Inhibition of Metastatic Tumor Growth in Nude Mice by Portal Vein Infusions of Matrix-targeted Retroviral Vectors Bearing a Cytocidal Cyclin G1 Construct

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Abstract

Tumor invasion and associated angiogenesis evoke a remodeling of extracellular matrix components. Retroviral vectors bearing auxiliary matrix-targeting motifs (i.e., collagen-binding polypeptides) accumulate at sites of newly exposed collagen, thus promoting tumor site-specific gene delivery. In this study, we assessed the antitumor effects of serial portal vein infusions of matrix-targeted vectors bearing a mutant cyclin G1 (dnG1) construct in a nude mouse model of liver metastasis. The size of tumor foci was dramatically reduced in dnG1 vector-treated mice compared with that in control vector- or PBS-treated animals (P = 0.0002). These findings represent a definitive advance in the development of targeted injectable vectors for metastatic cancer.

Introduction

Retroviral vectors are currently used in over 70% of human cancer gene therapy trials: more than 3000 patients have received a gene therapy vector in over 300 approved Phase I/II gene therapy protocols (1). Thus far, it appears that retroviral vectors are relatively safe and have minimal risk of insertional mutagenesis or oncogenesis (2). For cancer gene therapy, a built-in safety feature provided by retroviral vectors is the selective transduction of actively dividing cells (3), thus sparing normal nondividing cells. Retroviral transduction of target cells is initiated by viral binding to cell surface receptors (4, 5), followed by fusion of viral and cellular membranes (6) and core entry.

Cells, Cell Cycle Conditions, Plasmids and Vectors Bearing Marker, and Cell Cycle Control Genes. NIH3T3, 293T, and human pancreatic cancer MiaPaca2 cells were supplied by American Type Culture Collection. NIH3T3 and 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (D10; Biowhittaker). The pcgp plasmids containing the viral gag pol genes and a retroviral vector, pcnBg, expressing a nucleus-targeted β-galactosidase construct were kindly provided by Drs. Paula Cannon and Ling Li, respectively (University of Southern California Gene Therapy Laboratories, Los Angeles, CA). The plasmid containing VSVG env protein was kindly provided by Dr. Theodore Friedmann (University of California, San Diego, CA). A truncated (amino acids 41–249) cyclin G1 construct (dnG1) was cloned into the retroviral expression vector pRex.

Production of Matrix-targeted Retroviral Vectors Bearing Mutant Cyclin G1 Constructs. High-titer vectors were generated using a transient three- or four-plasmid cotransfection system (7) in which the packaging components gag-pol and a chimeric murine leukemia virus-based env bearing a vWF-derived collagen-binding (matrix-targeting) motif expressed from the cytomegalovirus promoter were placed on separate plasmids, each of which contained the SV40 origin of replication. The vectors expressed without WT env were named Bv1 or Hs2 (Bv, bovine vWF-derived; Hs, human vWF-derived; LF or 1, linkers derived from natural vWF sequences; LS or 2, standard linkers). To further increase viral titer, a fusogenic VSVG env protein (21) was coexpressed with Bv1 or Hs2 env proteins in a four-plasmid cotransfection protocol.

Viral titers in murine NIH3T3 cells were determined as described previously, based on expression of the β-galactosidase or neomycin phosphotransferase resistance (neo+) gene (22). Viral titer was expressed as the number of G418-resistant cfu/ml and ranged from 106–108 cfu/ml, depending on the nature and amount of plasmid DNA used in the transfection protocol.

In Vitro Efficacy Studies. To assess the cytocidal/cytostatic effects of the dnG1 vector, the transduced cells were evaluated for their proliferative potential by counting the number of viable cells in each culture at serial intervals (up to 4 days) after transduction without G418 selection. Western analysis of transduced NIH3T3 cells was performed using antibodies directed against the chromatin-binding protein H3 (Upstate Biotechnology Inc., Lake Placid, NY) and a rabbit polyclonal antibody against human cyclin G1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The cell viability was assessed by the MTT assay (Promega, Madison, WI).

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The abbreviations used are: ECM, extracellular matrix; VSVG, vesicular stomatitis virus G; WT, wild-type; vWF, von Willebrand factor; cfu, colony-forming unit(s); SA, surface area; CdK, cyclin-dependent kinase.
metastasis simulating the route of dissemination of human colon cancer was established in nude mice. Briefly, $7 \times 10^5$ tumor cells were infused slowly into the portal vein via an indwelling catheter that was kept in place for 14 days. Intracatheter infusions of either a low- or high-dose dnG1 vector (titers, $3 \times 10^6$ or $9 \times 10^8$ cfu/ml at 200 µl/day), a high-dose control vector bearing a $\beta$-galactosidase gene (titer, $8.5 \times 10^8$ cfu/ml), or PBS (pH 7.4) were begun 3 days later and continued daily for a total of 9 days. The mice were sacrificed by cervical dislocation 1 day after completion of treatment.

**Histological and Morphometric Analysis.** The liver lobes were excised, fixed in 10% formalin, labeled (A, right and caudate lobes; B, left lobe; C, median lobe), processed separately, and embedded in paraffin blocks. The antitumor efficacy of dnG1 vector treatment was assessed as follows:

- **Fig. 1.** A, transduction efficiency of matrix-targeted retroviral vectors in MiaPaca2 cells. $\beta$-galactosidase-expressing cells are shown with blue-stained nuclei. B, cytostatic effects of matrix-targeted retroviral vectors bearing mutant cyclin G1 constructs in MiaPaca cancer cells. The number of cells/well (plotted on the vertical axis) is expressed as a function of time (days after transduction), which is plotted on the horizontal axis. D10, control medium; Null, vector bearing only the neo' gene; AS 587 and AS 693, vectors bearing antisense cyclin G1 constructs; dnG1, dnG1 vector bearing a deletion in the NH₂ terminus of human cyclin G1. C, Western analysis of human cyclin G1 protein expression in dnG1 vector-transduced versus null vector-transduced cancer cells without G418 selection. Immunoreactive dnG1 (cyclin G1 DN41) is detected as a distinct band in the region of $M_r$ 20,000 (Lanes 3), whereas the endogenous cyclin G1 protein is seen as an intensely stained band in the region of $M_r$ ~30,000 (Lanes 1–4).

- **Fig. 2.** X-Gal immunostaining for detection of the $\beta$-galactosidase transgene in metastatic tumor nodules. A, H&E- and X-Gal-stained tissue section of liver from a $\beta$-galactosidase vector-treated mouse model of liver metastasis ($\times$40). B, X-Gal-stained tissue section of A counterstained with nuclear fast red stain ($\times$40). C, higher magnification of B ($\times$200). $\beta$-Galactosidase-expressing tumor cells (cells with blue-stained nuclei) near a disrupted hepatic venule (v) are indicated by arrows. D, H&E-stained tissue section of liver from a $\beta$-galactosidase vector-treated mouse model of liver metastasis ($\times$40) showing the tumor nodule undergoing active angiogenesis. E, X-Gal-stained liver sections counterstained with nuclear fast red stain ($\times$40). F, higher magnification of E ($\times$200). $\beta$-Galactosidase-expressing tumor stromal and endothelial cells are indicated by arrows.
H&E-stained tissue sections were examined by light microscopy; and
the SAs of representative liver sections and tumor foci from lobes A, B, and
C were measured by morphometric analysis using an Optimas image
analysis system. Evaluation of retroviral safety included assessment of the
integrity of the liver architecture and examination for the presence of
hepatocellular swelling or necrosis, inflammatory infiltrates, cholestasis,
and/or thrombosis. Tissue sections were also immunostained to assess the
presence of β-galactosidase transgene (20), cytokeratin, apoptosis, PAS,
vimentin, and CD68.

Statistical Analysis. For the in vivo efficacy study, four treatment groups
were compared: (a) low-dose Bv1/dnG1 (titer, 3 × 10^9 cfu/ml); (b) high-dose
Hs2/VSVG/dnG1 (titer, 9.5 × 10^9 cfu/ml); (c) high-dose control vector bear-
ing a β-galactosidase (nBg) gene (titer, 8.5 × 10^9 cfu/ml); and (d) PBS control.
Initially, 12 mice were studied; 4 were treated with a high-dose dnG1 vector,
4 were treated with a high-dose control vector, and 4 were treated with PBS.
Subsequently, four additional mice were treated with a low-dose dnG1 vector.
The response variables (total SA of the liver, total SA of the tumor, tumor
SA:liver SA ratio, and mean SA of tumor foci) were log-transformed before
formal analysis. A repeated measures analysis with lobe as the repeated
measures factor was used to determine whether or not the treatment had an
effect on each of the response variables. Pairwise comparisons were also
performed for the outcome variables with overall F test Ps of <0.05 between
groups.

Results and Discussion

In 1994, our laboratory cloned a human G-type cyclin (CYCG1),
which was markedly overexpressed in a subset of osteosarcoma cells
(23). Since then, the retroviral transfer of an antisense cyclin G1
construct has been repeatedly shown to induce down-regulation of
cyclin G1 expression and apoptosis of target cells, inhibit proliferation
of numerous cancer cells in vitro (22, 24, 25), and, subsequently,
arrest tumor xenograft growth in vivo (24, 25). Recently, a mutant
human cyclin G1 (dnG1) was created with a deletion in the cyclin box,
a conserved region among cyclins that helps determine cyclin-Cdk

Fig. 3. H&E-stained tissue sections of liver reveal the
tumor foci in the PBS-treated control group (A and C;
×40 and ×100) and the dnG1 vector-treated animals (B
and D; ×40 and ×100). Apoptosis in the tumor foci of
the PBS-treated control group (E; ×100) and the dnG1
vector-treated animals (F and H, ×100 and ×200; ar-
rows) is depicted as reddish-brown immunostaining in
an ApopTagPlus peroxidase in situ apoptotic assay. G,
negative staining control without the terminal de-
oxynucleotidyltransferase enzyme; t, tumor foci; H,
hepatocytes in liver parenchyma.

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association that induces Cdk activation.\(^4\) Initially shown to exhibit antiproliferative properties in vascular smooth muscle cells, dnG1 may act to inhibit the function of WT cyclin G1 or form inactive complexes with target Cdk molecules. Hence, the performance of a series of cytological mutant cyclin G1 constructs were tested \textit{in vitro} to determine the optimal construct for additional \textit{in vivo} studies.

A human undifferentiated cancer cell line of pancreatic origin was selected as the prototype of a metastatic gastrointestinal cancer. Retroviral transduction efficiency in these cancer cells was excellent, ranging from 26–85%, depending on the multiplicity of infection (multiplicity of infection = 4 and 250, respectively; Fig. 1A). For selection of an optimal therapeutic gene, cell proliferation studies were conducted in transduced cells using vectors bearing various cyclin G1 constructs. Fig. 1B shows the cytoidal/cytostatic effects of mutant and antisense cyclin G1 retroviral vectors in transduced cancer cells. Under standard conditions, the dnG1 vector consistently exhibited the greatest antiproliferative effect, concomitant with the appearance of immunoreactive cyclin G1 at the region of Mr 20,000, representing the dnG1 protein (Fig. 1C). The expression of the dnG1 protein in cancer cells results in unscheduled cell death; hence, the low-level expression of dnG1 protein in cell lysates from transduced unselected cell cultures. Based on the increased cytoidal activity of the dnG1 vector (Fig. 1B) in transduced MiaPaca2 cancer cells, the dnG1 vector was used in subsequent \textit{in vivo} efficacy studies.

Considering the overall mass of the liver, the physiological dilution factors involved, and the practical limitations of vector volume (~10% of blood volume), the efficiency of matrix-targeted gene delivery by portal vein infusions was remarkably high, ranging from 1–3% (20) to ≈50% in focal areas (Fig. 2). Under these conditions, repeated injections of a high-dose matrix-targeted retroviral vector bearing a β-galactosidase marker gene were required to detect an appreciable gene transfer in the tumor foci. As shown in Fig. 2, enhanced transduction of metastatic tumor cells (≈50%) was observed not only within established tumor nodules as described previously (20) but also at points of tumor exit from the circulation (Fig. 2, A–C). Moreover, significant transduction (≥50%) of tumor-associated stromal cells and endothelial cells was observed in some tumor foci undergoing active angiogenesis (Fig. 2, D–F). No evidence of transduction of neighboring hepatocytes was apparent, indicating the selective retroviral transduction of actively dividing tumor cells.

Histological and immunohistochemical evaluation of metastatic tumor foci from mice treated with either PBS or the low-dose dnG1 vector was performed and evaluated by morphometric analysis with an Optimas imaging system. Histological examination of liver sections from control animals revealed substantial tumor foci with attend areas of angiogenesis and stroma formation (Fig. 3, A and C); the epithelial components stained positive for cytokeratin, and the associated tumor stromal/endothelial cells stained positive for vimentin and FLK receptor (data not shown). This heterogeneous population of tumor cells, stromal cells, and endothelial cells constituted the tumor nodule. In contrast, the mean size of tumor foci in the low-dose dnG1-treated animals was significantly reduced compared with that in PBS controls (Fig. 3, B and D, arrows; P = 0.001), simultaneously revealing a focal increase in the density of apoptotic nuclei (Fig. 3, F and H, arrows) compared with that in the PBS-treated control group (Fig. 3E). Furthermore, infiltration by PAS+, CD68+, and hemosiderin-laden macrophages (Fig. 3D, arrow) was observed in the residual tumor foci of dnG1 vector-treated animals, suggesting active clearance of degenerating tumor cells and tumor debris by the hepatic reticuloendothelial system.

Morphometric analysis of tumor foci confirmed that the targeting strategy for therapeutic gene delivery was effective in that portal vein infusions (via indwelling catheter) of high-dose matrix-targeted dnG1 vectors induced dramatic reductions in the sizes of tumor foci when compared with the PBS- and control vector-treated animals based on all response variables (P < 0.0002; Tables 1 and 2). In pairwise comparisons for the three outcome variables, a dose-dependent tumor response to dnG1 vector treatment was apparent, and additional studies are currently underway to better determine tumor responsiveness to various vector doses in terms of tumor shrinkage versus complete

\(^4\) Liver SA, total surface area of liver section, mm\(^2\) (mean of lobes A, B, and C); Tumor SA, total surface area of tumor foci per lobe, mm\(^2\) (mean of lobes A, B, and C); Tumor SA: liver SA × 100, percentage of liver lobe infiltrated by tumor; Mean Tumor SA, mean surface area of each tumor foci in lobes A, B, and C, mm\(^2\).

\(^a\) CI, confidence interval.
disappearance of the tumor foci and to predict the minimum effective vector dose that could achieve the desired tumor response in Phase I/II gene therapy trials. Importantly, no evidence of hepatocellular damage, necrosis, thrombosis, or cholestasis was detected in tissue sections from dnG1 vector-treated animals, indicating that the matrix-targeted dnG1 vector (cumulative dose, $10^6$–$10^7$ cfu) may have a wide margin of safety. Whereas our previous studies showed that the level of retroviral transduction of tumor nodules in vivo was only 1–3% (20), the efficiency of gene delivery was dramatically enhanced by increasing the number of vector infusions (Fig. 2). A heterogeneous population of cells within the tumor nodule was transduced, with the greatest number of transduced tumor cells and tumor stromal and endothelial cells at sites of tumor invasion and in areas of active angiogenesis. An appealing concept is that tumor stromal and associated endothelial cells were transduced by the dnG1 vector simultaneously with the tumor cells, resulting in disproportionate tumoricidal effects caused by a decrease in vascular supply and/or growth factor stimuli. We have shown previously that blockade of cyclin G1 function using an antisense cyclin G1 (22) or a dnG1 vector (data not shown) induces apoptosis in ~35% of transduced cancer cell cultures. The simultaneous increase in the incidence of apoptotic nuclei in the tumor foci of dnG1 vector-treated mice indicates that dnG1 inhibits tumor growth by evoking apoptotic mechanisms.

In conclusion, we conducted in vivo efficacy and safety studies in a unique nude mouse model of liver metastasis and established the proofs of principle that (a) therapeutic gene delivery can be achieved by repeated portal vein infusions (via an indwelling catheter) of matrix-targeted retroviral vectors bearing a cytidial mutant cyclin G1 construct, as evidenced by statistically significant reductions in the sizes of tumor foci in dnG1 vector-treated mice compared with those of tumor foci in control animals, and (b) matrix-targeted dnG1 vectors may be systematically administered with a wide margin of safety, as indicated by the absence of associated hepatocyte necrosis, thrombosis, or cholestasis. Taken together, these findings represent a definitive advance toward the development of targeted injectable gene therapy vectors for metastatic cancer.

Acknowledgments

We thank Drs. Fan Xu and Liqiong Liu for technical assistance in cloning of the dnG1 vector and the retroviral vector targeting constructs.

Table 2 Pairwise comparisons of the outcome variables in Table 1*

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<th>Tumor size</th>
<th>Tumor:liver size ratio</th>
<th>Mean tumor size</th>
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<td>0.001</td>
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<td>0.012</td>
<td>0.220</td>
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</table>

* Pairwise comparisons were performed for the outcome variables with overall F test; Ps were <0.05 between groups.

References


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