p12CDK2-AP1 Gene Therapy Strategy Inhibits Tumor Growth in an In vivo Mouse Model of Head and Neck Cancer
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Abstract

Purpose: To test the potential of p12CDK2-AP1 (p12), a cell cycle regulator and cyclin-dependent kinase-2-associating protein commonly down-regulated in head and neck squamous cell carcinoma (~70%), as a gene therapy in inhibiting head and neck squamous cell carcinoma growth in vivo.

Experimental Design: We addressed the effect of p12 expression on tumor growth by using a well-established squamous cell carcinoma VII/SF floor of mouth xenograft mouse model. The effect of therapy on tumor growth was determined for: (a) no treatment, (b) PBS, (c) vehicle (1,2-dioleoyloxy-3-trimethylammonium propane:cholesterol liposomes / 5% dextrose), (d) empty vector controls, and (e) p12-encoding vector experimental groups.

Results: p12 gene therapy significantly induced antitumor effects as compared with controls, including (a) size and weight of p12-treated tumors decreased by 51% to 72% compared with all controls (P < 0.02), (b) tumor growth rate post-therapy was inhibited by 55% to 64% compared with empty vector controls (P < 0.0001), and (c) p12 expression was higher in p12-treated than controls (P < 0.002) by two-tailed t test analyses. Mechanistically, p12 treatment affected cell turnover kinetics as assessed by apoptotic and cell proliferation indices. p12 therapy significantly increased terminal nucleotidyl transferase-mediated nick end labeling (P < 0.05) and morphology-based apoptotic indices (P < 0.05) as well as significantly decreased Ki-67 cell proliferation indices (P < 0.001) compared with controls, resulting in a net cell turnover reduction in p12-treated tumors.

Conclusions: We show that this novel therapeutic modality can significantly induce antitumor responses in vivo. These results support a role for p12 as a novel tumor growth suppressor gene therapy and suggest that optimization and/or combination with current therapies may hold considerable promise in preparation for clinical trials.

Head and neck cancer affects approximately 38,000 new patients in the U.S. yearly, more than half a million patients worldwide, and despite improvements in surgery, chemotherapy, and radiation therapy, the overall survival has not improved in the past 30 years (1). For all stages combined, about 84% of patients diagnosed with head and neck cancer survive 1 year after diagnosis and the 5- and 10-year relative survival rates are 57% and 45%, respectively (1). The local-regional biology of head and neck squamous cell carcinomas (HNSCC) and the persistent poor treatment outcomes make this disease an ideal target for gene therapy, including tumor suppressor gene delivery (2). Recently, combinations of novel gene-based therapies with current therapeutic modalities have been explored, including tumor suppressor replacement gene therapy, suicide gene therapy, immunotherapy, and oncogene suppression (3, 4). Recently, p53 tumor suppressor replacement has been combined with chemotherapy and radiation therapy as a successful treatment for head and neck and other cancers in preclinical and clinical settings (4–6) and other tumor suppressor therapies such as p16INK4A (p16) or retinoblastoma are currently in clinical development for head and neck after promising results from preclinical studies (7–9). Therefore, gene-based approaches are being examined for HNSCC treatment and of particular promise are combinations of current treatments with novel cell cycle–regulatory gene therapies.

Abnormalities in various components of the cell cycle–regulatory machinery have been associated with tumorigenesis. Molecular alterations frequently associated with head and neck cancer include cyclin D1 amplification or overexpression (30-50%), decreased expression of tumor suppressor genes p53 (50%) and retinoblastoma (50%), decreased expression of cyclin-dependent kinase (CDK) inhibitors p27KIP1 (36%), p21WAF1/CIP1/CAP (46%), and p16 (69-80%) in SCC (10, 11). More recently, decreased expression of a novel CDK2 inhibitor, p12CDK2-AP1 (p12) has been associated with HNSCC (12, 13).
p12 is a growth suppressor gene identified and cloned from the Syrian hamster oral cancer model. The human p12 cDNA has been mapped to chromosome 12q24 and partially characterized (14); the full-length human p12 cDNA is 1.6 kb in length, encoding 115 amino acids (M, 12,400). The rodent p12 protein has 97% identity with that of humans (14). p12 is also implicated in S phase–associated growth suppression, through binding with DNA polymerase α/primase and/or CDK2 (15, 16). Additionally, we have shown that the clinical behavior of p12 differs in normal and tumor oral mucosa, with reduced expression in 14.3% of oral dysplasias, and in 64% to 72% of HNSCC cases examined (12, 13). In combination, these findings have suggested that p12 loss or reduced expression might contribute to the multistep nature of oral carcinogenesis, and that its loss may be an event associated with tumor progression.

Several in vitro studies have suggested a role for p12 in regulating cell growth. Ectopic expression of p12 into 7,12-dimethylbenz[a]anthracene-induced hamster oral carcinoma HCPC-1 cells has been associated with growth suppression and a significant antiproliferative effect. Cells reverted to a normal cellular morphology, lost the ability to grow in soft agar, and displayed an increase in cell doubling time (17), consistent with a potential role for p12 as a tumor suppressor gene in oral keratinocytes. Suppression of cell growth in a similar manner was also observed when malignant human keratinocytes were transfected with p12 (16). Additionally, p12 transfection into HCPC-1 cells significantly increased the fraction of apoptotic cells in p12-transfected as compared with null vector or untransfected controls (18). Another study examining p12 expression levels in the hamster oral carcinogenesis model in vivo revealed that apoptotic cell indices were significantly reduced in malignant epithelium, perhaps reflecting the abrogation of critical pathway(s) governing cell death. Also, cell proliferation (proliferating cell nuclear antigen labeling index) was found to increase progressively through hamster oral malignant progression, whereas p12 expression levels progressively decreased through malignant progression (19).

Finally, recent data using murine p12-targeted ES knockout clones (p12−/−) showed that in the absence of p12 expression, cellular proliferation is increased, with an increase in S phase and a decrease in G2-M phase populations, and apoptosis is significantly reduced with cisplatin treatment (20). In combination, these in vitro data support the role of p12 in regulating tumor cell phenotype and growth; however, specific examination of p12 antitumor effects had not been evaluated previously in vivo. Given the encouraging preclinical data using the tumor suppressors p16, p53, and retinoblastoma in gene therapy of head and neck cancer (4–9, 21), we hypothesized that p12 gene therapy would induce antitumor activity in vivo.

In our present study, we assessed whether local expression of p12CDK2-AP1 altered tumor growth in a controlled model of HNSCC in vivo.

Materials and Methods

Plasmids and DNA: liposome complexes for gene delivery. The p12 gene construct contained the murine p12 cDNA (0.9 kb) sequence expression cassette under transcriptional control by the human cytomegalovirus major immediate early promoter/enhancer and the polyadenylation signal of the bovine growth hormone gene [pIRES-enhanced green fluorescent protein (EGFP), Clontech, Palo Alto, CA]. The p12 cDNA was cloned into the EcoRI and BamHI sites at the MCS, resulting in a 6.1 kb vector. The empty vector (control) was 5.2 kb in length and lacked the p12 sequence. The plasmids were purified by using the Endofree Mega Kit (Qiagen, Valencia, CA), and formulated in the In vivo GeneSHUTTLE (Q-BioGene, Carlsbad, CA) cationic lipid formulation containing 1:1, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) with cholesterol as a colipid to optimize plasmid delivery (22–24). DNA:liposome complexes were prepared according to the manufacturer’s instructions in 5% dextrose. The final formulation was delivered using a precision Hamilton syringe (Hamilton, Reno, NV) and sterile 26-gauge needles.

Animal model of head and neck carcinogenesis. The animal model used was a previously described syngeneic murine model for squamous cell carcinoma of the head and neck (25–27). The care and use of the animals was in accordance with the guidelines of the Animal Research Committee of the University of California at Los Angeles. To establish a tumor model of the floor of the mouth, 6- to 8-week-old C3H/HeJ female mice had 5 × 104 squamous cell carcinoma VII/SF (SCC-VII/SF) cells injected into the floor of the mouth through the neck skin using sterile technique at day 0. At day 5, the tumors were surgically exposed and measured in three dimensions. Because lack of efficacy has been reported for this murine model if a large tumor burden is treated with liposome-based gene therapy formulations (26), we did all treatments on tumors at day 5 post-transplantation. On day 5, small tumors ranging from 30 to 100 mm3 were surgically exposed and directly injected with therapies. These experimental groups therefore received: (a) no treatment (no therapy), (b) PBS (to control for injection/mechanical trauma to the tumors), (c) vehicle (liposomes in 5% dextrose, to control for the potential effect of liposomes and/or dextrose alone), (d) empty vector (to control for the potential effects of vector backbone), or (e) p12 vector (therapeutic gene tested). Therapies were administered on day 5 (intratumorally, via surgical exposure) and day 8 (intratumorally, via percutaneous route) post-transplantation. On days 8 and 10, tumors were measured in three dimensions. On day 10 (end point) local lymph nodes, liver, lung, small intestine, and tumor samples were saved or fixed in 10% buffered formaldehyde for morphologic observations. This experiment was done in triplicate and the final groups consisted between 7 and 12 mice per group.

Tumor growth kinetics calculations. Measurements of tumor volume were done on day 10 using vernier calipers in three dimensions, obtaining tumor length (L), height (H), and width (W) and used to calculate tumor volume in cubic millimeters (L × H × W; ref. 28). Tumor growth change values (Δtumor growth) were calculated from the forminula: ΔVF/VF (C %) = (VFt/VFC × 100 (29), where ΔVF and ΔC are changes in p12-treated or control tumor volumes from days 5 to 10. ΔC was determined from the average growth change of empty vector control tumors. ΔVF/C% values were then averaged for each p12-treated group. In the case of reduction of tumor volume, ΔVF/C values were calculated according to ΔVF/VF (C %) = (VFt/VF) × 100 (29). Tumor growth inhibition rates were obtained by deducting the tumor growth change from 100% (maximum growth). Tumor doubling time calculations were determined using the equation VT = V0 × et where t is the number of days between VT and V0 measurements (5 days). According to this formula, tumor doubling time (in days) equals 0.693/ k, where k = ln(VT) / Vt ref. 28. The cell turnover was calculated by obtaining the ratio of the TUNEL apoptotic labeling index to the Ki-67 proliferation labeling index for each tumor sample. Then, the relative cell turnover (cell turnover as a percentage of control or T/C % ) values were calculated from T/C% = T/C × 100, where T is each individual p12-treated cell turnover ratio, and C is the average cell turnover ratio for the empty vector group. Relative cell turnover values (as a percentage of control) were then averaged. The cell turn-over inhibition rate was obtained by deducting the relative cell turnover averages from 100% (maximum cell turnover). For all measures above, the averages (± SE) were obtained for each treatment group and analyzed for statistical significance using a two-tailed Student’s t test.

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analyses of p12\textsuperscript{CDK2-AP1} and marker gene expression in tumor explants.

Western blots were done using tumor tissue (60-100 mg) stored at -80\degree C. Tissues were homogenized to a total protein lysate using a Polytron probe (Brinkmann Instruments, Westbury, NY) in 2 to 3 mL lysis buffer [150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate, 2% SDS, and 50 mmol/L Tris-HCl (pH 8.0)] with protease inhibitors (50 mmol/L sodium fluoride, 200 \mu mol/L sodium orthovanadate, 2 \mu g/mL of aprotinin, 100 \mu g/mL of phenylmethylsulfonyl fluoride, and 1 \mu mol/L of a lactacystin \gamma-lactone) and boiled for 5 minutes in Laemmli-loading buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 0.2 mol/L DTT, 20% glycerol, and 0.003% bromophenol blue]. Protein lysates were centrifuged at 15,000 x g for 25 minutes at 4\degree C, and protein concentration in the resulting supernatant measured using the Bio-Rad DC Kit (Bio-Rad, Hercules, CA). Ten micromgs of protein from each sample were subjected to electrophoresis in a SDS-polyacrylamide gel (15% resolving gel and 5% stacking gel) and transferred to a polyvinylidene difluoride membrane (Bio-Rad), using ice-cold CAPS buffer (10 mmol/L cyclohexylaminopropanesulfonic acid, 10% methanol (pH 11.0)). The membrane was incubated with a polyclonal mouse anti-rabbit pAb3 anti-p12 antibody (12) at a dilution of 1:3,000 or polyclonal anti-rabbit anti-actin (Sigma, St. Louis, MO) antibody at a dilution of 1:5,000 overnight at 4\degree C. Membrane was washed 3 x 15 minutes in TBS-T and horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ) was used as the secondary antibody at 1:3,000 for 1 hour at room temperature. Signals were visualized using the enhanced chemiluminescence (ECL+ Plus) nonradioactive method according to the manufacturer's instructions (Amersham, Pharmacia Biotech). To test for transfecion efficiency by the liposome formulation in vivo, we examined the expression of the EGFP marker gene in tumor tissue by fluorescent microscopy in combination with 4,6-diamidino-2-phenylindole counterstaining for nuclei visualization. Controls were tumor tissue – untransfected with EGFP, images were merged using a NIH Image J 3.0 program and analyzed for percentage of EGFP+/ total cells.

Apoptosis analyses. To detect early DNA fragmentation associated with apoptosis, the ApopTag peroxidase in situ apoptosis detection kit and protocols were used (Chemicon International, Temecula, CA). Briefly, sections were deparaffinized and pretreated with 0.2 \mu g/mL proteinase K for 15 minutes at room temperature. Tissues were then treated with 3.0% hydrogen peroxide in PBS, and equilibration buffer was applied. The terminal deoxynucleotidyl transferase (TdT) enzyme was then used to add digoxigenin-labeled nucleotides to the 3’ hydroxide ends of fragmented DNA. Digoxigenin in DNA was detected by peroxidase-labeled antidigoxigenin antibody and developed in diaminobenzidine. The specimen was counterstained with hematoxylin (DAKO Cytomation, Carpinteria, CA) for 30 seconds, washed, and mounted with Permount (Fisher, Pittsburgh, PA). For control sections, TdT enzyme was omitted and no positive staining was observed. Positively stained cells were counted in 5 to 10 random high-magnification (400x) microscope fields and the number of positive cells expressed for at least 1,000 cells counted was termed the TUNEL apoptotic index. As an additional measure, we also determined the H&E morphology apoptotic bodies index. Morphologic features of apoptotic cells included characteristic features such as condensation of chromatin, condensation of cytoplasm, cell shrinkage, and cytoplasm and nucleus fragmentation (30). The significance of difference between treatment groups was determined by two-tailed Student’s t test. We also determined linear correlation between the TUNEL labeling index and apoptotic index using the Pearson’s correlation coefficient (r) analysis. Significance of correlation was determined as described (31).

Cell proliferation analyses. We examined the Ki-67 labeling index in treated and nontreated tumor tissue sections. Ki-67 is a nuclear antigen expressed in proliferating cells in late G\textsubscript{1}, S, G\textsubscript{2}, and M phases but is absent in resting cells (G\textsubscript{0}; ref. 32). We used the rabbit anti-human Ki-67 polyclonal antibody (Novocastra, Norwell, MA) for the demonstration of the Ki-67 antigen using a 1:2,000 dilution in antibody diluent (DAKO Cytomation). Antigen retrieval was done with slides immersed in 1 L of 10 mmol/L citrate buffer (pH 6.0) in a microwave pressure cooker with heating conditions of 15 minutes at 1,000 W, 15 minutes at 500 W, cooling at room temperature for 15 minutes, then two 5-minute washes in 1x TBS (pH 7.6). Immunostaining was done using the LSAB2 kit (DAKO Cytomation) according to the manufacturer’s instructions. Peroxidase activity was visualized by applying the diaminobenzidine chromogen, containing 0.05% hydrogen peroxidase. The sections were then counterstained with hematoxylin (DAKO Cytomation) for 30 seconds according to the manufacturer’s instructions, dehydrated in graded alcohols, cleared in xylenes, and mounted with Permount (Fisher). Negative control staining was done by substituting nonimmune serum for primary antibodies. To determine labeling indices, at least five high-powered fields (400x magnification) for each tumor section were analyzed and the fraction of labeled nuclei for at least 1,000 total nuclei were determined for each sample. The significance of difference between treatment groups was determined by the two-tailed Student’s t test.

Results

Increased expression of p12\textsuperscript{CDK2-AP1} in transfected tumors in vivo. SCC-VII cells were derived from a spontaneous murine tumor of squamous cell origin from a C3H mouse strain (25, 33) and have been found to express reduced levels of p12 (Fig. 1A). As expected, every animal that received SCC-VII cells developed floor of mouth tumors with previously described biological HNSCC characteristics, including rapid tumor growth, and adjacent skin invasion (25). Western blot analyses of p12 expression showed gene delivery efficiency in this mouse model.

![p12 expression in vivo](image-url)
An extruded DOTAP and cholesterol (DOTAP:cholesterol) lipid formulation was used to deliver the empty or p12-containing vectors intratumorally. This liposome formulation has been shown to enhance gene delivery in vivo compared with naked DNA or other nonviral formulations (22–24, 34). Tumors treated with p12CDK2-AP1 gene therapy showed overexpression of p12 protein compared with control groups (Fig. 1B) and this increase was statistically significant (P = 0.004, two-tailed t test) according to densitometry analyses. We also analyzed tumor tissue for expression of the marker gene EGFP by fluorescence microscopy and observed a range of 9% to 25% transfected cells. In average, tumors transfected with empty vector pIRES-EGFP showed an average of 16.4 ± 3.1%, whereas those transfected with p12-EGFP showed an average of 16.9 ± 3.6% transfected cells.

p12CDK2-AP1 induced a reduction in tumor size, weight, and growth rate. In the absence of any treatment, SCC-VII tumors develop and range from 30 to 100 mm³ in volume within 5 days of transplantation to the floor of mouth. This was in concordance with previously reported tumor dimensions for this xenograft model (25–27). Treatment of tumors with PBS, vehicle, or empty vector formulations did not affect tumor growth significantly as compared with the “no treatment” group (P > 0.05), whereas treatment with p12 gene therapy at all three doses, 6.25, 12.5, and 25 μg, significantly delayed growth compared as determined by volume and weight measurements (P < 0.02; Fig. 2). Significant inhibition of tumor growth was shown 5 days following p12 therapy, whereas no inhibition was observed in tumors injected with control formulations, which suggests that the effects of therapy on growth are p12-specific. Control-treated tumors had volumes (mean ± SE) of 1,332 ± 268 (no treatment), 989 ± 93 (PBS), 906 ± 127 (vehicle), and 784 ± 114 mm³ (empty vector) and were significantly larger than p12-treated tumors.

![Fig. 2. Change in tumor volume after therapy.](image-url)
The tumor volume in mm$^3$ (mean ± SE) and the average tumor doubling time (±SD) was calculated as described in Materials and Methods.

The $P$ value was calculated from comparing p12-treated with empty vector control tumors. The size of empty vector-treated tumors was not significantly different from no-treatment ($P = 0.07$), PBS ($P = 0.13$), or vehicle ($P = 0.38$) controls. The two-tailed $t$ test was used for all comparisons. $P \leq 0.05$ considered significant.

Table 1. Change in tumor growth and kinetics post-therapy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$n$</th>
<th>Dose ($\mu$g DNA)</th>
<th>Tumor volume, mm$^3$* ($P$)</th>
<th>Tumor doubling time, days*($P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>9</td>
<td>—</td>
<td>1.332 ± 268</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>PBS</td>
<td>7</td>
<td>—</td>
<td>989 ± 93</td>
<td>0.40 ± 0.09</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7</td>
<td>—</td>
<td>906 ± 127</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>Empty vector</td>
<td>9</td>
<td>12.5</td>
<td>784 ± 114</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>p12 Vector</td>
<td>8</td>
<td>6.25</td>
<td>338 ± 62 (0.007)$^*$</td>
<td>1.37 ± 0.49 (0.02)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>12.5</td>
<td>378 ± 53 (0.004)</td>
<td>1.06 ± 0.30 (0.03)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25</td>
<td>386 ± 83 (0.015)</td>
<td>1.41 ± 0.54 (0.046)</td>
</tr>
</tbody>
</table>

*The tumor volume in mm$^3$ (mean ± SE) and the average tumor doubling time (±SD) was calculated as described in Materials and Methods.

The $P$ value was calculated from comparing p12-treated with empty vector control tumors. The size of empty vector-treated tumors was not significantly different from no-treatment ($P = 0.07$), PBS ($P = 0.13$), or vehicle ($P = 0.38$) controls. The two-tailed $t$ test was used for all comparisons. $P \leq 0.05$ considered significant.

Table 2. Growth inhibition in p12-treated SCC tumors

<table>
<thead>
<tr>
<th>Treatment dose ($\mu$g)</th>
<th>$\Delta$Tumor growth, $\Delta T / C%$* ($P$)</th>
<th>Tumor growth inhibition rate (%)$^*$</th>
<th>Relative cell turnover, $T / C%$* ($P$)</th>
<th>Turnover inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>36.5 ± 11.6$^*$ (3.8E-05)</td>
<td>63.5</td>
<td>18.0 ± 2.6$^*$ (2.4E-02)</td>
<td>82</td>
</tr>
<tr>
<td>12.5</td>
<td>39.6 ± 9.8$^*$ (2.5E-06)</td>
<td>60.4</td>
<td>13.6 ± 1.5$^*$ (1.8E-02)</td>
<td>86.4</td>
</tr>
<tr>
<td>25</td>
<td>44.1 ± 13.9$^*$ (7.2E-06)</td>
<td>55.9</td>
<td>14.0 ± 2.7$^*$ (1.9E-02)</td>
<td>86</td>
</tr>
</tbody>
</table>

*The change in tumor growth (tumor growth) over time post-treatment was calculated as described in Materials and Methods.

* Tumor growth inhibition rate was determined as described in Materials and Methods.

* The cell turnover was calculated as described in Materials and Methods.

* Tumor turnover inhibition rate was obtained by deducting the relative cell turnover from 100% (maximum cell turnover).

* The change in tumor growth (tumor growth) over time post-treatment was calculated as described in Materials and Methods.

* The cell turnover was calculated as described in Materials and Methods.

* Tumor turnover inhibition rate was obtained by deducting the relative cell turnover from 100% (maximum cell turnover).

* Tumor growth inhibition rate was determined as described in Materials and Methods.

* The tumor volume in mm$^3$ (mean ± SE) and the average tumor doubling time (±SD) was calculated as described in Materials and Methods.
effective antitumor agent for the treatment of HNSCC in vivo. We next proceeded to examine the mechanism of p12-induced tumor reduction. As p12 is a growth suppressor and inducer of apoptosis in culture, we examined the effect of gene therapy on the apoptotic, cellular proliferation, and cell turnover indices in the transplanted tumors.

**p12 gene therapy induced increased apoptosis in vivo.** Having observed a marked reduction in tumor size and growth rate, we examined apoptotic indices in treated and control tumor tissues to determine whether apoptosis is induced and/or enhanced by p12 treatment. We examined both the morphologic features of apoptosis and the presence of apoptosis-related molecular markers by TUNEL analyses. p12 therapy showed significant increases in the TUNEL (Fig. 3A) index or apoptotic index using H&E staining (Fig. 3B) when compared with control treatments. Following immunohistochemical analysis, the number of cells staining positive for apoptosis were counted in 5 to 10 randomly selected fields at 400× magnification, and data were graphed (Fig. 3C). Controls had TUNEL labeling indices (mean ± SE%) of 3.5 ± 1.3% (no treatment), 2.9 ± 0.9% (PBS), 3.6 ± 1.9% (vehicle), and 3.7 ± 0.5% (empty vector). The TUNEL labeling index was not significantly different between control groups (P > 0.5), whereas p12-treated tumors displayed significantly increased indices compared with all controls (P < 0.05; Fig. 3C). More specifically, p12-treated tumors also displayed significantly increased apoptotic labeling indices as compared with empty vector controls, of 10.2 ± 1.6% (p12 6.25 μg; P = 0.001), 9.0 ± 1.4% (p12 12.5 μg; P = 0.00004), and 10.0 ± 1.3% (p12 25 μg; P = 0.0003). Finally, the apoptotic indices from the p12 treatment doses were not significantly different (P > 0.8) from one another.

We also assessed the apoptotic indices using morphologic criteria of apoptotic cell death (30) on adjacent serial H&E stained tissue sections for each tumor sample. Cells exhibiting apoptotic morphology were counted as described above, and data were graphed (Fig. 3B). Controls had apoptotic indices (mean ± SE%) of 3.8 ± 1.6% (no treatment), 3.3 ± 0.8% (PBS), 3.3 ± 1.5% (vehicle), and 3.6 ± 0.6% (empty vector).
The apoptotic index was not significantly different between control groups \((P > 0.5)\), whereas p12-treated tumors displayed significantly increased indices compared with all controls \((P < 0.05\); Fig. 3B\). More specifically, p12-treated tumors displayed significantly increased apoptotic indices as compared with empty vector controls, of 11.2 \(+\) 1.6\% \((p12 6.25 \mu g; P = 0.002)\), 8.3 \(+\) 1.3\% \((p12 12.5 \mu g; P = 0.00008)\), and 10.3 \(+\) 1.1\% \((p12 25 \mu g; P = 0.0004)\). Finally, the p12 treatment doses were not significantly different from one another \((P > 0.3)\). Therefore, all three doses are equally effective in significantly increasing either apoptotic TUNEL labeling indices or apoptotic indices in p12-treated tumors as compared with controls.

Moreover, we compared both measures of apoptotic cell death for each control or p12 treatment group and found no significant differences between these methods \((P > 0.5)\). We observed that all treatment groups showed a strong correlation between the two apoptotic measures. The Pearson’s correlation coefficient \(r\) values were: no treatment \((0.68)\), PBS \((0.8)\), vehicle \((0.8)\), empty vector \((0.86)\); p12: 6.25 \(\mu\)g \((0.8)\), 12.5 \(\mu\)g \((0.7)\), and 25 \(\mu\)g \((0.6)\). Although all groups exhibited strong correlations as a trend, the no treatment group correlation was not significant \((P = 0.1)\), whereas the PBS and vehicle controls were borderline significant \((P = 0.05)\). Strong and significant correlations between TUNEL labeling index and apoptotic index were observed for the empty vector \((P < 0.01)\) and p12 treated groups \((P < 0.03)\). Therefore, p12 therapy overall showed statistically significant increases in apoptosis compared with control indices using two independent measures of apoptosis. We suggest that one important mechanism for the effectiveness of p12 gene therapy on reducing tumor growth may be the induction of apoptotic cell death in tumor tissue and associated decrease in cell turnover. These results suggest that p12 inhibits tumor growth through stimulation of proapoptotic pathways in vivo.

p12 gene therapy induced a decrease on cell proliferation Ki-67 labeling indices in vivo. We examined cell proliferation indices in treated and control tumor tissues to determine whether cell proliferation is induced and/or enhanced by p12 treatment. Having observed a marked reduction in tumor size and growth rate, we assayed for the presence of the Ki-67 molecular marker, and following immunohistochemical analysis, the number of positive cells in all treatment groups was quantified at \(400\times\) magnification. This proliferation marker is expressed in the G2-M phase of the cell cycle and is commonly expressed in tumors and associated with progression of tumor or poor prognosis \((36, 37)\). p12 therapy caused a decrease in the fraction of cells expressing the proliferation marker Ki-67 (Fig. 4). Controls had Ki-67 labeling indices of 55.7 \(+\) 4.8\% \((no\ treatment)\), 55.2 \(+\) 5.1\% \((PBS)\), 50 \(+\) 14.1\% \((vehicle)\), and 61.8 \(+\) 3.6\% \((empty\ vector)\). The Ki-67 labeling index of p12-treated tumors was significantly different than all controls \((P < 0.001)\). The empty vector control index was not significantly different from all other controls as determined by a two-tailed \(t\) test \((P > 0.3)\). p12-treated tumors displayed significantly reduced Ki-67 labeling index as compared with empty vector controls, of 37.8 \(+\) 2.2\% \((p12 6.25 \mu g; P = 4E-05)\), 29.3 \(+\) 4.8\% \((p12 12.5 \mu g; P = 2E-05)\), and 28.8 \(+\) 3.9\% \((p12 25 \mu g; P = 5E-06)\), which suggests that p12 inhibits cell proliferation. The p12 treatment doses were not significantly different from one another \((P > 0.2)\), except for the lower 6.25 \(\mu\)g dose, which was significantly different from the highest 25 \(\mu\)g dose \((P = 0.02)\). Therefore, we suggest that a potential mechanism for the observed effectiveness of p12 gene therapy on reducing tumor growth is due to the suppression of cell proliferation in tumor tissue.

p12 treatment reduced cell turnover in vivo. Cell turnover indices (Ki-67 proliferation index/TUNEL apoptotic index) for control-treated tumor groups were high, reflecting a rapid growth rate and were on average: 27.4 \(+\) 11.7 \((no\ treatment)\), 29 \(+\) 9.2 \((PBS)\), 36.3 \(+\) 20.2 \((vehicle)\), 24.7 \(+\) 8.5 \((empty\ vector)\), whereas for p12-treated groups they were reduced to 4.4 \(+\) 0.7 \((6.25 \mu g)\), 3.0 \(+\) 0.5 \((12.5 \mu g)\), and 3.5 \(+\) 0.7 \((25 \mu g)\). The p12-treated cell turnover indices were then corrected for the empty control values, and on average were found to be significantly lower than the empty vector control cell turnover \((~15\%; P < 0.05; Table 2)\). Therefore, p12 treatment was found to reduce/inhibit tumor cell turnover rate by \(~85\%). This reduction in tumor cell turnover (proliferation/apoptosis ratio) may be an important mechanism accounting for p12’s effects as an antitumor agent in vivo.

Discussion

Despite significant progress in cancer therapy, the survival rate has not improved over the last four to five decades and as a result, a trend is evolving whereby existing therapies such as traditional chemotheraphy and radiation treatment are being combined with novel gene-based therapies. Locoregionally recurrent HNSCC is a logical target for direct delivery of gene therapy approaches and as such, therapy using tumor suppressor genes in neoplastic tissue already has been approached using p16, p53, or retinoblastoma, in preclinical and/or clinical studies \((4–7, 9, 21)\), with promising results. The present study focused on testing the hypothesis that the putative tumor suppressor and cell cycle regulator p12 could also act as an inhibitor of tumor cell growth in vivo. p12 has been shown to induce cell cycle arrest, growth suppression, repression of DNA replication, and apoptosis in vitro \((18, 20, 38, 39)\), and also to regulate cell growth by association with CDK2, DNA polymerase α/primase, and p14ARF \((15, 16, 40)\). The present study supports previous in vitro observations and suggests that delivery of a p12 murine gene suppresses tumor cell growth in vivo, with specific increases in TUNEL labeling and apoptotic indices and decreases in Ki-67 cell proliferation labeling indices as compared with controls, resulting in an antitumor effect in a manner that is consistent with a role as a tumor growth suppressor in vivo.

The development of a p12-based therapeutic modality to regulate tumor growth could be a promising new component of HNCS cancer treatment. Our findings are highly relevant in this regard because they emerge from a fully characterized in vivo model in which the evolution of tumors is very aggressive and difficult to halt. Previous investigations using the SCC-VII head and neck cancer model to examine the efficacy of liposome gene therapy have included several immunotherapy studies, where administration of DNA coding for either interleukin (IL)-2 or IL-12 reduced the size of treated tumors compared with empty vector controls by ~40% to 65% \((26, 35, 41)\). Another study involving gene therapy using adenoviral vectors resulted in a growth reduction of ~40% of IL-2-treated or thymidine kinase–treated...
tumors, and of ~5% in granulocyte-macrophage colony-stimulating factor–treated tumors (42). Other studies using SCC-VII implantation into the flanks of mice have included treatments with either endostatin or epidermal growth factor receptor antisense gene therapy, and each treatment reduced tumor sizes by ~40% to 50%, with complete inhibition of growth in combination (43), highlighting the importance of combining gene therapies with other modalities for enhanced treatment efficacy.

Tumor suppressor gene therapy is still in its infancy for HNSCC treatment. However, several reports of p53 gene therapy in preclinical studies employed adenoviral vectors or liposome-based therapies in human head and neck tumors established in nu/nu or severe combined immunodeficiency mice, successfully causing a reduction in tumor size of ~50% to 80% compared with controls (6, 44–46). Modified retinoblastoma gene therapy (retinoblastoma-94, a more efficient truncated form of retinoblastoma lacking the NH2-terminal 112 amino acids) has also been tested using adenoviral vectors in human head and neck tumors established in nu/nu mice, with a reduction in growth of ~30% to 50% in retinoblastoma-treated compared with control tumors (9). Finally, gene therapy with the tumor suppressor p16 using adenoviral vector delivery in human HNSCC xenografts has resulted in a ~36% to 60% reduction in size and a ~57% reduction in growth rate in p16-treated compared with mock infection control tumors derived from an oral tumor cell line, whereas treatment of tumors derived from a larynx tumor cell line resulted in a ~40% to 74% reduction in tumor size compared with mock infection controls (8). In summary, effective tumor size or growth rate reductions following gene therapy of head and neck in vivo typically range from ~30% to 80%, and therefore our results with p12 gene therapy (a ~37-44% reduction in growth rate and a ~51-75% decrease in tumor size in p12-treated) are comparable with most monotherapies using HNSCC mouse models. Decreases in tumor weight (~43-66%), tumor doubling time (~20-40%), and cell turnover (~20-30%) were also observed. In combination, these observations suggest that this molecule can be considered a promising candidate for combination therapy in further preclinical studies.

The SCC-VII tumor model has several advantages for the testing of novel therapies because these tumors have a very rapid growth rate and malignant potential, and even though this model is derived from malignant murine epidermal keratinocytes, it has been well-characterized and widely used as an immunocompetent mouse model that mimics human HNSCC development (26–28, 43). These tumors harbor alterations in several growth-regulatory pathways that may account for their uncontrolled growth phenotype and many of these alterations are identified in human HNSCC. For example, these tumors overexpress epidermal growth factor receptor (47), cyclin D1 (48), extracellular matrix proteins, insulin-like growth factor binding protein 3, apolipoprotein E, α, and β globins, platelet-derived growth factor-R, fibronectin (49), retain low levels of wild-type p53 (47, 50), and express high p21 levels (50), the latter suggesting a potential p53 pathway defect. Therefore, we believe that this model is relevant for proof-of-principle testing of novel gene therapies, although future studies will include testing of p12 gene
therapies in human HNSCC xenograft models. Finally, we have shown here that SCC-VII tumors retain p12 expression, although at lower levels, which raises an interesting hypothesis that perhaps p12 could be used in combination with chemopreventive agents to prevent tumor cell conversion and progression of preneoplastic lesions, prior to complete loss of p12 expression, such as in this SCC-VII model. Moreover, these tumors are highly resistant to radiation and chemotherapy (paclitaxel; refs. 47, 50, 51), and thus are an excellent model to study whether p12 can sensitize cells in order to achieve better efficacy, especially in tumors that are resistant to radiation or chemotherapy, and those studies are under way.

The mechanism(s) by which p12 may induce apoptosis could potentially involve p53-independent pathways, because SCC-VII have low levels of p53 (50), and of Bcl-2 and Bax proteins (51). These tumors are able to up-regulate p53 on radiation treatment, but fail to undergo apoptosis (50). In contrast, in vitro studies showed that p12 up-regulated pro-apoptosis elements following transfection of microsatellite-unstable colorectal cancer cell lines. Increased p12 expression was associated with significantly increased expression of caspase cascade genes, and bcl2/bax pathway genes (39). We are currently examining in detail other pathways affected by p12 expression. p12 overexpression up-regulates various cell cycle and cell growth regulators, down-regulates expression of cell cycle inhibitors, and most interesting up-regulates expression of cell death mediators. Although the biological significance of p12-induced apoptosis remains to be characterized further, the correlation between p12 expression and enhanced apoptosis in vitro shown here supports our previous in vitro observations. For instance, treatment with control formulations (PBS, vehicle, or empty vector) did not result in significantly enhanced apoptosis as compared with that of p12-containing vector therapy. These results thus strongly suggest that p12-induced effects are likely gene-specific and suggests that a cellular apoptotic pathway may be activated by p12 expression. This effect may be an interesting component in future in vivo and in vitro studies, where the biological significance and mediators of p12-induced apoptosis can be better understood. Our results in effectively inducing increased apoptotic indices in vivo with p12 therapy are comparable with those reported with other treatments using the same tumor model. Significant increases in in vivo TUNEL-labeling indices were reported for murine IL-2 (~7%) or cisplatin (~12%) monotherapies, whereas the no treatment group remained at ~4% (35). Similar trends were reported for IL-2 or radiation monotherapies using this tumor model (27). Therefore, although SCC-VII tumors have a relatively high basal apoptotic index, they are highly resistant to radiation- and chemotherapy-induced apoptosis and remain relevant models with which to test the effectiveness of therapies in vivo.

The significant antitumor effect seen with p12 gene therapy supports further investigation and development of this therapeutic strategy. Future studies should include dose variation and combination of p12 gene therapy with radiation therapy and/or chemotherapy, as well as testing of nonviral formulations previously employed in head and neck cancer clinical trials (DC-cholesterol; refs. 52, 53). Liposome formulation methods of gene delivery typically are efficient in transfecting only ~10% of the cells within a tumor. To address these limitations, we used the DOTAP-cholesterol formulation, which has been suggested to effectively transfect a higher percentage of cells (~25%) in human lung tumor xenografts following intratumoral administration in vivo (23, 24). We observed a smaller percentage of cells transfected with this formulation (~16.5%), which may reflect cell/tissue-specific transfection efficiency variations. Therefore, it still would be important to employ viral vectors in future studies in order to confirm the effectiveness of p12 on tumor growth observed in this study. Future studies will include i.v. administration of p12 gene therapy to test effectiveness in inhibiting tumor growth in preparation for translational applications, as the intratumoral administration route usually limits studies to being translated to phase I/II clinical trials. Regardless of the formulation or administration route chosen, one could still use p12 therapy as a means to negatively regulate growth, slowing tumor cell proliferation while at the same time increasing apoptosis to sensitize cells to the effects of radiation or chemotherapy, resulting in a potentially synergistic treatment modality in future preclinical studies.

In summary, this orthotopic model of HNSCC resembles the human disease very closely and this makes it a valuable model for the testing of novel therapies. The fact that p12 nonviral gene therapy resulted in an antitumor effect suggests that a more general application of p12-based gene therapy in other SCC mouse models (skin, esophageal, or cervical) may be warranted. Taken together, the data we present here strongly suggest that p12 inhibits cell turnover and tumor growth in vivo and as such, may be a potent therapeutic agent suitable for further development in cancer gene therapy, including future clinical testing. Although tumor regression was only observed in ~9.5% animals receiving standard doses of p12 therapy, the induction of tumor “dormancy” is considered to be a clinically useful outcome that might increase disease-free survival and survival time. Regarding the potential of this novel gene therapy for human application, the safety of nonviral gene therapy (DC-cholesterol) has been shown in phase I and II human trials for head and neck cancer (6, 52). For the treatment of cancer, gene therapy may or may not be curative but it could add to the armament of surgery, radiation therapy, and chemotherapy in prolonging and improving the quality of life for patients. Whereas additional studies using p12 therapy will allow optimization and assessment of survival, this novel strategy warrants consideration in the future for human clinical trials for head and neck cancer. Additional studies are under way to elucidate the molecular specifics of p12 activity and to test our hypothesis for its efficacy in a combination therapeutic modality for head and neck cancers.

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