Suppression of the Neoplastic Phenotype in Vivo by an Anti-ras Ribozyme

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Abstract

In this study, the efficacy of an anti-ras ribozyme in reversing the neoplastic phenotype was investigated. Murine NIH3T3 cells were transfected with cellular DNA from the FEMX-I human melanoma cell line expressing the activated H-ras gene. The transformed cells displayed the neoplastic phenotype in vitro and were tumorigenic in nude mice in vivo. When the transformants were transfected by a ribozyme designed to cleave only activated H-ras RNA, the transformed phenotype was abrogated. In contrast, expression of a mutant ribozyme, essentially acting only as antisense, into the transformed cells resulted in less dramatic changes in cell growth and tumorigenicity. These results reinforce the potential role of anti-oncogene ribozymes as suppressors of neoplastic growth, with possible implications for gene therapy.

Introduction

In recent years, a central duality has emerged to define the molecular basis of cancer. In the acquisition of the neoplastic phenotype, alterations in action of oncogenes, as well as loss of function of tumor suppressor genes have been postulated. Based on this dichotomy, strategies used to reverse the tumorigenic phenotype have included suppressing activated oncogenes or restoring normal suppressor gene function. Hammerhead ribozymes, or site-specific RNases, are a newly described agent for manipulation of gene expression (1, 2). Anti-oncogene ribozymes have been demonstrated previously to effectively suppress targeted gene expression, resulting in profound effects on malignancy of a human bladder carcinoma cell line in vitro, as well as reversing drug resistance in vitro (3, 4).

In this study, we used the FEMX-I human melanoma cell line to investigate the efficacy of anti-ras ribozymes in suppressing the neoplastic phenotype induced by FEMX-I cell DNA in 3T3 cells. Activated H-ras genes have been thought to play a central role in tumorigenesis in several model systems (5, 6). The FEMX-I cell line was shown to express a mutated H-ras gene in codon 12, in which the normally glycine-encoding GGC sequence is converted to GUC, encoding valine (7). This mutation has been shown to activate the p21 ras gene in other cell lines (5, 6). Fortuitously, the FEMX-I gene contains a target sequence for hammerhead ribozymes, which cleave 3' to XUX-C sequences (1). We demonstrate that transfection of the FEMX-I DNA renders 3T3 cells tumorigenic and expressed the activated H-ras gene of FMEX-1 cells (7). The anti-ras ribozyme and the mutant ribozyme, prepared from two synthetic single-stranded oligodeoxyribonucleotides, contained a 77-base pair sequence with flanking Sall (BRL) and HindIII (BRL) restriction sites in the multiple cloning site (4).

Transfection Studies. Subconfluent growing 3T3 cells were transfected by electroporation according to a protocol provided by IBI (New Haven, CT). Individual G418-resistant clones were screened for expression of the H-ras ribozyme by the semiquantitated polymerase chain reaction assay as previously described (4).

Northern Blot Analysis. RNA isolation using the guanidinium isothiocyanate method, electrophoresis on horizontal agarose gels, hybridization, and densitometric analysis (Ambis, San Diego, CA) were performed as described previously (3). The complementary DNA sequences were isolated as described previously (4). The probes (25ng) were labeled with [α-32P]dCTP (New England Nuclear), 109 cpm/txg, by using a random primers DNA labeling system (BRL) (8).

Materials and Methods

Genes and Oligonucleotides. The human c-H-ras1 (pT24-C3, No. 41000) and the human c-K-ras (No. 41026) were obtained from the American Type Culture Collection (Rockville, MD) as probes. Synthetic nucleotides and the sequences for the primers used in this study were described previously (4).

Cells. The human metastatic melanoma cell line FEMX-1 which has a H-ras codon 12 mutation has been described previously (7, 8). The NIH3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in Dulbecco’s modified essential medium (Gibco, Grand Island, NY) and supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and penicillin-streptomycin (Gibco). NIH3T3 cells were plated at 104 and 105 cells/35-mm dish in 0.3% agar and 1 to 20% fetal bovine serum. Colonies were counted 12 days later with Giemsa stain as described previously (4). Thymidine uptake studies were used to determine the rate of [3H]thymidine incorporation in acid-soluble material. NIH3T3 cells (2.5 × 106 cells/35-mm dish) were grown for 48 h and then incubated for 2 h with [3H]thymidine (106 dpm/dish; New England Nuclear), washed, acid precipitated, and counted as previously described (4).

Plasmid Construction. The plasmid pPHβ was obtained from Dr. L. Kedes (University of Southern California, Los Angeles, CA) (4). The H-ras ribozyme and the mutant ribozyme, prepared from two synthetic single-stranded oligodeoxyribonucleotides, contained a 77-base pair sequence with flanking Sall (BRL) and HindIII (BRL) restriction sites in the multiple cloning site (4).

Results and Discussion

DNA from FEMX-1 cells was transfected into murine NIH3T3 cells. DNA from the first generation of NIH3T3 transformants (3FI) was then transfected into nascent 3T3 cells, and the resulting transformants were designated 3FI cells, containing much less human melanoma DNA than the 3FI cells. However, the 3FI cells still possessed and expressed the activated H-ras gene of FMEX-1 cells (7). The resultant 3FI cells demonstrated transformed properties in vitro, including altered morphology (Fig. 1), as well as enhanced thymidine incorporation into DNA and colony formation in soft agar when compared to parental 3T3 cells transfected with the pHβ expression

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2 To whom requests for reprints should be addressed.

3 The abbreviation used is pHβ, pHBAP-1 neo.

vector alone [NIH3T3 pHβ cells (Table 1)]. In vivo, control 3T3 cells produced no tumors in nude mice with an inoculum of 5 x 10⁶ cells (Table 2). In contrast, 3FI cells yielded tumors in 100% of inoculated mice, regardless of route of administration (s.c. versus i.v.). When 3FI cells were injected i.v., tumors developed in the lung and resulted in accelerated mortality, with average survival of 30 days. Interestingly, 3FI cells exhibited significantly elevated expression of H-ras RNA versus control 3T3 cells, thereby more closely resembling FEMX-I cells (data not shown).

Table 1 Growth characteristics of 3T3 and 3FI cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Ribozyme expression*</th>
<th>[%]Thymidine incorporation (%)b</th>
<th>Colonies 1/5%20% serumc</th>
<th>(10⁴ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 3T3 pHβ</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>0/0</td>
</tr>
<tr>
<td>2. 3FI</td>
<td>-</td>
<td>-</td>
<td>226</td>
<td>12/143</td>
</tr>
<tr>
<td>3. 3FIrib 1</td>
<td>++</td>
<td>+</td>
<td>107</td>
<td>0/42</td>
</tr>
<tr>
<td>4. 3FIrib 5</td>
<td>+</td>
<td>+</td>
<td>165</td>
<td>10/108</td>
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<td>5. 3FIrib 1</td>
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<td>+</td>
<td>175</td>
<td>11/121</td>
</tr>
<tr>
<td>6. 3FIrib 4</td>
<td>+++ +</td>
<td>+</td>
<td>125</td>
<td>5/98</td>
</tr>
<tr>
<td>7. 3FIrib 8</td>
<td>+++ +</td>
<td>+</td>
<td>185</td>
<td>12/121</td>
</tr>
</tbody>
</table>

* Ribozyme expression was quantitated by Ambis and 3FIrib 4 was compared to the other clones.

b To determine the rate of [%]Thymidine incorporation in acid-soluble material, cells were grown for 48 h and then incubated for 2 h with [%]Thymidine, washed, acid-precipitated, and counted. The NIH3T3 cell control (100%) represented 1.5 ± 0.25 (SD) fmol/mg DNA/hr. These results were the mean of three separate experiments.

c The clones were grown in 0.3% agar with 1 or 20% fetal bovine serum (10⁴ or 10⁵ cells/35-mm² dish). Colonies were counted 12 days later with Giemsa stain. These growth studies represent the mean of three separate experiments.

Fig. 1. Morphology of (1) NIH3T3 pHβ cells, (2) NIH3T3 cells transfected with FMEX-I DNA (3FI), (3) 3FIrib clone 1 cells, (4) 3FIrib clone 4 cells (top). The cell lines were assayed for expression of the 118-base ras ribozyme using the polymerase chain reaction (PCR) assay. Northern blots were performed as described to demonstrate expression of the H-ras and K-ras genes. Quantitation of the Northern blots was performed by Radiologic Imaging Systems, Ambis, San Diego, CA. The vector only cells (Lane 2) were used to normalize the data. The values for gene expression are for the following lanes (Lanes 1–5, respectively): ribozyme RNA (XX, X, 1.0, and 1.8); H-ras RNA (1.0, 11.1, 2.1, and 4.9); K-ras RNA (1.0, 0.9, 0.9, and 1.1).

Table 2 Tumorigenicity of 3T3 and 3FI cells

<table>
<thead>
<tr>
<th>Cells (×10⁴)</th>
<th>Tumor take</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v. injections</td>
<td></td>
</tr>
<tr>
<td>3T3</td>
<td>50</td>
</tr>
<tr>
<td>3FI</td>
<td>2.5</td>
</tr>
<tr>
<td>3FIrib (clone 4)</td>
<td>5</td>
</tr>
<tr>
<td>3FIrib (clone 4)</td>
<td>2.5</td>
</tr>
<tr>
<td>3FIrib (clone 1)</td>
<td>2.5</td>
</tr>
<tr>
<td>s.c. injection</td>
<td></td>
</tr>
<tr>
<td>3T3</td>
<td>50</td>
</tr>
<tr>
<td>3T3</td>
<td>10</td>
</tr>
<tr>
<td>3FI</td>
<td>10</td>
</tr>
<tr>
<td>3FIrib</td>
<td>5</td>
</tr>
<tr>
<td>3FIrib (clone 4)</td>
<td>5</td>
</tr>
<tr>
<td>3FIrib (clone 1)</td>
<td>5</td>
</tr>
</tbody>
</table>

(4) was cloned into the pHβ expression vector and transfected into 3FI cells. As antisense control parallel transfections were conducted with a mutant ribozyme with a base alteration in the catalytic core, possessing minimal cleavage capacity. Expression of the ribozyme in transformants was semiquantitated by polymerase chain reaction analysis (Fig. 1). The ability of the transfected H-ras ribozyme to cleave its target in the cells is supported by previous studies detecting the products of the cleavage reaction by polymerase chain reaction analysis (4). Moreover, we have also demonstrated that the cellular H-ras ribozyme cleaves its target RNA in vitro (4). Interestingly, even though less ribozyme was present than its antisense counterpart, 3FIrib cells exhibited greater reductions in H-ras gene expression when compared to 3FIrib cells. As a control, there was no change in...
expression of K-ras in the cell lines examined (Fig. 1). In addition, the 3FIIrhib cells continued to express the activated H-ras gene.  

In vitro, 3FIIrhib cells expressing the ras ribozyme reverted to the 3T3 phenotype, with a flattened morphology and a more sluggish growth pattern as evidenced by reduced DNA synthesis and colony formation in soft agar (Table 1). The 3FIIrhib cells exhibited intermediate growth characteristics between 3FII cells and 3FIIrhib growth pattern as evidenced by reduced DNA synthesis and colony formation in soft agar (Table 1). Fifteen different ribozyme clones were analyzed for their growth characteristics and the 3FIIrhib clone 1 was used with the highest expression of ribozyme RNA for the mouse studies. In vitro, there was a direct correlation between the amount of ribozyme RNA expression and reversal of the malignant phenotype.

Expression of the ras ribozyme completely abrogated the tumorigenicity of 3FII cells in nude mice, because no tumors developed when 2.5 × 10^5 3FIIrhib cells were injected into nude mice (Table 2). Moreover, this complete suppression of tumorigenicity was achieved whether the mice were inoculated s.c. or i.v. In contrast, 3FIIrhib cells produced tumors in five of five mice s.c. and in two of five i.v. (Table 2). In these experiments, only 2.5 × 10^5 3FII or 3FIIrhib cells clone 4 were injected i.v. into mice because the animals died directly upon injection if a higher number of cells was used.

Genetically mediated suppression of the neoplastic phenotype has been previously demonstrated using expression of tumor suppressor genes (11-13), triplex DNA (14) or antisense RNA or deoxyribonucleotides (15-21) targeted against oncogenes. In cases where antisense was used, only incomplete or transient reversion of tumorigenicity has been achieved. In this study, we demonstrate that expression of a 48-base anti-oncogene ribozyme suppressed the neoplastic phenotype of 3T3 cells transformed with DNA from a melanoma cell line. Moreover, this phenotype was stable as the ribozyme-containing cells were propagated for at least 12 months prior to injection into nude mice. This reversal of tumorigenicity was shown using both s.c. and i.v. modes of administration, which has not been previously demonstrated with either antisense RNA or tumor suppressor genes. Moreover, the ribozyme exhibited greater efficacy than antisense both in vivo and in vitro in reversing 3FII cell tumorigenicity. This increased efficacy was demonstrated by a one-base alteration in the ribozyme yielding a mutant with minimal demonstrable cleavage capacity.

The results presented here parallel the studies demonstrating the ability of tumor suppressor genes to inhibit neoplastic growth (11-13). Taken together with ribozyme-mediated reversal of malignancy (4), these results reinforce anti-oncogene ribozymes as a novel class of tumor suppressor agents. Theoretically, the use of ribozymes may yield certain benefits in gene therapy protocols (22). The ribozyme does not encode a protein and operates at the RNA level, whereas successful expression of a wild-type tumor suppressor protein is required for effective antitumor action. Moreover, the size of a ribozyme (less than 50 bases) may allow it to integrate into the recipient cell genome with less complexity than an entire gene. Additionally, ribozymes targeted against activated H-ras genes may act as tumor-selective agents, especially since they have been shown to leave parental NIH3T3 cell growth unperturbed (23).  

These studies warrant further investigation of anti-oncogene ribozymes as tumor suppressor agents.

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References

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\textit{ras} Ribozyme


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