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High lysosomal activities in cystic fibrosis tracheal gland cells corrected by adenovirus-mediated CFTR gene transfer

W. Kammouni [a], D. Naïmi [b], W. Renaud [a], N. Bianco [c], C. Figarella [a], M.D. Merten [a,*]

[a] *Groupe de Recherche sur les Glandes Exocrines, Faculté de Médecine, 27 Boulevard Jean Moulin, F-13385 Marseille Cedex 05, France*
[b] *Laboratoire de Physiologie, Institut des Sciences de la Nature, Université de Constantine, Constantine, Algeria*
[c] *Department of Pathology and Neuropathology, Hôpital de la Timone, Marseille, France*

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Abstract

Human tracheal gland serous (HTGS) cells are now believed to be a major target of cystic fibrosis (CF) gene therapy. To evaluate the efficiency of adenovirus-mediated gene transfer in these cells we tested the adenovirus construction containing β-galactosidase cDNA. We observed that the endogenous β-galactosidase activity in cultured CF-HTGS cells was too strong to allow us to detect any exogenous β-galactosidase activity. Immunohistological study on sections of human tracheal tissue confirmed the presence of β-galactosidase in the serous component of the submucosal glands. We then looked for other lysosomal activities in normal and CF-HTGS cells. We showed that normal cells already have elevated enzyme values and that CF-HTGS cells contained 2–4-fold more β-galactosidase, α-fucosidase, α-mannosidase and β-glucuronidase activities than normal cells. An analysis of their kinetic constants has shown that this difference could be attributed to a lower K_m of CF lysosomal enzymes. More importantly, these differences are eliminated after adenovirus-mediated CFTR gene transfer and not after β-galactosidase gene transfer.

Keywords: Airways; Lung; Submucosa; Gene therapy; Cell culture

1. Introduction

Cystic fibrosis (CF) is the most common hereditary disease in the Caucasian population. This defect consists of mutations in the CFTR (Cystic Fibrosis Transmembrane conductance Regulator) protein that leads to its inability to transport chloride ions in response to agents that elevate intracellular cAMP in epithelial cells of exocrine glands [1]. As a consequence, airway mucous secretions may become thick due to incorrect hydration altering bacterial adhesivity and favouring persistent infection of the airways by *Pseudomonas aeruginosa* (review: [2]).

Interestingly, it was demonstrated that the serous component of the submucosal gland cells expresses a very high level of CFTR in comparison to the other bronchial epithelial cell types [3]. The serous cells from the submucosal glands participate strongly in the local defence of the airways by secreting antibacterial proteins such as lactoferrin and lysozyme [4]. We have developed techniques to isolate and culture human tracheal gland serous (HTGS) cells [5,6]. At

* Corresponding author. Fax: (33) 4 91 78 68 95; E-mail: grge@medecine.univ-mrs.fr

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confluency, HTGS cells were shown to have retained epithelial and secretory characteristics similar to those the cells have in vivo: presence of cytokeratins, desmosomes and tight junctions, a constitutive secretion of lactoferrin, lysozyme and secretory proteinase leukocyte inhibitor (SLPI), and an ability to respond to adrenergic and cholinergic agonists. Cultured HTGS cells were also shown to highly express CFTR [7] and to have an active cAMP-dependent chloride transport [8].

Recent developments in molecular biology render gene therapy for CF now possible in principle. Numerous studies have been performed showing safe gene transfer into the airway surface epithelium in animals and human [9–11]. In this context, adenovirus-mediated β-galactosidase gene transfer using Ad-β-Gal vectors was demonstrated to be an accurate method to assess gene transfer efficiency. In addition, transfection by Ad-CFTR has shown enough surface epithelial cells to be corrected to normal transepithelial membrane potential [12]. However, no studies have been performed in order to quantify the efficiency of gene transfer into HTGS cells.

In the present work, we examined the effects of Ad-β-Gal on CF-HTGS cells in vitro. The data presented here show that normal and CF-HTGS cells basically express high lysosomal enzyme activities hindering any estimation of gene transfer efficiency. Interestingly, the significantly higher lysosomal activities found in CF-HTGS cells appear to be corrected after CFTR gene transfer. This correction was not due to the adenovirus per se since adenovirus-mediated β-galactosidase gene transfer did not modify the high lysosomal enzyme activities shown in CF-HTG cells.

2. Materials and methods

2.1. Materials

The adenoviral construction shuttle plasmids Ad-β-Gal and Ad-CFTR were built and kindly provided by Transgene (Strasbourg, France). 4-Methylumbelliferyl-α-L-fucoside, 4-methylumbelliferyl-α-L-mannoside, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-β-D-glucuroside and 4-methylumbelliferone were from Koch-Light Laboratories (Aitbrooks, UK). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 4-methylumbelliferyl phosphate, *Escherichia coli* β-galactosidase, mouse monoclonal antibodies to *E. coli* β-galactosidase (clone GAL 40) and Dulbecco's modified Eagle's/Ham's F12 mixture (DMEM/F12) were from Sigma (L'Isles d'Abeau Chesnes, France). Ultroser G was from Biosepra (Villeneuve La Garenne, France). All other reagents were of cell culture grade.

2.2. Cell culture

Three different HTGS and CF-HTGS (two carrying the ΔF508/ΔF508 and one ΔF508/unknown) cultures were carried out as previously described [6,13]. Briefly, cells were maintained in DMEM/F12 medium supplemented with 1% Ultroser G, glucose and pyruvate made up to 8 g/l and 0.22 g/l respectively. Epinephrine (2.5 μM) was routinely added to the cell culture medium in order to provide optimal growth and differentiation [14]. Type 1 collagen-coated, Falcon disposable tissue culture flasks were used. Cells were passaged using 0.025% trypsin, 1% polyvinylpyrrolidone and 0.02% EDTA. Replicate cultures were used at the third passage, 8 days after confluency had been reached.

2.3. Immunohistochemistry and cytochemistry

Paraffin-embedded sections (6 μm) of human tracheal tissue were fixed in methanol for 10 min at −20°C. Sections were then incubated for 1 h with anti-*E. coli* β-galactosidase (1:500 in PBS). Immunoperoxidase staining was performed using biotinylated rabbit anti-mouse IgG (1:500) and a Vecstatin Elite ABC kit (Vector Labs, Biosys). Specimens were counterstained with Mayer Hemalun.

Third passage cells grown on collagen-coated coverslips were washed with PBS, fixed for 10 min with 2% paraformaldehyde at 4°C and then incubated with a PBS (pH 7.2) solution containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1.3 mM magnesium chloride and 1 mg/ml X-Gal. Cells were examined by light microscopy 12 h after staining, where the blue staining indicates positive β-galactosidase activity.

2.4. Gene transfer with Ad-β-Gal and Ad-CFTR into CF tracheal gland cells

Confluent cultures of CF-HTGS cells were rinsed three times with serum-free culture medium and then exposed for 24 h to 10 MOI (multiplicity of infection) of either Ad-β-Gal or Ad-CFTR in complete culture medium. Supernatants were then harvested and flasks frozen until use.

2.5. Measurements of CFTR transcript levels

Total RNA was purified from cellular pellets of HTGS and CF-HTGS cells (approximately 10^7 cells) following the technique of Chomczynski et al. [15]. Total RNA was quantified by absorbance at 260 nm (1 OD = 40 mg/l) and the quality of the preparations was monitored by agarose gel electrophoresis in the presence of formaldehyde. Quantification of CFTR mRNA transcripts in the cells was performed by dot-blot analysis using a ^{32}P-labelled CFTR probe coding sequence cloned in pTG 4560 (generously given by Transgene, Strasbourg, France). The length of the insert was 2136 bp. Dot-blot assays were performed according to the method of Renaud et al. [7]. Sequential dilutions (5 μg to 0.156 μg) were spotted on nitrocellulose membranes and RNAs transcript levels were quantified by densitometric measurement of the autoradiograms using CFTR and GAPDH probes. The mRNA concentrations were obtained in arbitrary units per μg of total RNA from the slopes of the linear regression lines of the dot scans. The coefficient regression, r^2, was always >0.98. Results are determined as means ± S.D. from three experiments performed from the different CF-HTG cell cultures and expressed as a percentage of control. The intra-assay variation coefficients were $<2\%$ and the inter-assay coefficient variations were $<6\%$. The GAPDH cDNA probe was kindly provided by Dr M.R. Hirsh (Centre d'Immunologie de Luminy, Marseille, France) and the length of the insert into pBr322 was 1200 bp.

2.6. RT-PCR detection of the β-galactosidase mRNA encoded by Ad-β-Gal in CF-HTG cells 24 h after Ad-β-Gal challenge

After treatment of the total RNA with RNase-free DNase I (Boehringer Mannheim, Germany), polymerase chain reaction (PCR) amplification of mRNA (after conversion to cDNA) was performed using the Gene AMP RNA PCR kit (Perkin Elmer/Cetus) following the protocol of Bout et al. [16]. Thirty cycles of amplification were performed using the sense primer OTG 5347 (LacZ nt 4284–4308, 5′-AGCCCGTCAGTATCGGCGGAATTC-3′) and the reverse primer OTG 5349 (SV40 nt 2631–2608 [polyA]; 5′-TTGTGAAATTTGTGATGCTATTGC-3′). Thirty additional cycles of amplification were performed using OTG 5347 and the nested primer OTG 4741 (SV40 polyA, nt 2663–2639; 5′-GTAACCATTATAAGCTGCAATAAAC-3′) as internal probe. Cycle times were as follows: (i) denaturation for 3 min at 94°C, (ii) primer annealing for 2 min at 60°C and (iii) extension for 3 min at 72°C. An aliquot of the final amplification solution was analysed after ethidium bromide staining of a 1% agarose gel to assess the size of the amplified fragments.

Table 1
pH dependence of the specific activities of β-galactosidase in HTG cells, CF-HTG cells and CF-HTG cells incubated with Ad-β-Gal (in nmol 4-methylumbelliferone generated/mg protein/min)[a]

β-Galactosidase	HTG	CF-HTG	*E. coli*	CF-HTG+Ad-β-Gal
pH 4.0	100 ± 15	100 ± 6	4.3 ± 0.01	100 ± 4
pH 5.0	66 ± 8	69 ± 1.9	21 ± 1	–
pH 6.0	40 ± 8	35 ± 5	100 ± 2.5	–
pH 7.2	4.2 ± 0.2	4.4 ± 0.8	50.5 ± 3.9	5.1 ± 0.23

[a]Confluent CF-HTG cells were incubated for 24 h with 10 MOI of recombinant Ad-β-Gal. Protein extraction and enzyme activities as in Fig. 4. Assays were performed in triplicate for each culture. Specific activities represent the mean ± S.D. for all cell cultures for each treatment and are expressed as the percentage of the maximal activity, which was found to be at pH 4.0 for endogenous cellular β-galactosidase and at pH 6.0 for the *E. coli* enzyme.

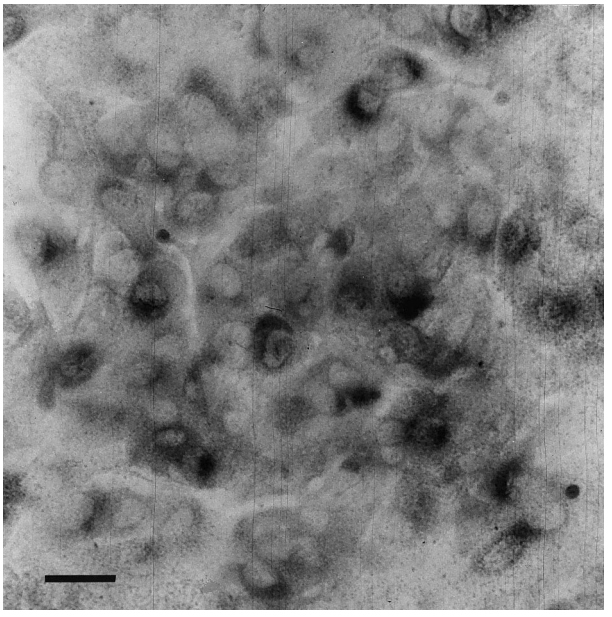

Fig. 1. Cellular β-galactosidase activity in human tracheal gland cells in culture. Eight days after cells reached confluency, cells were fixed with 4% paraformaldehyde and β-galactosidase activity was revealed using X-Gal as substrate. General appearance showing that staining was cytoplasmic. Bar = 2 μm.

2.7. Lysosomal enzyme activity measurements

Confluent cultures of HTGS or CF-HTGS cells were rinsed four times with phosphate buffer saline (PBS) and frozen at −80°C until use. Cell extracts were made by adding H_2O, scraping the cells, sonication for 5 s, and centrifugation at 10 000 rpm for 3 min to remove cell debris. The protein concentration in the cell extracts was evaluated using the Bradford method [17]. Measurement of lysosomal enzyme activity was performed using specific fluorescent substrates according to Figarella et al. [18]. Specific activities were determined by comparison with a 4-methylumbelliferone calibration curve and related to the initial protein concentration.

3. Results

3.1. β-Galactosidase activity in CF-HTGS cells after adenovirus-mediated β-galactosidase gene transfer

24 h after transfection by Ad-β-Gal, CF-HTGS cells showed high β-galactosidase activities as shown by the intense blue staining in the cells (Fig. 1). Staining was only found in cells at confluency but not during the growth phase. However, similar high β-galactosidase activity was also found in control CF-HTGS cells without transfection. The staining was found around the nucleus in the cytoplasmic compartment. No differences were found in this localisation between HTGS and CF-HTGS cells, but CF-HTGS cells showed a stronger staining than normal HTGS cells (not shown). Although the β-galactosidase cDNA is under a strong promoter and possesses a signal for nuclear localisation, we were not able to differentiate between nuclear and cytoplasmic activities in the cultured cells (at confluency as well as in the growth phase) after transfection with Ad-β-Gal.

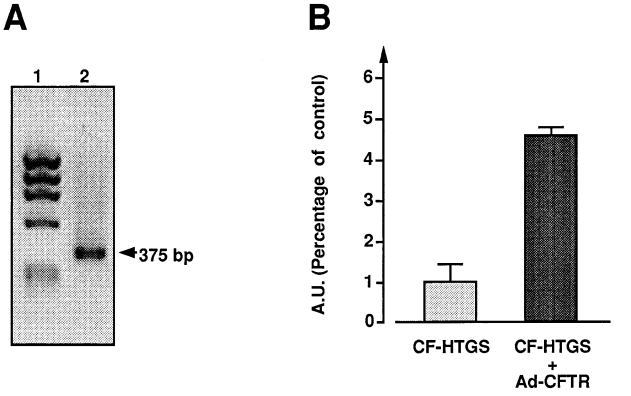

Fig. 2. Expression of β-galactosidase and CFTR in CF-HTG cells after β-galactosidase- and CFTR-mediated gene transfer. (A) Expression of β-Gal mRNA in CF-HTG cells 24 h after Ad-β-Gal administration. The resulting cDNA was subjected to nested PCR amplification using OTG 5347 as sense primer and OTG 5349 followed by OTG 4741 as reverse primers. The amplification products were visualised after ethidium bromide staining of a 1% agarose gel. As compared with the molecular weight marker ΦX174/*Hae*III (lane 1) the amplification products (lane 2) migrated at the expected size of 375 bp. (B) Relative quantitation of CFTR mRNA in CF-HTGS cells. Cells were harvested at confluency and cytoplasmic RNA was isolated. Increasing amounts of total RNA of CF-HTGS and CF-HTGS transfected with Ad-CFTR were loaded onto filters and hybridised with a radiolabelled specific probe. Spots were scanned and analysed by least-squares regression. Results have been normalised to the respective GAPDH levels and are reported as percentages of mRNA present in untreated CF-HTGS cells. Light bar: control CF-HTGS cells, dark bar: CF-HTGS cells treated for 24 h with 10 MOI of Ad-CFTR.

3.2. Expression of passenger genes of adenovirus vectors

To assess whether the CF-HTGS cells could be effectively transfected, we performed RT-PCR to detect expression of β-galactosidase of the adenoviral construction by using primers spanning a region between β-galactosidase and a SV40 polyA region [16] present in the adenoviral construction. The presence of an amplification product at 375 bp (Fig. 2A) indicates expression of the exogenous β-galactosidase into the cells. We also incubated the cells with Ad-CFTR in the same conditions as performed with Ad-β-Gal. As shown in Fig. 2B, the level of CFTR mRNA transcripts was significantly increased (4.7 ± 1.3 times more, $P < 0.01$) in CF-HTGS cells after Ad-CFTR gene transfer.

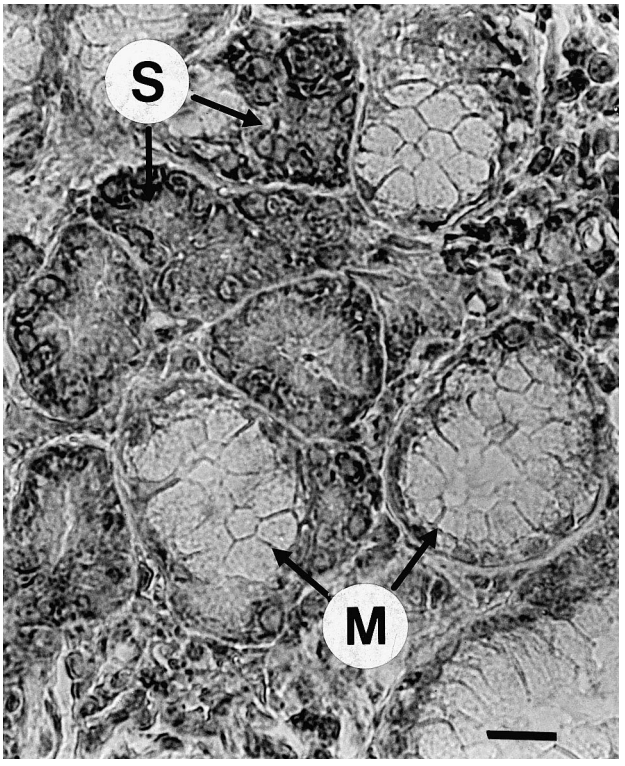

Fig. 3. Immunoperoxidase micrographs of transverse section of human trachea labelled with antibodies to *E. coli* β-galactosidase. Detail of staining of tracheal glands. Note the staining intensity preferentially located in the serous (S) gland cells. Mucous gland cells (M) are not stained. Bar = 20 μm.

3.3. Immunocytochemical demonstration of β-galactosidase in human trachea

Paraformaldehyde-fixed sections of human tracheas were then examined by immunoperoxidase microscopy and anti-*E. coli* β-galactosidase antibodies. Fig. 3 shows that β-galactosidase immunoreactivity was found in the serous component of the tracheal glands. Mucous gland cells and surface epithelial cells (data not shown) were found to be negative. No staining was observed when anti-*E. coli* β-galactosidase antibodies were omitted in control experiments.

3.4. Lysosomal enzyme activities in normal and CF-HTGS cells

These above findings prompted us to assay β-galactosidase and other lysosomal activities in cultured HTGS and CF-HTGS cells. Fig. 4 presents data showing the values of the specific activities of β-galactosidase, α-fucosidase, α-mannosidase and β-glucuronidase in cellular extracts of HTGS and CF-HTGS cells. Comparison with white blood cells showed that, except for β-glucuronidase, activities of these hydrolases are 10–260-fold more elevated in HTGS and CF-HTGS cells (data not shown). The cell cultures were all used at passage 3 and 8–12 days after having reached confluency and comparison of culture age showed no influence on the data when cell monolayers were used during this time interval. These data demonstrate the difference between the specific activities of these four acid hydrolases in the normal and CF cellular extracts of HTGS cells: CF-HTGS cells contained 2–4.2-fold more acid hydrolases than normal cells ($P < 0.01$). In addition, incubation of CF-HTGS cells with Ad-CFTR is able to decrease the levels of the acid hydrolase activities to values comparable to those found in normal HTGS cells. By contrast, incubation with Ad-β-Gal did not change the specific activities of the acid hydrolases in CF-HTGS cells, showing that the correction was not due to adenovirus per se.

Kinetic analysis of the activities from the double reciprocal plot presented in Fig. 5 showed that V_{max} values were similar in HTGS and CF-HTGS cells whereas the K_m value (approximately 57 μM) of the CF β-galactosidase activity was about three times

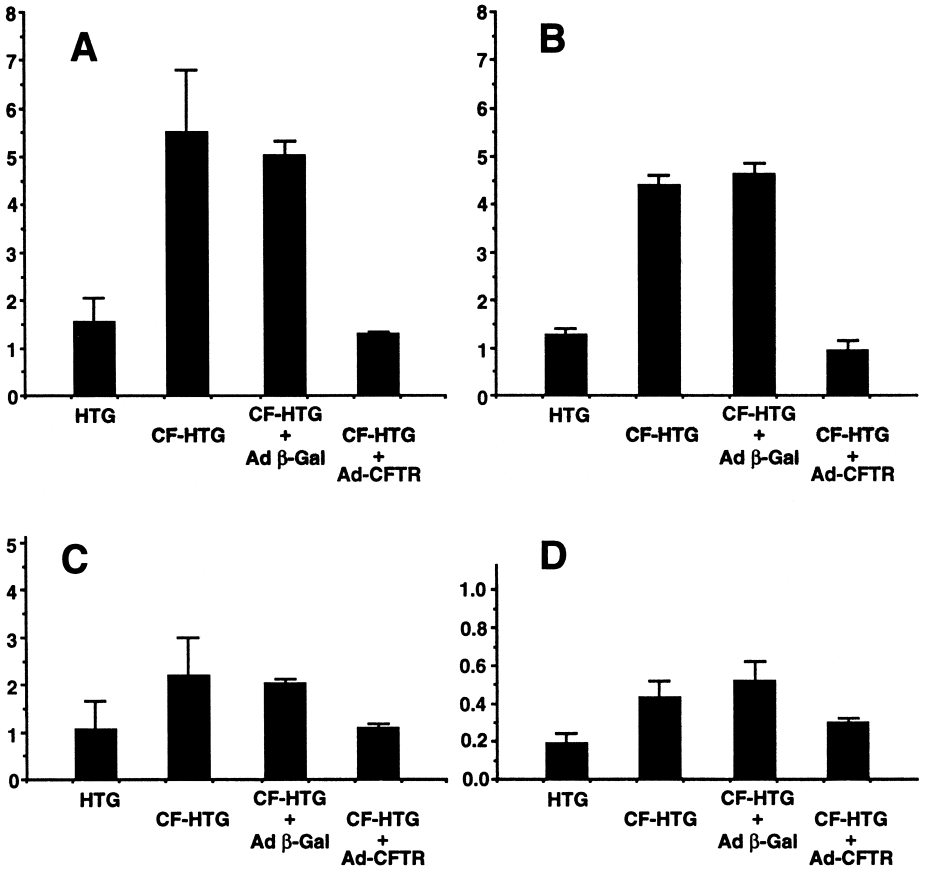

Fig. 4. Specific activities of β-galactosidase (A), α-fucosidase (B), α-mannosidase (C), and β-glucuronidase (D) in HTG cells, in CF-HTG cells, and in CF-HTG cells incubated with Ad-CFTR or with Ad-β-Gal. (in nmol ± S.D. of 4-methylumbelliferone generated/mg protein/min). Measurements have been performed on four different cultures of HTG and CF-HTG cells. Confluent CF-HTG cells were incubated for 24 h with 10 MOI of recombinant adenovirus. All flasks were confluent at the time of harvest and contained 0.1–0.8 mg protein/ml. Cell cultures, protein extraction and enzyme activity determinations are described in Section 2. Assays were performed in triplicate for each culture. Specific activities represent the mean ± S.D. for all cell cultures in each category or for each treatment.

less than that of the HTGS (approximately 167 μM). This is consistent with a similar amount of enzyme but with elevated affinity for its substrate.

3.5. *pH dependence of the specific activities of cellular and E. coli β-galactosidases*

The absence of an apparent increase in β-galactosidase activities in CF-HTGS cells after Ad-β-Gal incubation may be due to the different pH optima between the cellular and the exogenous *E. coli* enzymes. Thus, we compared the specific activities of β-galactosidase in HTGS, CF-HTGS, CF-HTGS incubated with Ad-β-Gal and a purified enzyme from *E. coli*, at different pH values. Table 1 shows that cellular β-galactosidase activity rapidly decreases when the pH increases. At pH 7.2, which is the pH used in the literature to detect β-galactosidase activities after adenoviral transfection, only about 4% of the initial cellular but 50% of the *E. coli* activities could be detected. At that pH value, we observed a slight but not significant increase in total β-galactosidase activity after incubation of CF-HTGS cells with Ad-β-Gal.

4. Discussion

Current gene therapy technologies have proved to be highly efficient in transferring a correcting gene to

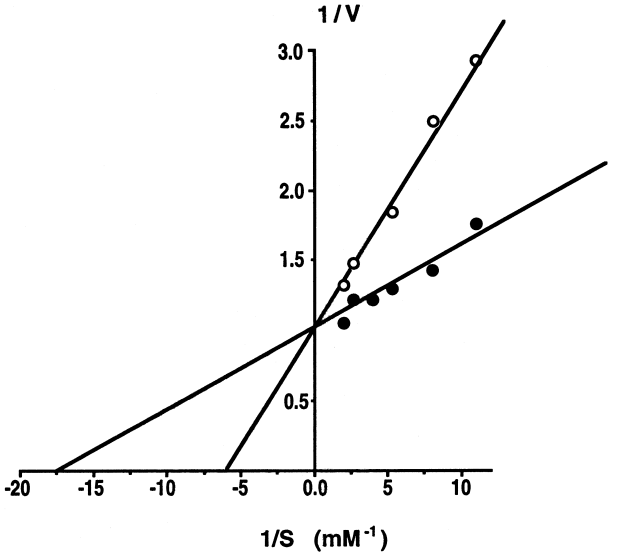

Fig. 5. Kinetics of β-galactosidase from normal and cystic fibrosis HTGS cells. Lineweaver-Burk plot of β-galactosidase activities of the cell extracts of HTGS (○) and CF-HTGS cells (●). The velocities are expressed in μmol of 4-methylumbelliferone generated/mg of protein/h.

diseased cells or organs. Since HTGS cells strongly express CFTR amongst all the other epithelial cell types present in the human bronchus [2], it was of primary importance to examine whether these cells were also correctable by gene transfer. Therefore our work was primarily carried out in order to determine gene transfer efficiency by using the adenoviral construction Ad-β-Gal.

We were unable to observe any exogenous β-galactosidase activity in CF-HTGS cells after β-galactosidase gene transfer whilst expression of the transferred β-galactosidase gene was shown to be effective. This is possibly due to the fact that the endogenous lysosomal β-galactosidase activity that we observed was too high therefore giving much artefactual side effect. In fact, by comparison with white blood cells, not only the intracellular activity of β-galactosidase was elevated but also those of α-fucosidase, α-mannosidase and β-glucuronidase, indicative of a general high quantity of these lysosomal enzymes within HTGS cells.

We observed a higher activity of these lysosomal enzymes in CF than in normal HTGS cells. A difference in lysosomal enzyme activities was also found in other CF cellular systems (mainly lymphoblasts and fibroblasts) or in CF patient sera by some authors [19–21] but not by others [22–24]. This diversity of these data has led to the conclusion that results may depend on cell types and on many external factors [25]. However, a question arises: were these models accurate since the cell types used are now known to express CFTR at a very low level [26]? The increased activity we observed seems to be due not to an increased amount of enzyme but to an increase in catalytic efficiency. This is consistent with possible structural changes of the enzymes. These could arise from changes in enzyme glycosylation as described for glycopeptides [27]. It has been proposed that a CFTR defect can modify the internal pH of some organelles rendering it more alkaline [28,29]. One can therefore hypothesise that a pH modification in the Golgi may alter lysosomal enzyme glycosylation in turn altering their intrinsic activities. The increase in the observed catalytic efficiency could also be related to possible regulatory changes of the CF enzyme. In lysosomes, β-galactosidase forms a high molecular weight complex with neuraminidase and the protective protein cathepsin A which protects β-galactosidase against intra-lysosomal degradation and stabilises the enzyme [30]. It is also tempting to hypothesise here that a possible change in glycosylation may modify the affinity between β-galactosidase and cathepsin A, changing in turn the enzymatic activity of β-galactosidase. Further studies will obviously be necessary to evaluate all these hypotheses.

Lysosomal and *E. coli* β-galactosidases have a different optimal pH activity. We were not able to detect any significant *E. coli* β-galactosidase activity in CF-HTGS cells after *E. coli* β-galactosidase gene transfer even at its optimal pH. This is probably due to a high residual lysosomal enzyme activity. However, it is likely that gene transfer was efficient since Ad-CFTR loaded in identical conditions (1) induced dramatic elevations in CFTR mRNA levels and (2) corrected the altered lysosomal enzyme activities in CF-HTGS cells. Thus, this correction may be due to CFTR gene transfer. The correction of the enzyme activities was complete in these conditions but we were unable to determine transfer efficiency in terms of the number of transfected cells. The question remains whether all the cells need to be transfected or, as is the case for the surface epithelial cells, only a small percentage of transfected cells is suffi-

cient to correct all the monolayer [11]. To answer this, it would be necessary to use a recombinant adenoviral construction containing another reporter gene encoding a protein whose activity is measurable but absent in HTGS cells. This construction is currently not available.

In conclusion, HTGS cells contain high lysosomal enzyme activities including β-galactosidase, which are found to be yet higher in CF cells. The high activities of lysosomal β-galactosidase in HTGS cells did not allow the use of adenovirus-mediated *E. coli* β-galactosidase gene transfer in CF-HTGS cells in order to evaluate gene transfer efficiency. However, and more importantly, adenovirus-mediated CFTR gene transfer was possible in the CF-HTGS cells and showed a total correction of the elevated lysosomal activities observed in CF cells.

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