%) were detected in all samples when transfection was performed 3 days after seeding. Gene expression subsequently decreased when cells are transfected at later postseeding times. This leads to the assumption that the loss of efficiency in gene transfer is based on the cellular differentiation process. Recent observations clearly indicate that Caco-2 cells exist in different stages of differentiation in culture: homogeneously undifferentiated (at subconfluence) and polarized and enterocyte-like (after confluence) (13,19–23). Characteristics like cell surface glycoprotein and

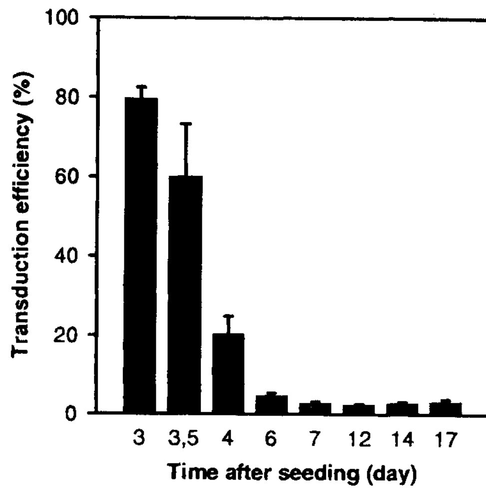

Fig. 1. Dependence of reporter gene expression in Caco-2 cells on degree of differentiation. Cells were seeded in six-well culture plates at a density of 100,000 cells/well and transduced with Ad.RSVntlacZ (10 lfu/cell) at different times after seeding. Error bars indicate standard deviations (n = 3).

receptor expression, enzymatic activity, occurrence of tight junctions and barrier function differ with the degree of differentiation and Caco-2 cells become very similar to enterocytes in mature monolayers. The following experiments were undertaken to determine the factors that are responsible for the reduced transduction efficiency in differentiated Caco-2 cells but that allow efficient gene transfer in undifferentiated cells.

Fluorescence-Labeling of Ad to Detect Binding on Caco-2 Cells

To visualize attachment of the viral particles on Caco-2 cells by fluorescence microscopy, tetramethylrhodaminylisothiocyanate (TRITC) was covalently linked to Ad virions as described previously (24) with slight modifications. Rhodamine labeled virions (Ad-Rh) bind intensely to undifferentiated, subconfluent Caco-2 cells 3 days after seeding (Fig. 2a and b). The virions are attached to the entire surface of the cells with preference to intercellular spaces and cell borders that lack neighboring cells (Fig. 2c and d). In areas where the cells became already confluent and smaller in diameter, less viral particles are attached to the membrane (Fig. 2b). When confluent Caco-2 cells (7 days after seeding) are incubated with Ad-Rh, the binding of Ad-Rh to the cell membrane is significantly decreased and almost undetectable as shown in Fig. 3a. Interestingly, increased virion binding is found on the top of so-called 'domes'. Domes occur in monolayers containing differentiated cells where small areas in the cell monolayer are detached from the dishes in the form of fluid-filled half-blisters (Fig. 3b to d).

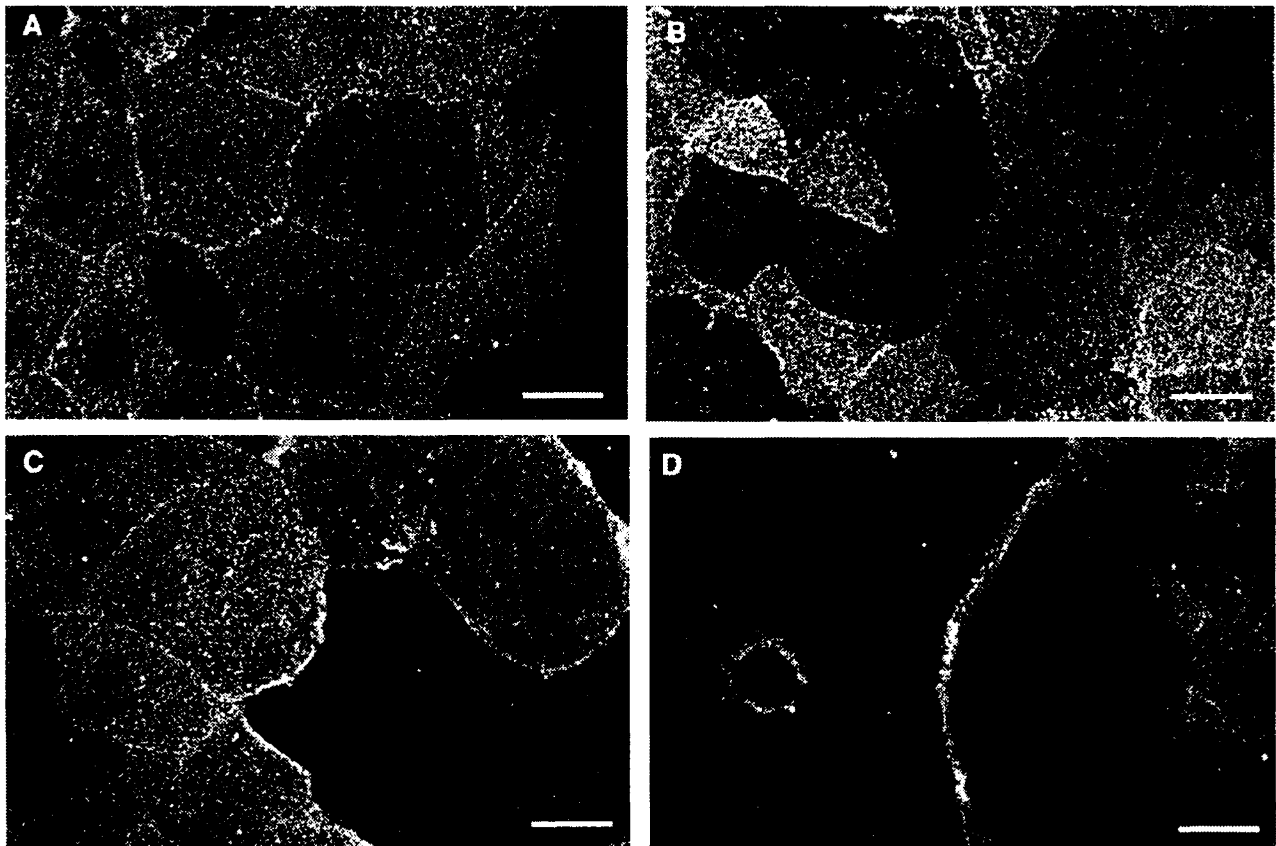

Fig. 2. Attachment of rhodamine-labeled Ad5 virions (Ad-Rh) to undifferentiated, subconfluent Caco-2 cells 3 days after seeding. Ad-Rh was added (10^6 particles/cell) at 4°C for 60 min. Samples were fixed and studied in a confocal laser scanning microscope. (bar 50 μm).

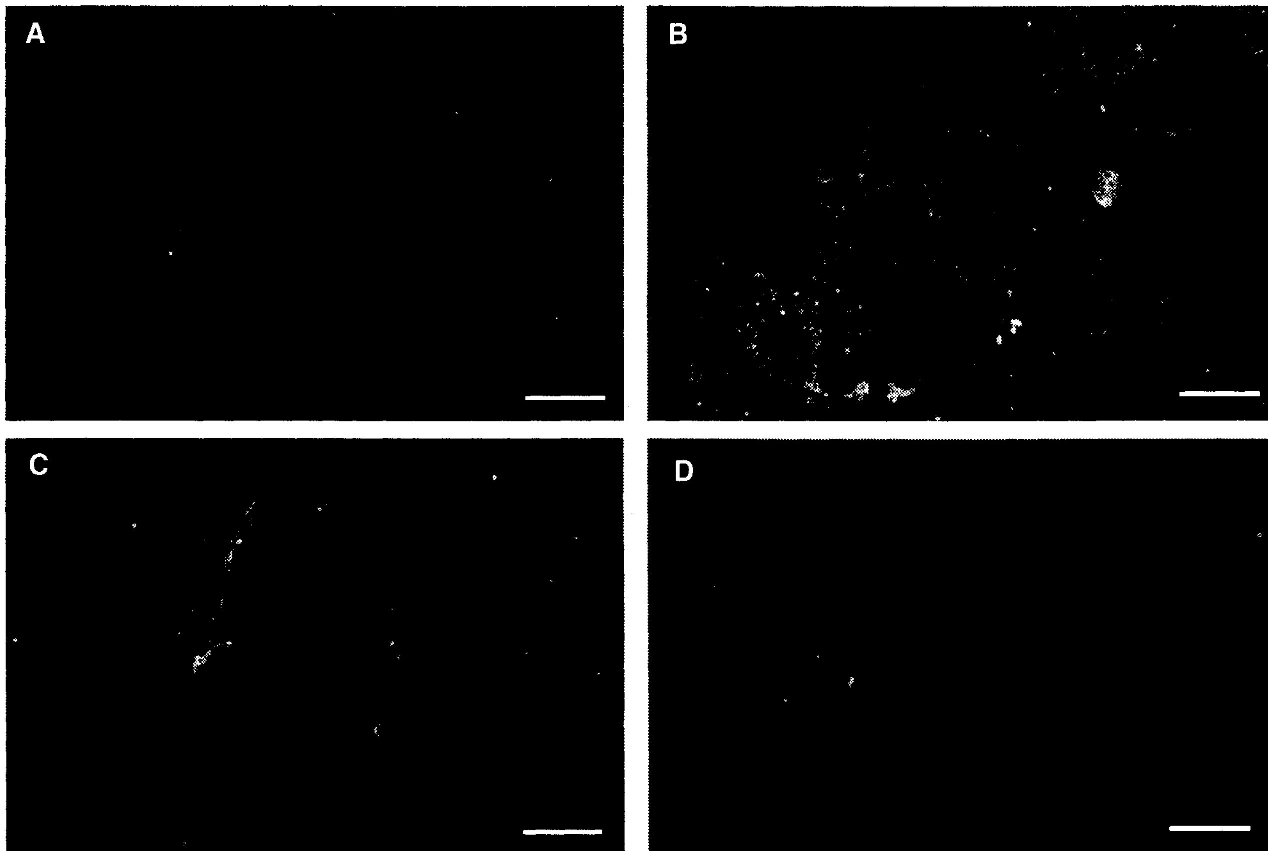

Fig. 3. a) Attachment of rhodamine-labeled Ad5 virions (Ad-Rh) to confluent Caco-2 cells 7 days after seeding. Ad-Rh was added (10^6 particles/cell) at 4°C for 60 min. Samples were fixed and studied in a confocal laser scanning microscope. Virion binding is found on domes with focus b) on the top, c) on the middle and d) on the bottom of the dome (bar 50 μm).

A similar behavior was observed after β-glactosidase gene transfer to confluent Caco-2 monolayers. The majority of cells showing gene expression are slightly detached from the substrate but still integrated in the monolayer in the form of domes (Fig. 4).

Vitronectin Receptor ($\alpha_v\beta_5$ Integrin) Expression in Caco-2 Cell Monolayer

Caco-2 cells were grown on glass coverslips and checked for integrin expression at the brush border membrane during growth at (i) subconfluent, (ii) undifferentiated and confluent and (iii) differentiated stages. This was done by using a monoclonal antibody that binds to accessible $\alpha_v\beta_5$ integrin receptors and therefore visualizes the presence of receptors available for adenovirus internalization. $\alpha_v\beta_5$ Integrin is strongly expressed on subconfluent undifferentiated Caco-2 cells (3 days after seeding), predominantly at cell borders which lack neighboring cells (Fig. 5a and b). Five days after seeding, the cells become confluent, but are still undifferentiated. Integrin receptors could not be detected any more on the cell surface of confluent monolayers (Fig. 5c and d), but are significantly expressed on cells that detach slightly from the substrate but still integrated in the cell layer in form of domes (Fig. 5e and f). A similar pattern of integrin expression was seen in 15 day old cells (Fig. 5g and h).

The Effect of Trypsin on Ad.RSVntlacZ Transduction of Caco-2 Cells

From the previous experiments it is evident that viral binding to cell surfaces as well as the occurrence of internalization receptors are drastically decreased in differentiated tissue. Internalization receptors are thought to be distinct from the receptors which are responsible for virus attachment to the cell surface (8). In a further study we checked the importance of both steps for transduction efficiency. Differentiated Caco-2 monolayers were incubated with Ad.RSVntlacZ under conditions that would allow binding as well as internalization of the virus (8). Hence, monolayers were intensively washed to remove non-bound virions and incubated with 2 mg/ml trypsin for 10 min. Trypsinizing the cells after incubation with virus leads to dramatically increased transduction rates compared to control samples (Table 1). Through the washing and trypsinization steps any unbound virus was removed and transduction rates up to 52.6% refer to virus that was bound to Caco-2 cell surfaces during incubation. Due to the trypsin treatment, differentiated Caco-2 cells detach from the substrate, the monolayer disintegrates and form a cell suspension with isolated cells. We showed in a previous study that intact virus is not influenced by high concentrations of trypsin (12). From these results we conclude that a sufficient amount of virus is attached to the surface of differentiated Caco-2 cell monolayers but

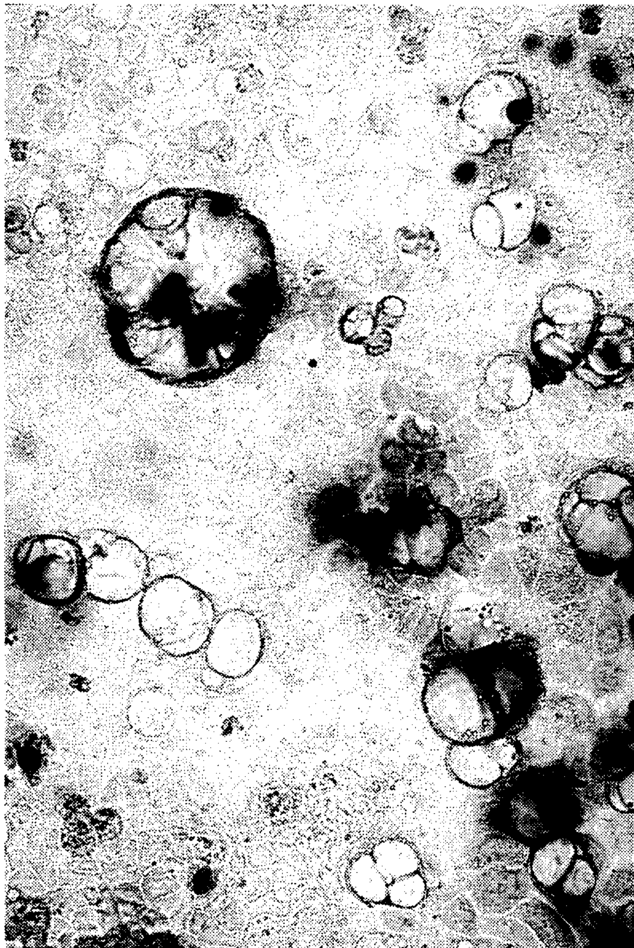

Fig. 4. β-Galactosidase expression in differentiated Caco-2 cells. Cells were seeded in six-well culture plates at a density of 100,000 cells/well and transduced with Ad.RSVntlacZ (100 lfu/cell) 16 days post-seeding (×200).

can not be internalized into the cells. Only trypsinisation and therefore disintegration of the cell monolayer allows internalization of the virions and access to the cellular synthetic machinery. Furthermore, Caco-2 cells grown on glass coverslips show higher transduction rates than cells grown on plastic dishes (Table 1). The underlying substrate may also play a role on integrin expression on cell surfaces.

DISCUSSION

Successful gene transfer to differentiated tissues seems to be dependent on the availability of α_v-integrin receptors for viral internalization. Despite decreased fiber receptor expression, growth-factor stimulated monocytes were more susceptible to virus infection because of upregulation of α_v-integrin expression (9). Ad5 efficiently transduced only immature muscle cells but not mature muscle fibers in vivo which correlates with a higher surface density of α_v integrins in immature muscle cells (10). Goldman *et al.* (11) found that within human airway epithelium, undifferentiated cells exposed to the lumen were substantially more infectable than fully differentiated ciliated or secretory cells. Immunocytochemical studies suggested a strong correlation between expression of α_v integrin and infection with recombinant Ad.

With the Caco-2 cell culture system we used a unique tool which can model intestinal epithelial cells in great detail. The differentiation process that occurs under normal growth conditions offers a system in which cells can be studied at different stages of differentiation. Several reports have confirmed that the general integrin expression on Caco-2 cells agrees well with the integrin localization in the human intestine, in which: (i) α_5 and α_6 subunits are expressed at the base of all enterocytes: (ii) the α_3 subunit is expressed in the basolateral domain of enterocytes: (iii) the α_3 and α_5 subunits are expressed in the lateral domain and are involved in cell-to-cell contacts between enterocytes (11,26,27). Vachon *et al.* (28) found that β_1 integrin is expressed at the basolateral surface with preference for the basal domain in fetal and adult intact human intestinal epithelium as well as in Caco-2 co-cultures with fibroblasts. However, Caco-2 cells grown on plastic exhibit only diffuse cytoplasmic/basolateral expression (28).

Conconnier *et al.* (25) showed that $\alpha_5\beta_1$ integrin is intensively expressed by proliferative undifferentiated Caco-2 cells entirely covering the cell surface. When cell confluence occurs, $\alpha_5\beta_1$ integrins are masked and become inaccessible as receptors, because they are immediately redistributed, lining cell-to-cell contacts (25). Bacterial internalization of *Y. pseudotubercolosis* into Caco-2 cells is dependent on $\alpha_5\beta_1$ integrin expression on the cell membrane and therefore, these receptors on Caco-2 cells are not accessible for *Y. pseudotubercolosis* infection after differentiation.

This is comparable with our findings on adenoviral gene transfer. Results from adenoviral binding studies, integrin immunofluorescence detection and gene expression are in good agreement and indicate that binding as well as uptake of Ad.RSVntlacZ almost disappear in fully differentiated cells. Nonetheless, viral attachment to differentiated Caco-2 cells seems to occur to a sufficient degree to cause efficient gene transfer after trypsinizing the cell monolayers. From our results we suggest that internalization of the virions into the cells can

Fig. 5. (*opposite*) a) Cell surface expression of $\alpha_v\beta_5$ integrin on subconfluent undifferentiated Caco-2 cells 3 days after seeding. Immunofluorescence staining is mainly expressed at cell borders without neighboring cells. b) Phase contrast micrograph displays the same section as Fig. 5a. c) Cell surface expression of $\alpha_v\beta_5$ integrin on confluent undifferentiated Caco-2 cells 5 days after seeding. Immunofluorescence staining is absent on confluent cell monolayers. Background fluorescence that indicates the shape of the cell is due to autofluorescence in the cytoplasm that occurs in older cells. d) Phase contrast micrograph displays the same section as Fig. 5c. e) Cell surface expression of $\alpha_v\beta_5$ integrin on confluent undifferentiated Caco-2 cells 5 days after seeding. Immunofluorescence staining is intensely expressed in cells that form domes. Background fluorescence that indicates the shape of the cell is due to autofluorescence in the cytoplasm that occurs in older cells. f) Phase contrast micrograph displays the same section as Fig. 5e. g) Cell surface expression of $\alpha_v\beta_5$ integrin on confluent differentiated Caco-2 cells 15 days after seeding. Immunofluorescence staining is absent in confluent monolayers, but is intensely expressed in cells that form domes. Background fluorescence that indicates the shape of the cell is due to autofluorescence in the cytoplasm that occurs in older cells. h) Phase contrast micrograph displays the same section as Fig. 5g (×1000).

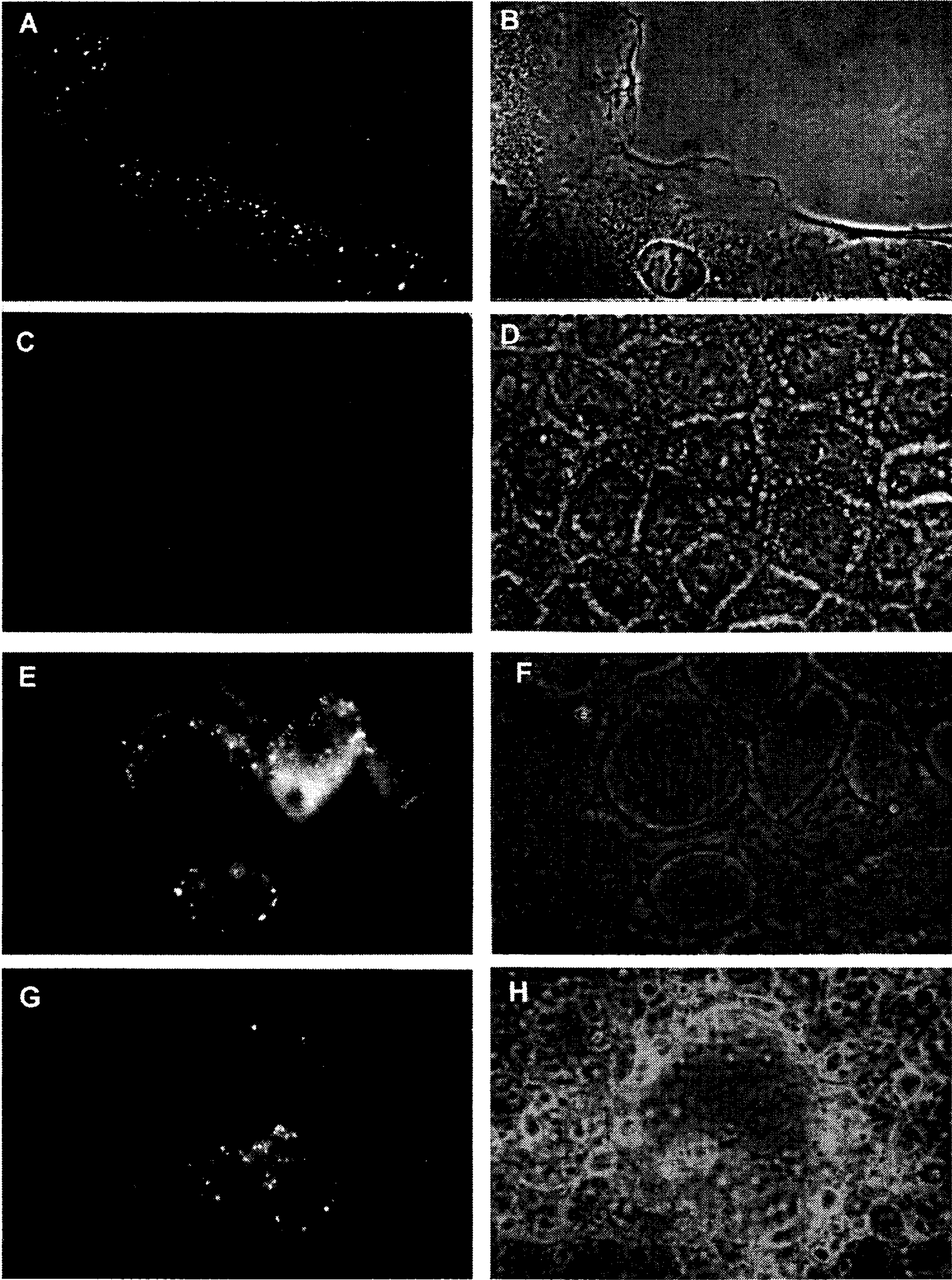
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Table 1. Influence of Trypsinizing Differentiated Cells After Virus Attachment on Transduction Efficiency

Day after seeding	Substrate	% Transduced cells[a]	
		Control	Trypsinized cells
23–24	polystyrene	2.02 (0.02)	18.7 (1.4)
25	polystyrene	1.36 (0.14)	23.4 (2.9)
19–28	glass	6.11 (0.50)	52.6 (5.89)

[a] Results for duplicate samples are given as mean (deviation).

be seen as the critical step and does occur to a limited extent in differentiated cells. In confluent Caco-2 monolayers, integrin receptors are inaccessible, because they are engaged in cell-to-cell and cell-substrate contacts and can not be detected by binding experiments with monoclonal antibodies (25). Trypsinizing the cell monolayers destroys intercellular as well as cell-substrate contacts and therefore unmasks integrin receptors. Thus, integrin receptors become accessible for virion internalization and therefore higher transduction efficiencies can be achieved. We found also that the underlying substrate seems to be important for the transduction rates and therefore, the situation could be different in vivo where the intestinal epithelium is connected to underlying tissue. Consequently, $\alpha_v\beta_5$ integrin receptor expression, accessibility and distribution in intestinal epithelial cells in vivo will likely have an important influence on the efficiency of gene transfer to the intestinal tract.

If efficiency of gene transfer is limited by the lack of available α_v integrin receptors, strategies will have to be designed to enhance virus uptake into epithelial cells. One approach would be to upregulate the expression of the essential integrin prior to administration of virus (9). Another approach could be the exploitation of pathways for viral entry that are independent on α_v integrins receptors (11). Several serotypes of human adenoviruses, such as adenovirus type 40, are missing RGD motifs in the penton base (29), suggesting α_v integrin-independent entry or interaction of the virus with other cell surface molecules. Alternatively, a viral vector may be used like Ad40 that is independent on integrin expression of the host cell or Ad5 may be conjugated to proteins that increase the uptake rate by receptor-mediated endocytosis (transferrin, lectins, etc.).

CONCLUSIONS

In summary, there is strong evidence that transduction efficiency of adenoviral vectors is dependent on the accessibility of $\alpha_v\beta_5$ integrin receptors on the cell surface. Vitronectin receptors enable the internalization of Ad5 into the cell and gene transfer is almost abolished when the receptor is not accessible. In Caco-2 cells, cell confluence and differentiation down-regulate the accessibility of vitronectin receptors because they are engaged in cell-to-cell and cell-substrate contacts. It is not fully clarified yet, if the spontaneous enterocytic differentiation of Caco-2 cells in culture mimics the situation found in the small intestine with regard to vitronectin receptor expression. Future work should include the characterization of accessible vitronectin receptor occurrence in intestinal tissue and the search for alternative approaches to improve gene transfer to differentiated epithelia.

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