Expression of α-gal epitopes on HeLa cells transduced with adenovirus containing α1,3galactosyltransferase cDNA

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α1,3Galactosyltransferase (α1,3GT) synthesizes α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on glycoconjugates in nonprimate mammals but not in humans. Transduction of α1,3GT gene into human HeLa cells by an adenovirus vector allowed for accurate kinetics studies on the appearance of α1,3GT and of its product, the α-gal epitope, in the transduced cells. Mouse α1,3GT cDNA was inserted into a replication-defective adenovirus vector. This viral vector, designated AdαGT, could be propagated in human 293 cells that have the viral E1 complementing gene. Transduction of HeLa cells resulted in immediate penetration of ~20 AdαGT copies into each cell and the appearance of α1,3GT mRNA after 4 h. Catalytic activity of α1,3GT was first detected in the cells after 6 h. The initial appearance of α-gal epitopes (~6 × 104/cell) on cell surface glycoconjugates was detected 10 h posttransduction, whereas 24 h posttransduction each cell expressed 2 × 106 epitopes. The activity of α1,3GT in cells transduced with approximately two copies of AdαGT was eightfold lower than that in cells transduced with ~20 AdαGT copies; however, the number of α-gal epitopes/cell remained closely similar. This implies that increased α1,3GT activity above a certain saturation level does not result in a corresponding increase in the carbohydrate product, possibly because of competing glycosyltransferases.

Key words: α1,3galactosyltransferase/α-gal epitope/adenoavirus transduction/anti-Gal/Golgi

Introduction

The glycosylation enzyme α1,3galactosyltransferase (α1,3GT) catalyzes the synthesis of Galα1-3Gal β1-4GlcNAc-R (α-gal epitope) on glycoproteins and glycolipids of various mammals (Basu and Basu, 1973; Blake and Goldstein, 1981; Betteridge and Watkins, 1983; Blanken and Van den Eijnden, 1985; Cummings and Mattox, 1988) in the following reaction:

Galβ1-4GlcNAc-R + UDP-Gal → Galα1-3Galβ1-4GlcNAc-R + UDP
N-acetylgalactosamine α-gal epitope

The enzyme was found to reside within the trans-Golgi network in the same compartment as α2,3sialyltransferase (Smith et al., 1990). The α1,3GT cDNA was cloned initially simultaneously from mouse (Larsen et al., 1989) and bovine (Joziasse et al., 1989) cDNA libraries and the organization of the α1,3GT gene was elucidated in the mouse genome (Joziasse et al., 1992). Analysis of the α-gal epitope expression and of α1,3GT activity in a large number of species revealed a striking pattern of distribution. This enzyme and its product are abundant in nonprimate mammals, prosimians, and New World monkeys (Galili et al., 1987a, 1988; Thall et al., 1991). In contrast, nonmammalian vertebrates, Old World monkeys, apes, and humans lack α1,3GT and α-gal epitopes. These findings suggested that α1,3GT appeared early in mammalian evolution, as it is found both in marsupial and placental mammals, and it was inactivated in ancestral Old World primates (Galili et al., 1988). Nevertheless, the α1,3GT gene is present in humans, apes, and Old World monkeys as a pseudogene that does not undergo transcription (Joziasse et al., 1989, 1991; Larsen et al., 1990; Galili and Swanson, 1991). Comparative studies on the sequences of the pseudogene in primates suggested that the α1,3GT gene was inactivated in ancestral Old World primates 20–25 million years ago (Joziasse et al., 1991; Galili and Swanson, 1991; Galili and Andrews, 1995).

Whereas humans, apes, and Old World monkeys lack α1,3GT and α-gal epitopes, they produce large amounts of a natural antibody against the α-gal epitope (Galili et al., 1987a). This antibody, designated anti-Gal, constitutes as much as 1% of circulating antibodies (Galili et al., 1984), and it binds specifically to α-gal epitopes on glycoconjugates (Galili et al., 1985, 1987b). This distribution of anti-Gal and α-gal epitopes in mammals has generated a major barrier for the transplantation of mammalian organs (e.g., pig heart or kidney) into humans (xenotransplantation). This is because the binding of circulating anti-Gal to α-gal epitopes on pig cells results in the rapid immune rejection of pig organs transplanted in humans, or in monkeys (Good et al., 1992; Galili, 1993; Sandrin et al., 1993; Collins et al., 1995).

We have proposed to exploit this interaction between anti-Gal and α-gal epitopes for increasing the immunogenicity of autologous tumor vaccines in humans by engineering human tumor cells to express α-gal epitopes (LaTemple et al., 1996; Galili and LaTemple, 1997). This increased immunogenicity occurs because the in situ binding of the natural anti-Gal antibody to α-gal epitopes de novo expressed on the vaccinating autologous human tumor cells targets these cells for effective uptake by antigen-presenting cells. This, in turn, may result in the generation of an effective immune response against tumor-associated antigens and the subsequent destruction of metastatic tumor cells. The efficacy of autologous tumor vaccines engineered to express α-gal epitopes was recently...
demonstrated in the experimental animal model of α,1,3GT knockout mouse and with the highly tumorigenic mouse melanoma cell line B16-BL6 (LaTemple et al., 1999).

One of strategies we have explored for expression of α-gal epitopes in human tumor cells involves the transduction of such cells with a replication defective adenovirus vector containing the open reading frame (ORF) of mouse α,1,3GT gene. The experience gained in transduction of glycosyltransferases by adenovirus vector has been limited to the transduction of fucosyltransferase (Nagasaka et al., 1998; Baboval et al., 2000) and of sialyltransferase (Abe et al., 1999) to increase their expression in cells that already have these enzymes as endogenous enzymes. The present study, demonstrating transduction of α,1,3GT into human HeLa cells, is novel in that the transduced cells completely lack the enzyme as an endogenous glycosyltransferase. Because the viral genome enters rapidly into the transduced cells, we could closely follow for the first time the kinetics of the appearance of α,1,3GT mRNA, the production of the catalytically active enzyme within the cells and initial appearance of the product of this enzyme, that is, the α-gal epitope, on cell surface glycoconjugates. The information gained from this study enabled us to construct a precise timeline for the expression of glycosyltransferase genes and the biosynthesis and transport of their carbohydrate products to the surface of the cell.

Results

Production of α,1,3GT mRNA in transduced HeLa cells

HeLa cell monolayers were incubated with replication defective adenovirus vector containing α,1,3GT ORF (designated AdoGT) at a concentration of 10^10 multiplicity of infection (MOI)/ml at 37°C. The cultured cells were detached at the different time points by phosphate buffered saline (PBS) containing 1 mM ethylenediamine tetra-acetic acid (EDTA), washed eight times with PBS/1 mM EDTA to remove unbound AdoGT and assayed for the presence of the viral α,1,3GT gene and of α,1,3GT mRNA within the transduced cells.

Viral α,1,3GT DNA. Transduced HeLa cells were obtained after 10, 30, 60, and 120 min of incubation with the virus vector, and the presence of the viral α,1,3GT gene was determined by polymerase chain reaction (PCR) of the ORF (1118 bp). Whereas the DNA from untransduced HeLa cells did not yield a PCR product, DNA from HeLa cells incubated for the short period of 10 min yielded a positive PCR product (Figure 1A). These findings imply that AdoGT binds to its corresponding receptor on HeLa cells very fast. Thus, it is probable that the virus genome including α,1,3GT gene penetrates into the cell by the first hour of incubation.

α,1,3GT mRNA. To analyze the transcription of α,1,3GT within the transduced cells, the extracted RNA from 2 × 10^6 cells was treated with endonucleases to cleave the DNA, then subjected to reverse transcriptase (RT)-PCR with the same primers as those used above for the amplification of ORF of α,1,3GT. As shown in Figure 1B, a positive RT-PCR product was observed only with RNA preparations obtained 4 h or more post-transduction. Two controls were used in the experiment: (1) Analysis of the quality of extracted RNA: the cultures incubated for 3 h or less had sufficient amounts of mRNA for the template, as indicated by the amplification of the β-actin mRNA (Figure 1B). (2) Analyses for false-positive RT-PCR reactions: to determine the complete digestion of DNA template, the RNA preparations were assayed for uncleaved α,1,3GT DNA by direct PCR. No PCR products were detected following direct PCR of any of the mRNA preparations (data not shown). These findings imply that despite rapid entry of AdoGT into the cells, completion of α,1,3GT gene transcription appears to occur ~ 4 h after the initial exposure of the cells to the transducing virus vector.

Appearance of α,1,3GT enzyme in the transduced cells

The catalytic activity of the de novo produced α,1,3GT within transduced HeLa cells was analyzed in cell lysates with a sugar acceptor that is similar to the physiologic intracellular sugar acceptor, that is, N-linked carbohydrate chains that have terminal N-acetyllactosamine units on solid-phase asialofetuin and UDP-Gal as sugar donor (LaTemple et al., 1996). The de novo synthesized α-gal epitopes were identified by the subsequent specific binding of the monoclonal anti-Gal anti-
body M86 (Galili et al., 1998). This assay was found to be very sensitive, as it could detect the enzyme activity in a lysis of as few as 7.5 × 10^4 cells/ml, 24 h posttransduction (Figure 2). In contrast, original HeLa cells completely lacked any enzyme activity. Transduced HeLa cells assayed for α,1,3GT activity up to 5 h posttransduction also were completely devoid of enzyme activity. However, α,1,3GT appeared in the cells 6 h posttransduction (Figure 2). At this time point the activity of the enzyme was approximately 60-fold lower than that observed 24 h posttransduction.

Expression of α-gal epitopes on transduced HeLa cells

The lectin Bandeiraea (Griffonia) simplicifolia IB4 (BS lectin) has been shown to interact specifically with α-gal epitopes (Wood et al., 1979). Staining of untransduced HeLa cells resulted indeed in no binding of the lectin as demonstrated by flow cytometry (Figure 3A). In contrast, HeLa cells tested 24 h posttransduction with AdGT expressed various amounts of α-gal epitopes per cell as indicated by the broad histogram of binding of the fluorescein (FITC)-conjugated lectin. Expression of these epitopes on transduced HeLa cells could be further demonstrated by the binding of the human natural anti-Gal antibody, which readily bound to the transduced cells (Figure 3B). This staining of α-gal epitopes with either lectin or anti-Gal was not sensitive enough, however, for clear detection of the initial appearance of small numbers of the epitopes on the transduced cells.

We have used the enzyme-linked immunosorption assay (ELISA) inhibition assay (Galili et al., 1998; Stone et al., 1998; Chen et al., 2001) to determine the time point for the initial appearance of the de novo synthesized α-gal epitopes and to quantify these epitopes on the transduced HeLa cells. This assay, which is analogous to a radioimmunoassay, is based on binding of the monoclonal anti-Gal antibody M86 at a high dilution to α-gal epitopes on the assayed cells. The cells and bound M86 are removed by centrifugation, and the amount of unbound M86 remaining in the supernatant is determined by ELISA with synthetic α-gal epitopes linked to bovine serum albumin (α-gal BSA) as a solid phase antigen (see Materials and methods). The extent of M86 removal at various cell concentrations (defined as % inhibition of M86 binding) is inversely proportional to the number of α-gal epitopes per cell. Comparison of the % inhibition of M86 binding at various concentrations of transduced HeLa cells with that of rabbit red blood cells (standard cells expressing 2 × 10^4 α-gal epitopes/cell) enables the quantification of α-gal epitopes on the tested cells. This assay is highly sensitive and can detect as few as 5 × 10^4 epitopes/cell (Galili et al., 1998).

As shown in Figure 4, HeLa cells transduced with the control adenovirus vector (1 × 10^10 MOI/ml), lacking α1,3GT cDNA, and assayed 24 h posttransduction, bound no M86 (i.e., no inhibition of M86 binding to α-gal BSA), implying complete lack of α-gal epitopes. Similarly, HeLa cells transduced with AdGT at a concentration of 1 × 10^10 MOI/ml for up to 8 h

Fig. 2. Analysis of α1,3GT activity in transduced HeLa cells, as assayed in cell lysate supernatants at various cell concentrations. Synthesis of α-gal epitopes on solid-phase asialofetuin by the de novo produced α1,3GT was detected by ELISA measuring the binding of the monoclonal anti-Gal antibody M86. (open circles) Enzyme activity in control HeLa cells or in transduced cells 1, 2, 3, and 5 h posttransduction; (closed circles) enzyme activity in cells 6 h posttransduction; (squares) enzyme activity in cells 24 h posttransduction. Representative data from three different experiments.

Fig. 3. Expression of α-gal epitopes on HeLa cells transduced by AdGT (1 × 10^10 MOI/ml), as measured by flow cytometry of cells stained with BS lectin (A), or with human anti-Gal (B). Closed histograms, untransduced cells; open histograms, cells transduced with AdGT.

Fig. 4. Expression of α-gal epitopes on transduced HeLa cells as determined by ELISA inhibition assay. Data expressed as % inhibition of M86 binding to α-gal BSA. The transduced cells were obtained at different time points. (open circles), HeLa cells posttransduction with a control adenovirus vector lacking α1,3GT cDNA and AdGT transduced HeLa cells assayed 1–8 h posttransduction; (closed circles) AdGT transduced HeLa cells 10 h posttransduction; (triangles) 12 h posttransduction; (closed squares) 24 h posttransduction; (diamonds) 48 h posttransduction; (open squares) rabbit red cells as standard cells expressing 2 × 10^4 α-gal epitopes/cell. Data representative of three experiments.
expressed no α-gal epitopes. However, α-gal epitopes appeared on the transduced cells 10 h posttransduction, as indicated by the distinct inhibition of M86 binding to α-gal BSA at 40 × 10⁶ cells/ml. This inhibition was higher in cells assayed 12 h posttransduction and further increased after 24 h, implying increased expression of α-gal epitopes on the cells.

The actual number of α-gal epitopes expressed on the cells at different time points could be determined by comparing the % inhibition of each of the tested cells with that of rabbit red cells. The % inhibition observed with the cells 10 h posttransduction was approximately 32-fold lower than that of rabbit red blood cells (Figure 4), reflecting the expression of ~6 × 10⁸ α-gal epitopes/cell at that time point. After 2 h more the number of these epitopes on the transduced cells increased fourfold to a level of ~2.4 × 10⁸ α-gal epitopes/cell. These epitopes reached an expression level similar to that observed with rabbit red blood cells, by 24 h posttransduction, implying the expression of ~2 × 10⁹ α-gal epitopes/cell. The maximal number of epitopes per cell was observed 48 h posttransduction and was twice as high as that observed 24 h posttransduction, that is, 4 × 10¹⁰ α-gal epitopes/cell.

**Correlation between the number of AdoGT copies, α1,3GT activity, and α-gal epitope expression in transduced HeLa cells**

To determine whether there is a direct correlation between the number of AdoGT copies, the activity of α1,3GT within the transduced HeLa cells, and the number of α-gal epitopes expressed per cell, the cells were transduced with AdoGT at different concentrations of the vector (i.e., 1 × 10⁹, 1 × 10⁸, 1 × 10⁷, and 1 × 10⁶ MOI/ml). After 4 h incubation, fresh medium was added to the cells, which were further cultured for a total period of 24 h. At the end of incubation, the cells were harvested and studied for the number of AdoGT copies, the activity of α1,3GT, and the number of α-gal epitopes/cell. The number of AdoGT copies was determined by limiting dilution PCR of exon 9 of the mouse α1,3GT gene and comparing the end point to that of mouse DNA-yielding PCR product. As shown in Figure 5, cells transduced with the original suspension of 1 × 10⁹ MOI/ml virus vector contained ~ 20 copies of the α1,3GT gene per cell. This is based on the finding that the end point of DNA amount yielding a PCR product (0.1 ng) was ~10-fold higher than that found with mouse DNA (i.e., DNA containing two copies of the gene per cell). The number of AdoGT copies decreased proportionally to the concentration of the AdoGT vector in the transducing suspension. It was found to be ~ 2 copies/cell in cells incubated with 1 × 10⁸ MOI/ml of the adenovirus vector, ~ 0.2 copies/cell in cells incubated with 1 × 10⁷ MOI/ml (i.e., one copy per five cells), and ~ 0.02 copies/cell (i.e., one copy per 50 cells) in cells incubated with 1 × 10⁶ MOI/ml (Figure 5).

The activity of α1,3GT in the transduced cells was found to be proportional to the extent of transduction by AdoGT. Comparison between α1,3GT activity in cells transduced with 1 × 10⁹ MOI/ml with that in cells transduced with 1 × 10⁸ MOI/ml demonstrated an ~ eightfold decrease in the activity of the enzyme (Figure 6A). The overall enzyme activity further decreased an additional 16-fold in cells transfected with 1 × 10⁷ MOI/ml (Figure 6A), as not all cells contained AdoGT (Figure 5). In contrast to the observations on the enzyme activity, the extent of decrease in α-gal epitope expression did not correlate with the decrease in concentration of transducing virus vector. Whereas cells transduced by a virus suspension containing 1 × 10⁹ MOI/ml expressed 2 × 10⁹ α-gal epitopes/cell (Figure 4), those transduced by AdoGT at a 10-fold lower concentration express only a twofold lower number of epitopes, that is, 1 × 10⁸ α-gal epitopes/cell (Figure 6B). Accordingly, cells transduced by AdoGT at a 100-fold lower concentration (i.e., 1 × 10⁷ MOI/ml) expressed ~10-fold less epitopes per cell (i.e., ~0.2 × 10⁸ epitopes/cell) (Figure 6B). Taken together with the data in Figure 5 implying that only ~20% of the cells incubated with 1 × 10⁸ MOI/ml were transduced, it is probable that the transduced cells in the population expressed 1–2 × 10⁶ epitopes/cell. The data in Figures 5 and 6...
further suggest that whereas the amount of the enzyme produced was proportional to the number of Adα1,3GT copies per cell, there was no direct correlation between the concentration of the α1,3GT in the Golgi apparatus and the amount of produced α-gal epitopes. The enzyme produced in cells incubated with $1 \times 10^{10}$ MOI/ml Adα1,3GT vector seemed to be at a concentration that was at a saturation level in regard to the number of α-gal epitope production. Thus, an eightfold decrease in the enzyme activity resulted in only twofold decrease in the number of synthesized α-gal epitopes.

**Stability of the transduced α1,3GT gene**

The genome of Adα1,3GT lacks the ability to replicate independently because of the absence of the E1 viral gene in the replication-defective vector (Gao et al., 1996; Davis et al., 1998). This viral vector resides within the nucleus of the transduced cell without integrating in the cellular genome. Therefore, it was of interest to determine whether Adα1,3GT undergoes replication along with the replication of the cellular DNA and whether the transduced DNA is resistant to endonucleases. This was assessed by follow-up of α-gal epitope expression in transduced cells and the number of Adα1,3GT copies per cell, after different numbers of cell divisions. HeLa cells were detached 24 h posttransduction with Adα1,3GT, washed, counted, and cultured for 5, 9, and 14 days. Subsequently, the cells were detached, counted for determining the number of cell divisions, and subjected to analysis of α-gal epitope expression by the ELISA inhibition assay. Cells cultured for 5 days were found to undergo 3 divisions; cells cultured for 9 days underwent 5 divisions; and cells cultured for 14 days underwent 10 divisions. DNA was extracted from the cells of the various cultures and assayed by PCR at different amounts of template for the presence of α1,3GT gene.

As shown in Figure 7, the cells that underwent three cell divisions (i.e., 5-day cultures), expressed 75% less α-gal epitopes than the original number of epitopes observed 24 h posttransduction. Thus, proliferation of one cell into eight cells resulted in the in decrease in α-gal epitope expression. This suggests that the initial number of α1,3GT gene copies introduced into the HeLa cells by Adα1,3GT is not maintained in the proliferating cells but decreases along with the cell divisions. An additional fourfold decrease in the number of α-gal epitopes was further observed in cells undergoing 5 divisions, whereas no epitopes were observed after 10 divisions, that is, proliferation of each cell into 1000 cells within 14 days.

The decrease in α-gal epitope expression on cultured cells correlated with the α1,3GT cDNA within the cells. DNA was extracted from the transduced proliferating cells and the presence of the catalytic domain of the mouse α1,3GT (i.e., exon 9 of this gene) (Henion et al., 1994) was determined by PCR with primers that are specific to mouse exon 9 and do not amplify the human homologue. DNA samples at various amounts, starting at 100 ng, from cells cultured for 5, 9, and 14 days were subjected to PCR; the end point was determined and compared with that observed in transduced HeLa cells cultured for 1 day. As determined in Figure 5, 24 h posttransduction the cells contained ~ 20 copies of the transducing α1,3GT gene. The DNA samples from 5 and 9 days posttransduction contained ~ 10- and 100-fold less α1,3GT template, respectively (Figure 8). The amount of α1,3GT cDNA template in 100 ng of DNA from 14-day culture was too low for detectable amplification. This suggests that the number of gene copies indeed decreases in correlation with cell division, and there was no apparent destruction of the transducing viral α1,3GT by endonucleases.

**Discussion**

The study on transduction of HeLa cells by Adα1,3GT provides information about the kinetics of the appearance of a glycosyltransferase and its corresponding carbohydrate product within a mammalian cell. This type of analysis has been feasible because α1,3GT is completely absent in human cells. In accord with previous observations on the internalization of wild-type adenovirus (Greber et al., 1993), the internalization of Adα1,3GT into HeLa cells is a highly efficient process in which the transducing α1,3GT gene can be detected within 10 min of incubation with the
adenovirus vector. We could further demonstrate by the use of RT-PCR that the α1,3GT mRNA appeared within the transduced cells 4 h after the penetration of AdαGT into the cells. Analysis of α1,3GT in the transduced cells indicated that the catalytically active enzyme appears 2 h after α1,3GT mRNA is detected in the cells, that is, 6 h posttransduction. The initial appearance of the product of α1,3GT, that is, the α-gal epitope on cell surface glycoconjugates, could be determined accurately by the highly sensitive ELISA inhibition assay. The α-gal epitope was detected on the transduced HeLa cells 4 h after the identification of α1,3GT within the cells, that is, 10 h posttransduction (Figure 9). This rapid expression of the transduced enzyme is many times faster than that observed when the cellular α1,3GT gene undergoes induction for expression. Cho et al. (1996) reported that mouse teratocarcinoma cells display a fourfold increase in α1,3GT expression only 36 h after incubation of the cells with retinoic acid.

The appearance of α1,3GT within 2 h after the detection of the corresponding mRNA suggests that, at that early time point, the enzyme is likely to be present within the rough endoplasmic reticulum (RER). Although α1,3GT is a glycoprotein with one N-linked carbohydrate chain (Larsen et al., 1989), it can be catalytically active in the absence of the carbohydrate chain. This is based on our previous demonstration of recombinant α1,3GT activity following the expression of the enzyme in bacteria (Galili and Anaraki, 1995), that is, in the absence of N-linked carbohydrate chains. Thus it is probable that once the enzyme appears in the RER, its activity can be readily detected. This initial activity detected after 6 h was found to be ∼ 60-fold lower than that measured in the cells after 24 h. Nevertheless, the sensitive assay using solid-phase asialofetuin as sugar acceptor and the subsequent detection of the de novo synthesized α-gal epitopes by binding of the monoclonal anti-Gal antibody M86 clearly demonstrated the activity of α1,3GT in cells 6 h posttransduction and the complete absence of the enzyme in cells incubated for shorter periods.

To synthesize α-gal epitopes on glycoconjugates, this de novo produced enzyme has to be transported to the trans-Golgi network. Previous studies on transport of proteins from the RER to the Golgi apparatus demonstrated that this process takes 2–3 h (Rothman and Orci, 1992). Thus it is probable that α1,3GT reaches the trans-Golgi network ∼ 8 h posttransduction. The detection of cell surface α-gal epitopes 10 h posttransduction, further suggests that the first glycoconjugates carrying de novo synthesized α-gal epitopes within the trans-Golgi network reach the cell surface within ∼ 2 h after the synthesis of this carbohydrate epitope (Figure 9).

Whereas the activation of the α1,3GT gene within AdαGT by CMV promoter is much faster than that of the cellular α1,3GT gene by retinoic acid (Cho et al., 1996), it is probable that the translation of the mRNA and the synthesis of α-gal epitopes are independent of the source of the gene. Therefore, the timeline in Figure 9 is likely to reflect the general timeline for production of glycosyltransferases from mRNA and the subsequent synthesis of the corresponding carbohydrate units within the Golgi apparatus.

Although the α1,3GT cDNA introduced by the AdαGT vector does not integrate into the genomic DNA, it is stable within the nucleus for more than 1 week and does not seem to be affected significantly by endonucleases within the cell. This is implied from detection of the transduced α1,3GT gene, 9 days posttransduction. Nevertheless, the AdαGT vector does not seem to replicate but is divided between the daughter cells. This is suggested by the observation that the expression of α-gal epitope decreases from 2 × 10⁶ epitopes/cell in the transduced cells, to 0.4 × 10⁶ epitopes/cell on each of the eight daughter cells generated from three successive divisions of a cell. The subsequent decrease of α-gal epitopes expression after 5 divisions and the lack of such epitopes after 10 divisions (i.e., the original cell proliferating into ∼ 1000 cells), further supports this assumption.

The extent of transduction by AdαGT correlated with the enzyme activity within the HeLa cells. The relative amount of α1,3GT within the Golgi apparatus of cells incubated with AdαGT at 1 × 10¹⁰ MOI/ml was approximately 10-fold higher than that in cells incubated with AdαGT at 1 × 10⁹ MOI/ml and 100-fold higher than that in cells incubated with AdαGT at 1 × 10⁸ MOI/ml, respectively. This further correlated with the number of transducing copies of the vector per cell as well. Nevertheless, the number of α-gal epitopes/cell was significantly higher than that expected if there would have been direct correlation between the enzyme activity and carbohydrate expression. Thus HeLa cells transduced by the vector at 1 × 10⁶ MOI/ml had half the number of epitopes on cells transduced with 1 × 10⁸ MOI/ml. These data suggest that the concentration of α1,3GT may decrease within the Golgi apparatus by several-fold with no parallel decrease in the expression of the α-gal epitope on the cell membrane. Thus the amount of α1,3GT seems to be above a saturation level for maximum synthesis of α-gal epitopes. It is also probable that the competition between sialyltransferase and α1,3GT within the trans-Golgi network (Smith et al., 1990) also limits the number of produced α-gal epitopes.

The study of transduction by AdαGT may also be of clinical significance because the transduced cells expressing α-gal epitopes bind human natural anti-Gal IgG molecules. It remains to be determined whether similar transduction of freshly obtained tumor cells from cancer patients, by the AdαGT vector, results in expression of a sufficient number of α-gal epitopes to enable effective in vivo binding of anti-Gal. Hypothetically, if the de novo expression of these epitopes is sufficient, such cells may be considered for studies as autologous tumor vaccines, in which the in situ binding of anti-Gal to these α-gal epitopes, targets the vaccinating cells to antigen-presenting cells. Such targeting may ultimately result in increased immune response against tumor antigens on the vaccinating cells and the possible destruction of metastatic cells expressing these antigens (LaTemple et al., 1996, 1999; Galili and LaTemple, 1997).
Materials and methods

Reagents and cells

Restriction enzymes and other enzymes used for molecular cloning were from New England BioLabs (Beverly, MA). UDP-Galactose was received as a generous gift from Neose Pharmaceuticals (Horsham, PA). Fetuin from fetal bovine serum was purchased from Sigma (St. Louis, MO). The monoclonal anti-Gal antibody designated M86 was obtained in tissue culture supernatants of the hybridoma M86 cells, as previously described (Galili et al., 1998). The natural human anti-Gal was isolated from human AB sera by affinity chromatography on columns with synthetic α-gal epitopes (ChemBioMed, Edmonton, Canada), as previously described (Galili et al., 1984, 1985, 1987b). Horseradish peroxidase (HRP)-conjugated anti-mouse IgM was purchased from Axcell (Westbury, NY), Phycoerythrin-conjugated anti-human IgG from Pharmingen (San Diego, CA) and FITC-conjugated BS lectin was purchased from Vector Laboratories (Burlingame, CA).

The study was performed with HeLa cells, which originate from a human cervical carcinoma and purchased from American Tissue Typing Cell Collection. The cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics. All cell cultures with the viral vector were performed in this medium, as well.

Construction of AdαGT vector

The ORF of the mouse α1,3GT cDNA was inserted into a replication defective adenovirus vector, in which the early genes E1 and E3 were deleted as previously described (Gao et al., 1996). This was achieved by the use of a pAd shuttle plasmid containing the α1,3GT cDNA that allows for homologous recombination of the cDNA into the replication defective adenovirus vector. In addition, the cells that propagated the virus were of the human kidney cell line 293 (ATCC) which contains the E1 complementing viral gene. To generate pAd shuttle plasmid containing α1,3GT cDNA, the ORF of α1,3GT mRNA was amplified by RT-PCR of mouse RNA, based on the sequence described by Larsen et al. (1989). This PCR product was ligated into pAdCMVlink by the use of Hind III and blunted Spe I sites. This plasmid contains adenovirus terminal repeat and the packaging signal, a CMV promoter for transcription in the transduced cells, a multiple cloning site, an SV40 polyadenylation site, and flanking regions to the E1 genes of adenovirus (m.u. 9–16) which includes the 5′ inverted terminal repeat and the packaging signal, a CMV promoter for the effective expression of the inserted gene in mammalian cells, a multiple cloning site, an SV40 polyadenylation site, and flanking regions to the E1 genes of adenovirus (m.u. 9–16) for homologous recombination (Gao et al., 1996; Davis et al., 1998). The pAdCMV-α1,3 GT plasmid was cotransfected into 293 cells with Cla I–digested H5.030CMVEGFP viral backbone containing deletions in map units 1–9 of the E1 region and in E3 region (Davis et al., 1998) to create a hybrid viruses by homologous recombination of the regions flanking the viral E1 gene. Recombinant plaques were initially isolated through green/white selection (Davis et al., 1998). The positive plaque was plaque-purified and grown up in 293 cells for CsCl gradient purification (Gao et al., 1996). Subsequently, each of the viral clones was assayed for presence of α1,3GT cDNA that produces catalytically active enzyme, by transduction of 293 cells and analysis of α-gal epitope expression after 24 h by flow cytometry following BS lectin binding, as described below. Like other human cells, 293 cells also lack α-gal epitopes. Expression of this epitope following transduction indicated that the assayed adenovirus vector contained the intact α1,3GT cDNA. The viral clone proven to induce expression of α-gal epitopes was designated AdαGT and was further propagated in the 293 cells and used for transduction of HeLa cells, as described below.

The concentration of AdαGT was determined as MOI units per ml and defined as the highest dilution of adenovirus vector that displayed cytopathic effects in 293 cells, 6 days postinfection. The assay was performed in 24 well plates containing 293 monolayers and incubated with AdαGT suspensions at 10-fold serial dilutions. The cytopathic effects of the replicating virus within the cells was determined as rounding of the cells and detachment from the well followed by cell lysis.

Transduction of HeLa cells by AdαGT

HeLa cell monolayers were incubated with AdαGT at various concentrations of the viral vector for 4 h at 37°C. Subsequently, culture medium was added to dilute the AdαGT suspension fivefold and cell monolayers were incubated for additional 20 h. In monolayers incubated for several days, the culture medium was changed 24 h posttransduction.

PCR analysis of transduced α1,3GT DNA and mRNA

The transduced α1,3GT cDNA was detected by PCR with the following primers that amplify the ORF of the mouse gene (Larsen et al., 1989): 5′ATGAATGTCAAGGGAAAAAG and 3′TCAGACATTATTTCTAACCA. For this purpose DNA was extracted from cells and subjected to 100 ng to PCR with these primers for 40 cycles, each including 30 s at 94°C, 20 s at 62°C, and 20 s at 72°C. The first cycle included 5 min at 94°C to achieve complete denaturation of the DNA. For quantifying the number of AdαGT copies within the transduced cells, the extracted DNA was subjected to PCR at different amounts of DNA template, and the end point was compared to that observed in mouse cells containing two copies of this gene in their genome.

For analysis of α1,3GT transcription in the transduced cells, the RNA was extracted and the contaminating DNA was destroyed by endonuclease. cDNA was generated by the use of reverse transcriptase with random hexamers. Subsequently, PCR was performed, as described above. To confirm complete elimination of any contaminating AdαGT DNA, the isolated RNA was shown to lack any template for direct PCR with the two primers described above.

Estimation of the number of AdαGT copies in transduced cells

Cells were detached 24 h posttransduction and DNA extracted. PCR was performed with DNA at different amounts. The primers used, amplified exon 9 of the mouse α1,3GT gene, which corresponds to the catalytic domain of the gene (Joziasse et al., 1992; Henion et al., 1994). The primers used to amplify exon 9 were 5′AGACTTTCTGGAGTCTGCTGACAT and 3′TCAGATATTAAAGTGTCACAGGT. The DNA amounts used for PCR ranged from 100 ng to 0.01 ng per reaction at serial 10-fold dilutions, and the end point for PCR product was determined. The results were compared to PCR products obtained with various amounts of mouse genomic DNA. The number of copies of AdαGT was estimated by comparing the lowest amount of DNA from transduced cells

Transduced HeLa cells expressing α-gal epitopes
that yielded a positive PCR, with the lowest amount of mouse DNA yielding PCR, that is, DNA containing two copies of the gene.

Analysis of α1,3GT activity in transduced HeLa cells

The activity of α1,3GT produced in the transduced cells was assessed in an assay that simulates the synthesis of α-gal epitopes on glycoproteins within the Golgi apparatus, as we have previously described (LaTemple et al., 1996). The assay was based on the ability of the enzyme to transfer galactose from the sugar donor UDP-Gal to terminal N-acetyllactosamine residues on N-linked carbohydrate chains of asialofetuin. Fetuin is a protein obtained from fetal calf serum that carries nine N-acetyllactosamine residues that function as sugar acceptors. Synthesized α-gal epitopes were identified by a nonradioactive assay in which the monoclonal anti-Gal antibody M86 (Galili et al., 1988). The removal of these sialic acid units exposes nine N-acetyllactosamine residues that function as sugar acceptors. Synthesized α-gal epitopes were identified by a nonradioactive assay in which the monoclonal anti-Gal antibody M86 (Galili et al., 1998) binds to the de novo synthesized epitopes and the binding is measured by ELISA. Desialylation of fetuin was performed by incubation of fetuin in 50 mM sulfuric acid at 80°C for 2 h and subsequent repeated dialysis (LaTemple et al., 1996). Asialofetuin (20 µg/ml) in carbonate buffer (pH 9.5) was used to coat microtiter ELISA plates. Subsequently, the wells were blocked with 1% BSA in carbonate buffer.

HeLa cells transduced by AdαGT were detached from the tissue culture flasks by 1 mM EDTA in PBS, washed with saline, and frozen as pellets each containing 2 × 10^6 cells. The pelleted cells were lysed by resuspension in 0.1 ml of enzyme buffer (25 mM 2-[N-morpholino]ethane sulfonate in saline, 25 mM MnCl₂, and 1 mM UDP-Gal, pH 6.2) containing 0.5% Triton X-100. The cells were incubated for 20 min at 37°C with occasional vortexing for achieving complete lysis. Subsequently, the lysates were centrifuged to remove the particulated material. The supernatants were assayed for α1,3GT activity by serial twofold dilutions in enzyme buffer as above, which contained 0.05% Triton X-100 instead of 0.5% of the detergent. The diluted supernatants of the cell lysates were incubated in the ELISA plates coated with asialofetuin at 50 µl/well for 2 h at 37°C. The plates were then washed and incubated for 2 h with the monoclonal anti-Gal antibody M86 (Galili et al., 1998). At the end of incubation the antibody was removed, and the plates were washed and incubated with HRP-conjugated anti-mouse IgM secondary antibody. The enzyme activity was expressed as the optical density units measured in wells containing the enzyme within supernatants of cell lysates at various concentrations of the cells.

Flow cytometry analysis of α-gal epitope expression on transduced HeLa cells

HeLa cells were incubated at a concentration of 1 × 10⁶ cells/ml for 2 h at 4°C with 10 µg/ml FITC-BSA lectin in PBS containing 1% BSA. This lectin binds specifically to α-gal epitopes (Wood et al., 1979). Cells were then washed three times with PBS, fixed, and analyzed in FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), as we have previously described (Gorelik et al., 1995). Similar studies were performed with cells incubated with purified human anti-Gal (Galili et al., 1985, 1987a,b) (10 µg/ml), followed by phycoerythrine-conjugated rabbit anti-human IgG (Dako, Denmark).

Quantifying α-gal epitope expression on transduced cells

Detection of α-gal epitopes de novo expressed on transduced cells was performed by the ELISA inhibition assay, in which the number of α-gal epitopes per cell could be determined by the subsequent binding of the monoclonal anti-Gal antibody M86 (Galili et al., 1998; Stone et al., 1998; Chen et al., 2001). This assay, which is analogous to radioimmunoassays, is the most sensitive assay for quantifying α-gal epitopes on cells (Galili et al., 1998) and is a modification of the original assay for measuring α-gal epitope expression on soluble glycoproteins (Thall and Galili, 1990).

The assayed cells were brought to a concentration of 40 × 10⁶ cells/ml and subjected to serial twofold dilutions in 100-µl aliquots of PBS containing 1% BSA. The cells in each dilution were mixed with equal volume of monoclonal anti-Gal M86 at the final dilution of 1:100 of the antibody. This concentration of the antibody yields ELISA absorption results at the slope of the binding curve. The mixture was incubated overnight at 4°C with constant rotation to enable maximum binding of the antibody to α-gal epitopes. Subsequently, the cells with bound M86 IgM molecules were removed by centrifugation, and the activity of free M86 remaining in the supernatant was determined by ELISA with α-gal BSA as solid phase antigen, using HRP-conjugated goat anti-mouse IgM antibody as secondary antibody.

The cells expressing α-gal epitopes bind the antibody proportionally to the number of α-gal epitopes expressed on the cell. Therefore, the amount of free M86 antibody remaining in the supernatant is inversely proportional to the number of epitopes per cell. By comparing the binding of M86 to cells with known number of α-gal epitopes per cell (standard cells) to that of the antibody binding to the assayed cells, it is possible to determine the number of α-gal epitopes per cell. Rabbit red cells were used as the standard cells because they were previously shown to express 2 × 10^6 α-gal epitopes per cell (Galili et al., 1998). Thus, if the transduced cells are 10-fold less effective than rabbit red cells in the ELISA inhibition assay (i.e., 10-fold higher concentration of transduced HeLa cells than that of rabbit red cells is required for 50% inhibition of anti-Gal M86 binding to α-gal BSA in ELISA), the number of α-gal epitopes on the tested cells is approximately 2 × 10⁵ per cell.

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Abbreviations

α-gal epitope, Galα1–3Galβ1–4GlcNac-R; α1,3GT, α1,3galactosyltransferase; α-gal BSA, synthetic α-gal epitopes coupled to...
bovine serum albumin; AdetGt, adenovirus containing mouse α,3GT cDNA; anti-Gal, Ab to α-gal epitope; BS lectin, Bandeiraea (Griffonia) simplicifolia IB4; EDTA, ethylene-diamine tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein-isothiocyanate; HRP, horseradish peroxidase; MOI, multiplicity of infection; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RER, rough endoplasmic reticulum; RT, reverse transcriptase; UDP-Gal, UDP-galactose.

References


