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Receptor-mediated gene delivery employing lectin-binding specificity

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Selective targeting of malignant cells will be necessary to implement many of the gene therapy strategies being designed to combat cancer. Targeting can be achieved by transductional or transcriptional approaches. Transductional targeting can be accomplished by exploiting differences in the molecules or receptors expressed on the cell surface of malignant versus normal cells. Given that malignant cells can be distinguished from normal by differences in the expression of cell surface carbohydrates, we hypothesized that transductional targeting would be feasible by molecular conjugate vectors which achieve cell binding by virtue of lectins directed against the cell surface glycocalyx. We have shown that gene transfer can be accomplished by these novel lectin-targeted molecular conjugate vectors and that lectin binding specificities may serve as a means for potential targeting of cancer cells for the purposes of gene therapy.

Introduction

As methodologies for employing gene therapy for cancer develop, it is becoming clear that specific targeting of tumor cells will be essential for successful implementation of many of these approaches. In this regard, specific targeting can be achieved by strategies that can be grouped in terms of 'transductional' versus 'transcriptional' approaches. The former approach exploits differences in the expression of surface antigens and growth factor receptors (e.g. C-erbB-2 gene product epidermal growth factor receptor etc.) to target cancer cells. A monoclonal antibody or specific ligand is conjugated to the effector toxins, drugs, radionuclides or cytokines to be delivered. Targeting is thus achieved by specific binding of the vector with cognate cell surface molecules. The transcriptional targeting approach capitalizes on the observation that certain tumors are capable of differentially and specifically expressing selected genes (e.g. carcino-embryonic antigen; α-fetoprotein; etc.) and thus exploits this distinctive pattern of gene regulation. In this regard, aligning therapeutic or toxin genes under the control of promoters specific for these selected genes offers a potential mode for specific targeting by restricting expression to these tumor cell targets.

Of the vector systems available for gene transfer, molecular conjugates possess unique advantages in terms of a design plasticity that potentially permits transductional targeting [1–3]. This vector system consists of linked ligand and DNA binding domains which function in an integrated manner to mediate efficient gene transfer [4–6]. In this vector strategy, the ligand domain permits interaction with cell surface receptors that are constituents of cellular internalization pathways. This interaction allows the linked DNA to be transported into the cell by efficient pathways of receptor-mediated endocytosis. Thus, a physiologic cellular entry system responsible for macromolecular internalization is subverted to accomplish heterologous gene transfer. For applications of cell-specific gene delivery, it is the flexibility of design of the molecular conjugate vectors that permits the possibility of achieving targeted gene delivery. In this regard, altering the ligand moiety of the construct makes it possible to change the vector's binding specificity, and consequently the target cell type for the vector.

In terms of gene therapy for cancer, unique cancer-specific ligands are needed to accomplish transductional targeting of tumor cells. Although various tumors have been shown to overexpress certain cell surface receptors, unique receptor pathways have not been identified. Despite this potential limitation, two classes of agents have been exploited to achieve distinction between normal and malignant phenotypes based upon differences in cell surface markers: monoclonal antibodies and lectins. By virtue of their specificity, monoclonal antibodies and lectins can thus potentially serve as selective markers for targeting malignant cell types. To date, much work has focused on the exploitation of antibody specificity to achieve tumor targeting. For gene therapy applications, this has included the use of antibodies to achieve targeted gene delivery in the context of both viral and non-viral vector systems [7, 8]. As an alternative, we report here data that supports the hypothesis that selective

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transductional targeting can be achieved by exploiting differences in lectin binding to cell surface carbohydrates. Lectins, which are divalent or polyvalent ligands that recognize specific glycoconjugates expressed on the cell surface [9], have been widely used to differentiate phenotypic variants in tissues by virtue of their distinctive patterns of expression of surface carbohydrates [10–13]. The demonstration that lectins can mediate specific binding and endocytosis [14–16] underlies their potential to serve as carriers to mediate site-specific drug delivery and prompted us to consider their use as vectors for directed gene transfer [17]. By incorporating lectins as ligands into the design of molecular conjugate vectors, we capitalized on differences in the cell surface glycocalyx to accomplish gene transfer selectively into cells.

Results

Gene transfer mediated by lectin-targeted molecular conjugates to different cellular targets

To determine if lectin-targeted conjugates could accomplish gene transfer to a specific cell target, a panel of conjugates was derived with various lectin-targeting moieties and delivered to Lewis lung carcinoma (LLC) cells (Figure 1A). In this analysis, it could be seen that the concanavalin (ConA)-containing lectin conjugates possessed a greater efficacy of gene transfer into LLC cells than molecular conjugates incorporating other lectins. In contrast, when the same panel of lectin-targeted conjugates were delivered to IPEC-J_2 cells, an entirely different profile of gene transfer efficacy was noted (Figure 1B). This finding suggested that cell specific glycoconjugates dictated gene transfer efficiency based upon their interaction with specific lectins. Thus, it was likely that these differences in surface carbohydrates led to the observed differences in profiles of DNA transfer efficiency into the two cell types.

Optimization of lectin-targeted gene transfer

The conjugates used in the experiments above were constructed empirically employing a ratio of biotinylated lectin to anti-biotin antibody of 1:1 (i.e. 300 pmol biotinylated lectin/150 pmol aBAbpL). These constructs were hence designated as '1×' conjugates. To determine the optimum ratio of biotinylated lectin to antibody–polylysine complexes, conjugates were derived varying the amount of biotinylated lectin. Therefore, 0.1× conjugates had the same amount of plasmid containing reporter gene (3.0 μg DNA), but a biotinylated lectin/antibody ratio of 1:10. Similarly 0.2× conjugates had a fivefold excess of antibody and 0.5× conjugates had a biotinylated lectin/aBAbpL ratio of 1:2. In assessing the gene transfer efficiency of conjugates constructed in this fashion, analysis revealed that the optimum gene transfer was mediated by conjugates that had a biotinylated lectin/aBpL ratio of 1:10 or 1:5 (Figure 2). Notably, the relative selectivity of ConA conjugates in mediating higher efficiency DNA transfer into LLC cells was maintained even when conjugates were formulated as 0.1× complexes (data not shown). This empiric observation was only determined for the LLC cells and their optimum targeting lectin ConA. It may be that other cellular targets would have different optima. Alternatively, this observation may reflect aspects of the final conjugate configuration that would be more generally applicable.

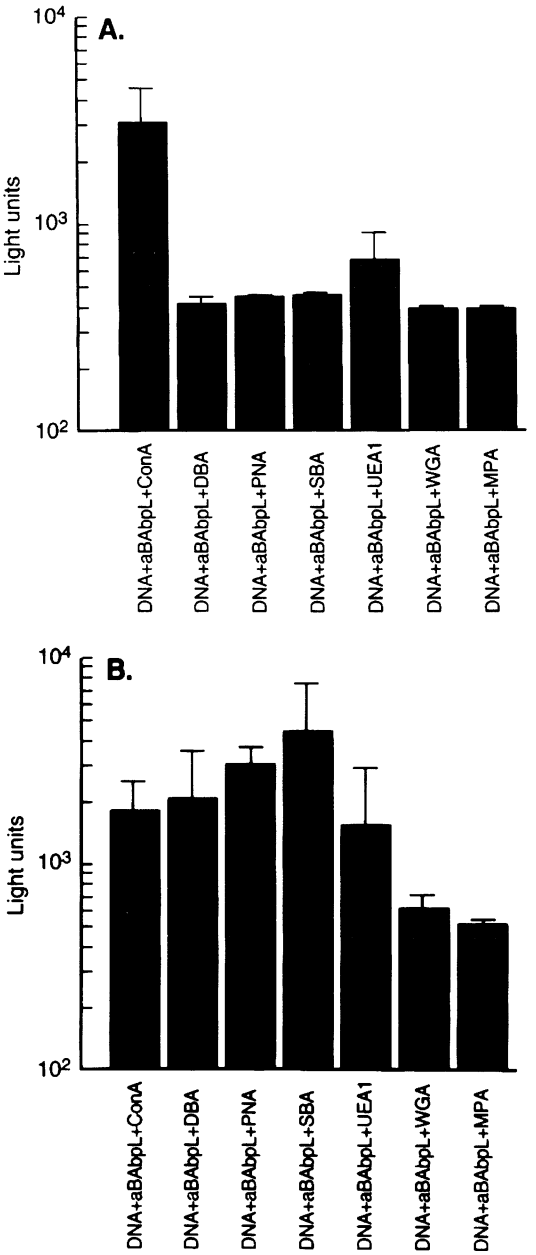

Figure 1 *Gene transfer into (A) Lewis lung carcinoma (LLC) cells and (B) IPEC-J_2 cells by various lectin-targeted molecular conjugate vectors. Cells were transduced with lectin-targeted molecular conjugate vectors containing 3.0 μg of reporter plasmid DNA pCMVL linked to 3.0 μg aBAbpL. The resultant DNA–aBAbpL complex was adjoined to 150 pmol biotinylated lectin to achieve molar equivalents of aBAbpL and biotinylated lectin and, thus, molecular conjugates in the '1×' configuration.*

Specific gene delivery mediated by cell surface–lectin interaction

To establish that gene transfer to LLC cells mediated by the lectin-targeted conjugates was a function of the

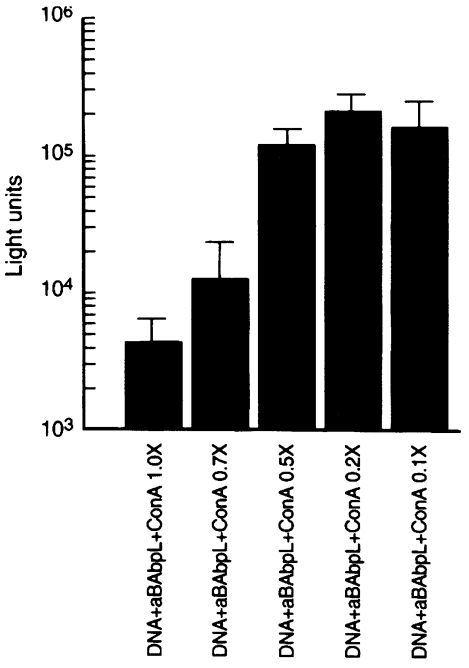

Figure 2 Optimization of gene transfer by lectin-targeted molecular conjugate vectors. LLC cells were transduced with lectin-targeted molecular conjugates containing variable amounts of ConA. For these complexes, the concentration of the biotinylated lectin was varied between molar equivalents of aBAbpL (1× conjugates) to a 10-fold molar excess of aBAbpL (0.1× conjugates).

biotinylated lectin, the conjugate components were evaluated for their utility for gene transfer (Figure 3). As shown, DNA + ConA or DNA did not accomplish detectable reporter gene expression. Although there was some non-specific transfer of DNA conjugated with antibody–polylysine (DNA + aBAbpL), which has been previously noted for polylysine–DNA complexes, the efficacy of gene transfer was significantly augmented when ConA was incorporated into the design of the construct. Importantly, this gene transfer could be attenuated when complexes were constructed containing an excess of the ligand, which would serve to compete for complex binding. This is consistent with our hypothesis that ligand–receptor interaction on the cell surface between the lectin and its ligand carbohydrate triggered conjugate uptake and expression of the reporter gene.

To ensure that incorporation of lectins into this design did not enhance non-specific DNA transfer by sterically altering conjugate complexes, control experiments were performed that demonstrated that lectins added to human transferrin–polylysine–DNA complexes (hTfpL–DNA) had no greater effect on gene delivery than hTfpL–DNA complexes alone. Moreover, when anti-biotin antibody–polylysine complexes were formed with biotinylated ConA versus non-biotinylated ConA, gene delivery as measured by luciferase gene expression was consistently at least fivefold higher with conjugate constructs that allowed for immunolinkage with the lectin. This confirms the crucial nature of the physical link between the targeting lectins and the polylysine-condensed DNA.

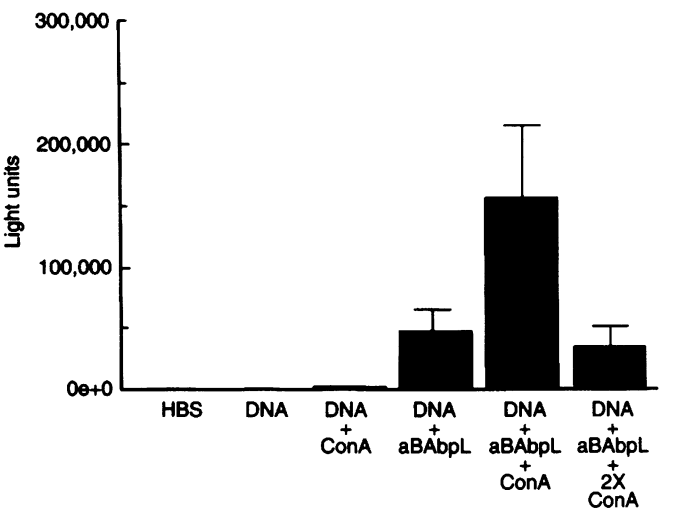

Figure 3 Specific gene transfer mediated by lectin-targeted molecular conjugates. LLC cells were transduced with components of the lectin targeted molecular conjugates for comparison to the complete conjugate.

To rule out the the possibility that the differences in luciferase gene expression observed with the various lectin conjugates were due to increased DNA delivery and not due to a secondary action of exogenous lectins acting via modulation of signal transduction, LLC cells with a stably integrated luciferase gene driven by the CMV promoter/ enhancer were mock-transduced with the various biotin-labeled lectins (0.1× and 1×). In this analysis, there was no difference in the expression of the luciferase gene product between the groups, suggesting the lectins were not acting via transactivation of promoters. Thus, the effect of the various lectins would appear to be on the basis of their ability to accomplish cellular delivery of the polylysine-complexed DNA. Finally, to investigate other methods of linkage of the lectin within the complex configuration, molecular conjugates were constructed using the streptavidin–biotin high affinity bond to link biotinylated lectins to polylysine. Comparison of gene transfer mediated by these lectin-targeted molecular conjugates demonstrated that there was no significant difference between gene delivery mediated by constructs linked by virtue of an anti-biotin–biotin relationship versus the streptavidin–biotin interaction (data not shown).

Discussion

Elegant molecular strategies have been proposed for destruction of malignant cells, while sparing normal cells. Many of these molecular strategies employ the techniques of gene transfer and, for most of these strategies, targeted intracellular delivery of polynucleotides is a prerequisite for success. Targeting can be accomplished by utilizing known differences between the normal and malignant phenotypes. These differences can lie within the expression of tumor antigens, or be in the atypical gene regulation of normal versus cancer cells. We have presented data for a strategy that exploits cell surface differences of cancer cells to deliver genes selectively into target cells. Lectins,

which recognize differences in cell surface architecture, have been employed as carriers of toxins and chemotherapeutic agents [18–20]. This use of lectins has exploited the observation that malignant transformation is frequently associated with characteristic and distinct changes in the cell surface glycocalyx. We have utilized lectins here as ligands within the context of molecular conjugate vectors to achieve gene delivery. We have shown not only that lectin binding affinities of different cell types can be discriminating, but also that those differences can be exploited for transductional targeting of selected cell populations. In doing so, we have established that lectins, like monoclonal antibodies, have the potential for mediating targeted gene delivery.

However, much needs to be learned about the function of cell surface glycoconjugates recognized by lectins before we can predictably use lectins as carriers of genes. The data reported here demonstrate that at least some of the surface carbohydrates recognized by lectins may serve as receptors and will allow specific endocytosis. In this regard, it is of note that there was heterogeneity in terms of gene transfer efficiency mediated by targeted lectin molecular conjugate vectors. Part of this heterogeneity may be attributed to the inconsistent efficacy of chloroquine in mediating functional disruption of lysosomes following internalization of conjugate vectors. Consequently, a significant portion of internalized conjugates was probably degraded within the intracellular vesicle system. It is also conceivable that immunolinkage of biotinylated lectin with the aBAbpL/DNA complexes was less than satisfactory and that a component of variability observed could be attributed to the inherent instability of this antigen–antibody interaction. Perhaps a stronger linkage of the ligand with the DNA binding domain could serve to overcome this variability, although preliminary data suggest otherwise.

Lastly, before one can generalize regarding the utility of lectin-targeted molecular conjugate vectors, their efficacy in mediating gene transfer in primary cell populations and, ultimately, *in vivo* must be demonstrated. In this regard, it is well recognized that cellular lectin-binding specificities change with the microenvironment of the cell, as well as with phenotypic transformation of the cell. Therefore, the selective targeting capabilities of a lectin molecular conjugate would reflect the experimental design used to assess its targeting efficacy. In no circumstance can lectins, or any targeting moiety yet developed, reliably discriminate between normal and malignant phenotypes with complete selectivity and specificity. However, whether the relative specificity afforded by recognition of altered glycocalyces will suffice in mediating site-specific gene delivery and targeting of cancer cells merits further investigations.

Materials and methods

Cells

LLC cells, a murine cell line derived from a spontaneously arising lung carcinoma, were acquired from the American Type Culture Collection (ATCC). IPEC-J_2 cells, a non-transformed (primary-passaged) intestinal epithelial cell line derived from neonatal piglets [21, 22], were obtained from Dr Helen Berschneider (NC State University, USA). The cell types were plated at a density of 1×10^5 cells/well in six-well (3.5 cm) tissue culture plates. IPEC-J_2 cells were maintained in Dulbecco's modified Eagle's medium/ F12 (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), insulin (5 mg/l), transferrin (5 mg/l), selenium (5 µg/l), penicillin (10 000 U/l), streptomycin (10 mg/l) (complete primary medium for IPEC-J_2 cells). LLC cells were maintained in DMEM supplemented with 10% FBS and penicillin (10 000 U/l), streptomycin (10 mg/l) (complete primary medium for LLC cells). The cells were allowed to reach a 50–70% confluency over 24–36 h following passage. At this point, complete primary media was replaced with 1.25 ml DMEM supplemented with 2% FBS and 100 µM chloroquine (Sigma) (transfection medium). To accomplish gene transfer, 250 µl of conjugate solution containing the complexes was added to the transfection medium within each well. Four hours later, the transfection medium was removed and the cells washed with ice-cold phosphate-buffered saline (PBS, pH 7.5). Complete primary medium was replaced onto the cells, which were then incubated for 24 h at 37°C prior to their harvest for analysis of reporter gene function.

Conjugate synthesis

Anti-biotin–polylysine conjugates (aBAbpL) were prepared in an analogous fashion as previously described [5]. To a solution of 5 mg (31 nmol) of goat anti-biotin antibody (Pierce) in 1.5 ml of Hanks' balanced salt solution (HBS; 150 mM NaCl, 20 mM HEPES buffer pH 7.9), 18 µl of a 10 mM ethanolic solution of *S*-pyridyldithiopropionate (Pharmacia) was added with vigorous mixing. After reacting for 2 h, purification was performed by gel filtration (Sephadex G25 PD10, Pharmacia; HBS buffer pH 7.9) to give 1.8 ml of a solution containing 31 nmol antibody modified with 120 nmol dithiopyridine linker. This solution was concentrated to a volume of 0.8 ml and mixed under an argon atmosphere with a solution of 37 nmol poly(L)lysine. The poly(L)lysine had an average chain length of 290 lysine monomers ($pLys_{290}$), and was modified with 110 nmol 3-mercaptopropionate groups in 360 µl 500 mM NaCl, 20 mM HEPES buffer pH 7.3. The reaction mixture was kept for 48 h at room temperature. The mixture was diluted with 1.7 ml 0.5 M NaCl and conjugates were isolated by cation-exchange chromatography (Pharmacia MonoS 5 × 50 mm column; gradient solution 20–100% buffer B/buffer A/50 mM HEPES pH 7.9 and buffer B/buffer A plus 3 M NaCl). The product was eluted at a NaCl concentration of approximately 2 M. Pooled product fractions were dialyzed against HBS (2 × 500 ml). As checked by ultraviolet and ninhydrin assays, 6.4 ml of conjugates containing 2.54 mg (17 nmol) anti-biotin antibody, modified with 32 nmol polylysine $pLys_{290}$, were obtained.

Molecular conjugate gene transfer vectors

Molecular conjugate vectors containing lectin-targeting domains were constructed by immunolinking

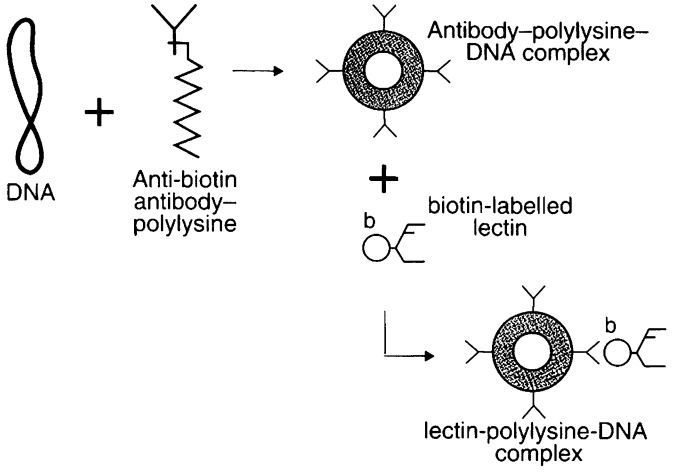

Figure 4 Construction of lectin-targeted molecular conjugate vectors. For derivation of conjugates, plasmid DNA was combined with the antibody–polylysine aBAbpL. This resulted in the formation of antibody–polylysine–DNA complexes. Addition of biotin-labeled lectins resulted in the formation of lectin–polylysine–DNA complexes.

biotinylated lectins with DNA complexed with polylysine (Figure 4). The targeting lectins were obtained as biotinylated derivatives: bConA, bWGA, bUEA, bMPA, bPNA, bDBA and bSBA, where ConA = concanavalin A, WGA = wheat germ agglutinin, UEA = *Ulex europaeus* agglutinin I, MPA = *Maclura ponifrea* lectin, PNA = peanut agglutinin, DBA = *Dolichos biflorus* agglutinin, SBA = soybean agglutinin (Vector Laboratories, Burlingame, CA, USA). The biotinylated lectins were reconstituted according to manufacturer recommendations in high performance liquid chromatography (HPLC) purified H_2O.

The monoclonal antibody achieved linkage to the targeting lectins by virtue of its specificity for biotin (Figure 4). To form complexes, 3.0 µg of plasmid DNA was added to 3.0 µg of aBAbpL in 175 µl HBS (150 mM NaCl, 5 mM HEPES pH 7.3), and incubated for 30 min at room temperature. Targetable lectin-containing vectors were then derived by varying the amount of biotinylated lectins added into the above mixture at molar ratios of lectin to antibody of 0.1:1.0 and the resultant complexes were incubated at room temperature in 250 µl of total volume for an additional 30 min. Subsequently, the complexes were added to cells in 1.25 ml of low serum, chloroquinated transfection media as described above.

The plasmid pCMVL was used as a reporter of gene transfer. In this plasmid, the reporter gene luciferase is under the control of the cytomegalovirus (CMV) early promoter/enhancer region [23]. Analysis of luciferase gene expression in transduced cells was accomplished by methods described elsewhere. Luciferase activity was detected following normalization of protein content in cell lysates 24 h after transfection. All transfection experiments were carried out in triplicate or quadruplicate.

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