Advances in Brief

Prevention of Orthotopic Human Lung Cancer Growth by Intratracheal Instillation of a Retroviral Antisense K-ras Construct

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Abstract

An orthotopic human lung cancer model in nu/nu mice was used to study the effect of an antisense K-ras (AS-K-ras) retroviral construct on tumor growth in vivo. A 2-kilobase genomic AS-K-ras DNA fragment linked to a ß-actin promoter was cloned into the LNSX retroviral vector. The recombinant construct was packaged into GP+envAm12 cells and titers greater than 10^6 colony-forming units/ml were obtained. Irradiated (350 cGy) nu/nu mice were first inoculated intratracheally with 10^3 H460a human large cell lung carcinoma cells which have a codon 61 mutation of the K-ras oncogene. Three days later they received intratracheal instillation of viral supernatant (5 x 10^6 colony-forming units/ml) from either LNSX, LNSX-AS-K-ras, LNSX-sense-K-ras producer cells, or medium daily for 3 days. At autopsy, 30 days after tumor cell inoculation, 90% of the control mice had tumors whereas 87% of mice treated with the LNSX-AS-K-ras viral supernatant were free of tumors. The efficacy of the viral supernatant was dose dependent. Intratracheal administration of retroviral LNSX-AS-K-ras supernatant prevents the growth of human lung cancer cells implanted orthotopically in nu/nu mice.

Introduction

The identification of specific genetic alterations in premalignant and malignant cells offers an opportunity to develop therapies and prevention strategies based on the correction of these lesions. Such strategies could involve elimination of activated oncogene products or the replacement of nonfunctioning with functioning wild-type tumor suppressor genes. Since the reversal of a single genetic lesion can have significant effects on tumor cell growth and tumorigenicity this approach is potentially useful even in cancer cells with multiple genetic lesions (1-3). A major obstacle to direct correction of genetic lesions in cancer cells, however, is the difficulty of efficiently delivering genetic constructs to the cells. Retroviruses have been extensively studied as delivery vehicles in gene transfer protocols (4-7). Because gene constructs transduced by retroviruses are integrated preferentially in dividing cells, this technique gives proliferating cancer cells a selective advantage for expressing the gene construct (8).

Retroviruses and cells modified by retroviral transduction have little acute toxicity, making multiple treatments with high-tier preparations feasible (9, 10). We report the development of an antisense K-ras retroviral expression vector and delivery technique that successfully prevents growth of orthotopic human lung cancer in a nu/nu mouse model. We previously showed that introduction of an antisense K-ras fragment was able to specifically inhibit expression of mutant K-ras protein in H460a human non-small cell lung cancer cells, which resulted in a reduced rate of cell proliferation and tumorigenicity in nu/nu mice (1). Subsequently, the AS-K-ras fragment has been incorporated into the retroviral expression vector LNSX to evaluate the potential of the vector as a delivery system for the AS-K-ras construct to human cancer cells in vivo. We found that i.t. instillation of retroviral supernatant could prevent the growth of H460a human lung cancer cells in the lungs of nu/nu mice.

Materials and Methods

Retroviral Vector Construction. A 2-kilobase K-ras genomic DNA fragment carrying exons 2 and 3 together with flanking intron sequences was subcloned into an Apr-1-neom' plasmid with a ß-actin promoter driving synthesis of antisense RNA. The promoter-K-ras segment was further subcloned into a retroviral vector (LNSX). Two constructs were obtained. In one, the ß-actin promoter was linked toward the 5' terminal repeat and positioned at 5'-3' orientation. In the other construct, the promoter was linked to the 3'-long terminal repeat and positioned at 3'-5' orientation. In both constructs, the ß-actin promoter drives antisense K-ras, but we observed optimal AS-K-ras expression in the latter orientation. A control construct was made with K-ras transcribed in sense orientation (S-K-ras).

Virus Production. Amphotropic packaging cell line GP+envAm12 was transfected with recombinant LNSX-AS-K-ras plasmid DNA by the calcium phosphate coprecipitation method (11, 12). Forty-eight hours later, the transfected cells were placed in medium containing G418 (400 µg/ml). Ten to 14 days later G418 resistant colonies were expanded into large cultures. The viral titer was tested by transducing NIH-3T3 cells (13). High-titer GP+envAm12 cells transfected by LNSX-AS-K-ras were cocultured with ecotropic packaging cell line Q12 to further increase the viral titer. Medium from NIH-3T3 cells transduced with the recombinant retroviruses was used to infect fresh NIH-3T3 cells to detect the presence of replication-competent retrovirus. The secondarily infected NIH-3T3 cells were either selected with 400 µg/ml G418 for 10-14 days or passaged continuously for 1 month prior to G418 selection to detect the presence of rare recombinants. No wild-type virus was detected. Integration and expression of the LNSX-AS-K-ras construct in H460a cells have been described.

Orthotopic Human Lung Cancer Model. H460a cells (10^3/mouse) were injected i.t. in irradiated (350 cGy) female nu/nu mice (14). On days 4, 5, and 6, mice were given i.t. injections of 0.1 ml of viral supernate mixed with 5 µg/ml of protamine. Thirty days after tumor cell inoculation mediastinal blocks were harvested from the mice and assessed for tumor growth. Tumors were measured with linear calipers in two orthogonal directions by the same observer without knowledge of the treatment groups. Tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Three micron histological sections (2/block) were made from the heart-lung blocks of mice treated with LNSX-AS-K-ras or medium control, stained with hematoxylin and eosin, and analyzed for the presence of H460a cells.

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Polymerase Chain Reaction and Southern Analysis for neo'. Total genomic DNA was isolated from normal and tumor samples (lung, trachea, spleen, liver, and ovary) after 3 washes with phosphate-buffered saline. One μg of DNA was used for amplification of the target gene by PCR. A 900-base pair neomycin phosphotransferase (neo') gene segment was amplified using 5' and 3' neo' primers (5'-CAAGATGGATTTCAAGCCAGG; 3'-GGGAATCCAGAAGAACTCCTG) and Moloney murine leukemia virus reverse transcriptase. A 395-base pair segment of the cDNA product was amplified by PCR using the primers described in Methods. "The mean volume is calculated only for the tumors detected. "The mean volume is calculated only for the tumors detected. The growth of a few small tumors in the group receiving LNSX-S-K-ras presented the opportunity to measure the expression of AS-K-ras and S-K-ras at the RNA level in tumor cells growing in vivo. Preliminary studies showed that both the sense and antisense sense K-ras fragments were expressed as unspliced mRNA. Thus, K-ras intron 3-specific sense or antisense primers which would recognize only the provirus-derived mRNA for K-ras and not the endogenous spliced K-ras mRNA could be used to reverse transcribe retroviral mRNA. Following reverse transcription, the resulting cDNA was amplified by PCR using primed intron 3 and exon 3 K-ras primers. Expression of the antisense and sense K-ras constructs was shown by RNA-PCR in tumors from mice treated with the respective constructs (Fig. 2B). The expression of the sense construct was greater than that of the antisense construct in these tumors. The amount of unspliced AS mRNA detected by this assay should be representative of the mRNA produced by the AS construct. The splice sites for intron 3 are not functional in the antisense sequence, and therefore, spliced transcripts cannot be produced. The synthesis of unspliced mRNA transcripts of the sense K-ras fragment is not surprising. About 50% of the transcripts produced by unmodified Moloney murine leukemia virus are unspliced and this percentage may increase with modification of the virus genome (15). Tumors treated with LNSX-S-K-ras and LNSX-S-K-ras which were removed 30 days later continued to express both the antisense and sense K-ras mRNA constructs, respectively. It appears that even in the absence of selective pressure the constructs can express the appropriate mRNA and mediate a biological effect. LNSX-S-K-ras transduced H460a cells selected with G418 normally produce high levels of AS K-ras transcripts as detected by Northern analysis. 4 It may be that in those small tumors that developed in the LNSX-S-K-ras-treated mice, reduction of expression of the antisense construct occurred because of the site of proviral integration. It is of course impossible to measure the level of AS-K-ras expression in H460a cells that did not form tumors. We hypothesize that high levels of antisense expression as reported in vitro inhibit tumorigenicity. 4 The efficacy of the LNSX-S-K-ras supernatant in preventing the growth of human lung cancer cells was somewhat surprising in view of the previous reports of low efficiency of retroviral gene transfer in vivo. Several factors probably contributed to this efficacy. The retroviral supernatants had high titers and, therefore, multiple local inoculations in the region of the tumor were sufficient to transduce a high percentage of tumor cells. Infection of H460a cells with fresh supernatant for 5 consecutive days in vitro will result in a 95% transduction efficiency. 5 The supernatants, therefore, when given multiple times, could theoretically transduce cells in unsynchronized populations by exposing cells to the virus as they traverse the cell cycle. This concept
is supported by the lack of integration of the retrovirus by nondividing normal cells, which suggests that retroviruses may have an inherent advantage in targeting rapidly dividing tumor cell populations and that toxic effects to normal cells may be avoided. Administration of the supernatant had no apparent acute toxic effects on the mice. In another experiment, i.p. injection of 1 ml of the supernatant had no visible toxic effects on mice observed for up to 8 months (data not shown). Antitumor effects were seen even at relatively low multiplicities of infection as shown in the dose titration experiment.

Integration of the retrovirus was not detected in normal tissues by DNA-PCR analysis. The limit of sensitivity of this assay in our laboratory is detection of 1 transduced cell in a mixture of 10^5 non-transduced cells. Furthermore, no histological changes were seen in the normal cells of mice receiving the retroviral supernatants. Previous studies have shown that integration of AS-K-ras into cells without a K-ras mutation does not affect the viability or growth rate of the cells in culture (1). Thus, even if integration of the K-ras gene had occurred at low levels, it is doubtful that acute toxicity would occur. This may be due to redundancy within the ras gene family. Oncogenes are frequently derived from multigene families, the gene products of which may have redundant function. Additional support for this concept comes from a recent study by Soriano et al. (16) in which transgenic mice were created that lacked a functional c-src protooncopogene. The resulting developmental defect in the mice was osteopetrosis. The ubiquity of c-src, its high degree of conservation among species, and its role in mitosis suggest that inactivation would be lethal, but this was not the case; viable mice were recovered. A possible explanation is that other closely related nonreceptor tyrosine kinases such as yes and lyn can compensate for loss of c-src. Our antisense construct has been shown to specifically inhibit K-ras while preserving expression of p21 proteins produced by other members of the ras family (1). The presence of H-ras and N-ras p21 proteins may be sufficient to preserve function despite the absence of the K-ras p21. This of course does not eliminate the possibility that the construct may cause long-term alterations in cell growth patterns not detectable in the relatively short time frame of our study.

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**Table 2** Titration experiments with retroviral supernatant in nu/nu mice

<table>
<thead>
<tr>
<th>Experiment (virus: cell)</th>
<th>Treatment</th>
<th>No. mice with tumor/total (%)</th>
<th>Mean volume ± SEM (mm^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1.7:1)</td>
<td>LNSX vector only</td>
<td>4/5 (80)</td>
<td>49.05 ± 21.60</td>
</tr>
<tr>
<td></td>
<td>S-K-ras-LNSX</td>
<td>5/5 (100)</td>
<td>56.75 ± 19.36</td>
</tr>
<tr>
<td></td>
<td>AS-K-ras-LNSX</td>
<td>3/5 (60)</td>
<td>17.39 ± 5.53</td>
</tr>
<tr>
<td>B (3:3:1)</td>
<td>LNSX vector only</td>
<td>5/5 (100)</td>
<td>43.35 ± 7.05</td>
</tr>
<tr>
<td></td>
<td>S-K-ras-LNSX</td>
<td>5/5 (100)</td>
<td>44.30 ± 4.80</td>
</tr>
<tr>
<td></td>
<td>AS-K-ras-LNSX</td>
<td>2/5 (40)</td>
<td>21.70 ± 6.00</td>
</tr>
<tr>
<td>C (5:1)</td>
<td>LNSX vector only</td>
<td>5/5 (100)</td>
<td>47.04 ± 5.37</td>
</tr>
<tr>
<td></td>
<td>S-K-ras-LNSX</td>
<td>5/5 (100)</td>
<td>41.20 ± 8.91</td>
</tr>
<tr>
<td></td>
<td>AS-K-ras-LNSX</td>
<td>1/5 (20)</td>
<td>7.63 ± 2.00</td>
</tr>
</tbody>
</table>

*The mean volume is calculated only for the tumors detected.

"p < 0.05 by two-way analysis of variance when compared to each of the control groups.

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**Fig. 1.** Representative histological sections and mediastinal blocks of treated and control mice. A, histological section from the mediastinal block of a control mouse stained with hematoxylin and eosin. A peribronchial H460a tumor is present. B, histological section from the mediastinal block of a mouse treated with LNSX-AS-K-ras. No tumor cells are visible. In C, the mediastinal blocks (left) are from mice treated with medium alone; arrows, tumors. Right, mediastinal blocks from mice treated with the antisense K-ras supernatant that show no gross evidence of tumor.
To the best of our knowledge, these studies constitute the first reported use of retroviral supernatants to mediate antitumor effects and the first successful use of an antisense construct to mediate therapeutic tumor regression in vivo. This study has interesting implications for the prevention and therapy of lung cancer. Local tumor recurrence is still a major problem following surgery and primary radiation therapy for lung cancer. The i.t. administration of gene constructs to target residual cancer cells may be useful as adjuvant therapy. Oncogene and tumor suppressor gene mutations have been identified in premalignant lesions of the upper aerodigestive and gastrointestinal tracts (17–20). Correction of these lesions by i.l. administration of viral expression vectors should be considered as an investigative strategy for the prevention of invasive cancers.

References


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