Antisense DNA Inhibition of Tumor Growth Induced by c-Ha-ras Oncogene in Nude Mice

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ABSTRACT

Antisense DNA has shown an ability to target specific oncogene transcripts and inhibit their expression in cells, but the degree to which sustained treatment can suppress total levels of an oncogenic product and alter tumorigenesis in vivo remains to be determined. In this study, NIH-3T3 cells transformed by the activated c-Ha-ras oncogene from T24 human bladder cancer cells were treated for 3 consecutive days in vitro with an antisense DNA pentadecamer complementary to a target in the 5'-flanking region of the c-Ha-ras RNA transcript. Following antisense DNA treatment, a portion of the cells was lysed for measurement of RAS p21 while the remaining cells were evaluated for tumorigenicity by injection s.c. into athymic nude mice at a dose of 5 × 10⁶ cells/mouse. The 3 days of treatment with the anti-c-Ha-ras DNA reduced RAS p21 cellular levels by more than 90% while a nonspecific control DNA reduced p21 cellular levels by approximately 20%. Tumor growth of cells treated with anti-c-Ha-ras DNA was significantly reduced for up to 14 days following the end of treatment and implantation into the mice whereas the nonspecific control DNA had no significant effect. These effects on tumor growth were evident in two different strains of nude mice and in both males and females. It is suggested that the pronounced decrease in RAS p21 levels produced by anti-c-Ha-ras DNA resulted in a reversal of the transformed phenotype, and it is this reversal which accounts for the prolonged inhibition of tumorigenesis following antisense DNA treatment.

INTRODUCTION

Over the last decade, the existence of oncogenes has become a central concept in our understanding of the molecular biology of cancer, and more recently, oncogenes and their products have begun to play an important role in clinical diagnosis and prognosis (1–4). Oncogenes are also obvious targets for therapeutic drugs, but as yet, they have had almost no role in cancer therapeutics. All oncogenes and most of their protein products operate inside the cell, and they are therefore not accessible to protein-based drugs the specificity of which derives from antibody-ligand-related recognition.

However, antisense DNA is a class of compounds which is able to enter all cells relatively easily and has the potential specificity to target individual oncogenes (5, 6). Antisense DNA oligomers are short (10–50-base) sequences of modified or unmodified deoxynucleotides complementary to a site on the RNA transcript of a target gene. Hybridization of the antisense DNA and the target RNA by complementary base pairing provides high specificity and binding affinity, which is in many ways superior to that of antibody-ligand binding, and complementary sequences can be manufactured easily against any gene sequence. Binding of the antisense DNA can inhibit expression of the protein product of the transcript by several mechanisms, including blocking ribosomal translation of the RNA transcript (7), triggering RNase H degradation of the target RNA (8), and interfering with the processing of pre-mRNA (9). The efficacy of antisense DNA inhibition has been demonstrated in cell culture against a variety of genes (5, 6).

The ras oncogenes are an obvious potential target for antisense therapeutics since they are implicated in a variety of solid tumors in humans (10). Both unmodified and chemically modified antisense DNA oligomers complementary to various sites on c-Ha-ras RNA transcripts have been shown to inhibit RAS p21 synthesis in cell culture, and this inhibition is associated with a decrease in the cell proliferation rate and focus formation (11–13). The effect is sufficiently specific such that even a single mismatch substantially reduces the inhibition produced by the antisense DNA (14). It remains to be determined, however, whether antisense DNA treatment can have any sustained effect on tumor growth in vivo. In an attempt to address this question, we have examined tumor growth in athymic nude mice following implantation of transformed cells pretreated in vitro with anti-c-Ha-ras DNA. The DNA used was complementary to a site within an intron of the 5'-flanking region of the c-Ha-ras RNA transcript, and in previous experiments, it was shown to be the most effective at inhibiting RAS p21 synthesis of three different DNA oligomers targeted at various sites in the 5' flanking region of the transcript (11).

MATERIALS AND METHODS

Cell Lines. NIH-3T3 mouse fibroblasts transformed by the T24 human bladder cancer c-Ha-ras oncogene with a G12→V12 point mutation (15) were used as the experimental cell line. The original NIH-3T3 mouse fibroblast line was used as a nontransformed control cell line. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 µM glutamine and maintained at 37°C in an incubator with 5% CO₂. The Y13–259 rat monoclonal hybridoma cell line was maintained as described for the preceding cell lines except that the medium was supplemented with 10% heat-inactivated fetal bovine serum rather than calf serum. Filtered medium from the cells served as the source for the anti-RAS p21 Y13–259 antibody (16).

Antisense DNA Treatment. Two DNA pentadecamer sequences were used in this study. One sequence, termed RAS, has the sequence 5'-dCAGCTG-CAAACCGGACGC complementary to the human c-Ha-ras 5' flanking sequence beginning at nucleotide 1455 as denoted by Reddy (17). The second sequence, termed VSV, served as a nonspecific control and has the sequence 5'-dTTGG-GATAACACTTA complementary to the M protein of vesicular stomatitis virus beginning at nucleotide 17 (18). The antisense DNA oligomers were synthesized using standard phosphoramidite chemistry (19). Following n-butyl alcohol precipitation (20), the DNA was dissolved in deionized water and frozen at −80°C for storage. Purity was verified by high performance liquid chromatography using a reversed phase C18 column (21).

Four experimental groups were included in the in vitro DNA treatment of cells: (a) T24-T24/NIH-3T3 cells which received no antisense DNA treatment; (b) RAS-T24/NIH-3T3 cells which received the anti-c-Ha-ras DNA; (c) VSV-T24/NIH-3T3 cells which received the anti-VSV DNA; (d) T33-NIH-3T3 control cells which received no DNA treatment. All cells were first plated in 25-cm² flasks at a concentration of 1 × 10⁶ cells/flask. On the next 3 successive days, the old medium was removed, and fresh medium containing either no DNA or the appropriate antisense DNA at a concentration of 50 µM was added. On the fourth day, the cells were harvested using 0.1% trypsin in phosphate-buffered saline, washed twice with phosphate-buffered saline, and either placed in Hanks' balanced salt solution for immediate injection into nude mice or lysed for subsequent measurement of RAS p21 protein. A portion of cells...
from each flask was counted in a hemocytometer using trypan blue exclusion to identify viable cells. Cells of all four treatment groups evidenced greater than 90% viability based on trypan blue exclusion.

**Tumor Growth in Nude Mice.** Tumor growth was investigated in two experiments. In the first experiment, three female BALB/c athymic nude mice (6 weeks old)/treatment group were injected s.c. in the flank region with $5 \times 10^5$ cells. In the second experiment, 7 or 8 male C57BL/BALB/c athymic nude mice (6 weeks old)/treatment group were given similar injections. Tumor growth was monitored daily beginning several days after injection in a single blind protocol. Two perpendicular measurements of the diameter of any palpable nodule were obtained, and an estimated volume was calculated as:

$$V = \frac{4}{3} \pi r^3$$

In the case of multiple nodules, the total volume was recorded. The animals were sacrificed at the end of the experiment and examined for any intrusion of tumor through the body wall or evidence of metastases to various body organs. The tumors were removed, fixed in buffered formalin for 24 h, and imbedded in paraffin. For Experiment 2, 3 tumors were selected from each treatment group, sectioned at 5 μm, stained with hematoxylin/eosin, and examined for morphological characteristics.

**Measurement of RAS p21 Protein.** Evaluation of the M<sub>0</sub>, 21,000 protein products of both the mutated and wild-type c-Ha-ras genes was conducted by Western blot analysis of immunoprecipitated cell lysates. Approximately $5 \times 10^6$ cells were lysed in ice-cold lysis buffer (28 mM Tris-HCl, pH 7.1–100 mM NaCl, 1 mM MgCl<sub>2</sub>, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 6 μg/mL aprotinin) for 10 min, and the lysate was collected after centrifugation of particulate matter. Total protein in the lysate was measured on a Cobas Fara analyzer based on the method of Bradford (22). Cell lysate containing 500 μg total protein was immunoprecipitated using rat anti-RAS antibody Y13-259 followed by anti-rat IgG agarose conjugate. Controls included a negative control which contained no cell lysate but was immunoprecipitated in the same manner as the cell samples and an IgG control which contained untreated T24/NIH-3T3 cell lysate but was immunoprecipitated with 1 μg of rat IgG rather than Y13-259 antibody. After it had been washed, the immune complex containing RAS p21 and anti-RAS antibody was eluted from the agarose conjugate and denatured using Laemmli sample buffer and heat at 90°C for 5 min (23). The resulting sample was subjected to polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane using the method of Towbin et al. (24). Immunodetection of blotted p21 protein was accomplished using anti-RAS Y13-259 antibody, anti-rat IgG horseradish peroxidase conjugate, and horseradish peroxidase amplification (Blast amplification kit; NEN Research Products). Western blot analysis of immunoprecipitated cell lysates. Approximately 5 x 10<sup>6</sup> cells were lysed in ice-cold lysis buffer (28 mM Tris-HCl, pH 7.1–100 mM NaCl, 1 mM MgCl<sub>2</sub>, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 6 μg/mL aprotinin) for 10 min, and the lysate was collected after centrifugation of particulate matter. Total protein in the lysate was measured on a Cobas Fara analyzer based on the method of Bradford (22). Cell lysate containing 500 μg total protein was immunoprecipitated using rat anti-RAS antibody Y13-259 followed by anti-rat IgG agarose conjugate. Controls included a negative control which contained no cell lysate but was immunoprecipitated in the same manner as the cell samples and an IgG control which contained untreated T24/NIH-3T3 cell lysate but was immunoprecipitated with 1 μg of rat IgG rather than Y13-259 antibody. After it had been washed, the immune complex containing RAS p21 and anti-RAS antibody was eluted from the agarose conjugate and denatured using Laemmli sample buffer and heat at 90°C for 5 min (23). The resulting sample was subjected to polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane using the method of Towbin et al. (24). Immunodetection of blotted p21 protein was accomplished using anti-RAS Y13-259 antibody, anti-rat IgG horseradish peroxidase conjugate, and horseradish peroxidase amplification (Blast amplification kit; NEN Research Products). Visualization was achieved by chemiluminescence (Amersham) followed by X-ray film exposure, and the resulting bands were quantitated by optical scanning and analytical imaging using a Bio-Image Scanner (Millipore). The results yielded a total measure of mutated and wild-type RAS p21.

**RESULTS**

**Tumor Growth in Nude Mice.** Tumor growth in the BALB/c females given injections of cells from the four treatment groups (Experiment 1) is shown in Fig. 1. All females except one evidenced some tumor development in the 11 days following injection, and this one animal (from the untreated T24 group) was excluded from the results. Statistical analyses were not performed due to the small number of mice in the first experiment. In the second experiment, 3 tumors were selected from each treatment group, sectioned at 5 μm, stained with hematoxylin/eosin, and examined for morphological characteristics.

**Measurement of RAS p21 Protein.** A portion of the cells of Experiment 2 was lysed for subsequent measurement of RAS p21 protein on the day DNA pretreatment ended and cells were injected into the mice (Day 0). The resulting Western blot revealed a pronounced suppression of RAS p21 levels in the group treated with anti-c-Ha-ras DNA as compared to either the untreated T24 or VSV-treated group (Fig. 3). Scanning results indicated that the suppression amounted to a 95% reduction in total RAS p21 relative to the level in untreated T24/NIH-3T3 cells (Fig. 4). In fact, RAS-treated cells had p21 levels below that of NIH-3T3 cells. It should be noted, however, that in the case of the RAS-treated T24/NIH-3T3 cells the levels reflect mutated as well as normal p21, whereas NIH-3T3 cells contain
**DISCUSSION**

The results clearly demonstrate the pronounced effectiveness of anti-c-Ha-ras DNA in inhibiting tumor growth of c-Ha-ras-activated cells. The effect was evident in two different strains of nude mice and in both males and females. It was specific to the anti-c-Ha-ras DNA, since the nonspecific anti-VSV DNA had no effect. What is apparent in the results is not just the degree of inhibition but also its duration. Tumor growth was suppressed in both experiments throughout the period of observation. In Experiment 2, this was 14 days after DNA treatment was concluded and the cells were injected into the mice. Even after 14 days, the RAS-treated group still had substantially smaller tumors and a slower rate of tumor growth than either the untreated T24 or VSV-treated groups.

The effects of the anti-c-Ha-ras DNA on tumor growth are associated with its ability to suppress RAS p21 levels. Total RAS p21 was reduced by more than 90% with the 3-day treatment regimen. This is not surprising since comparable concentrations of the same antisense DNA were previously reported to produce a substantial inhibition of RAS p21 synthesis (11), and the 3-day treatment regimen is several times the reported 24-h cellular half-life of the RAS protein (25). The DNA presumably exerts its effect by binding specifically to the c-Ha-ras RNA transcript, and inhibition of synthesis is maintained as long as antisense DNA is present to bind the RNA transcript. Inhibition of RAS p21 expression should therefore have relaxed within a few days following the end of antisense DNA treatment since the intracellular half-life of DNA oligomers is on the order of 12 h (21); yet the effect on tumor growth extended for up to 14 days, suggesting more than a simple relationship between inhibition of RAS p21 and suppression of tumor growth. RAS p21 has been shown to affect the expression of a number of genes including MDR1, actin, fos, and transforming growth factor (26–28), and the sequences of at least two transcriptional elements have been identified which mediate some of the ras effects on transcription (29, 30). A sustained reduction of RAS p21 levels may therefore result in an extensive reorganization of cellular genetic expression and behavior, a reversal of the transformation phenotype of the cell. Such a reversal may interfere not just with cellular replication but also with such tumor-related processes as colonization, vascularization, invasion, etc. The time involved in reversing the transformation process as a result of RAS p21 inhibition and the subsequent retransformation following restoration of the protein levels may account for the prolonged duration of anti-c-Ha-ras DNA effects on tumor growth.

The possibility of reversing the transformation of cancer cells may be a critical advantage of drugs targeted specifically against oncogenes. Most current drugs affect only replication nonspecifically. An...
antisense DNA able to reverse the transformation process may produce a reorganization of cellular genetic expression which affects a number of neoplastic and malignant attributes. Even a partial reestablishment of normal cellular controls on growth could markedly alter the behavior of cancer cells. Many, if not most, neoplasms have identifiable oncogenes which may serve as targets for specific antisense DNA. Although the oncogenes presumably serve to trigger neoplastic behavior, what remains to be determined is the degree to which neoplastic and malignant characteristics of transformed cells remain dependent on particular oncogenes. In the case of c-Ha-ras oncogenes, Gilbert and Harris (31) reported data which indicated that the neoplastic behavior of some NIH-3T3 cells transformed by the T24 c-Ha-ras oncogene eventually became independent of c-Ha-ras expression itself. The authors suggest that the c-Ha-ras oncogene may act only to trigger cellular changes initially, and it is these changes which subsequently served to maintain neoplastic behavior independent of the oncogene. However, Feramisco et al. (32) demonstrated that microinjection of anti-RAS p21 antibodies directly into c-Ha-ras-transformed cells reversed transformation as measured by morphology and growth rate, and in the present study, anti-c-Ha-ras DNA was capable of suppressing tumor growth in c-Ha-ras transformed cells. These results indicate that neoplastic growth is dependent on continued expression of the c-Ha-ras oncogene. In the case of a different oncogene, nude mice that were given s.c. injections of human neuroepithelioma-derived cells containing an N-myc oncogene were treated in vivo with anti-N-myc DNA for 14 days. The antisense DNA not only reduced tumor growth but also altered the morphology of the neuroepithelioma cells (33), suggesting that both neoplastic growth and morphological characteristics of the cells were dependent on continued N-myc expression.

Antisense DNA has been shown here to be a very effective agent for in vitro treatment, and in another study, it has demonstrated some efficacy in vivo when administered at the relatively high concentration of 5 mm by a constant infusion pump (33). The high doses are necessary in vivo because of the susceptibility of normal DNA oligomers to rapid degradation by serum nucleases (34). More likely candidates for in vivo applications are modified oligonucleotides made resistant to nucleases by altering the nucleotide structure itself (e.g., alkylphosphatidates, phosphorothioates, 2′-O-alkylnucleotides) or by attaching protecting groups to the 3′ and 5′ ends of DNA oligomers (35). Which modifications most effectively balance cost, in vivo and intracellular mobility, stability, and most importantly, binding affinity and specificity remain to be determined. In any case, our findings here demonstrate the substantial potential of antisense DNA as a new class of anticancer drugs.

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