Specific Inhibition of K-ras Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA

Tapas Mukhopadhyay, Michael Tainsky, Adriana C. Cavender, and Jack A. Roth

Abstract

A human lung cancer cell line (H460a) with a homozygous spontaneous K-ras mutation was transfected with a recombinant plasmid that synthesizes a 2-kilobase genomic segment of the K-ras protooncogene in antisense orientation. Translation of the mutated K-ras mRNA in H460a cells was specifically inhibited, whereas expression of H-ras and N-ras was unchanged. A 3-fold growth inhibition occurred in H460a cells when expression of the mutated ras p21 protein was down-regulated by antisense RNA. However, cells remained viable despite the absence of K-ras expression. The growth of H460a tumors in nu/nu mice was substantially reduced by expressed K-ras antisense RNA.

Introduction

A wide spectrum of human cancers harbor ras genes activated by a single point mutation (1–12). Despite considerable knowledge of the structural aspects of the ras gene product, the functional role in physiological and pathological processes remains elusive (1). Cellular location and structural and biochemical similarities to G proteins suggest that ras gene products are involved in signal transduction (13, 14). We used an antisense RNA construct to block selectively the production of the mutated protein in the human non-small cell lung cancer (NSCLC) cell line NCI-H460A and examined the direct contribution of the mutated p21 protein to the malignant phenotype.

Materials and Methods

H460, H322, H226, and H522 NSCLC cell lines were generously provided by Drs. J. D. Minna and A. F. Gazdar (National Cancer Institute Naval Medical Oncology Branch, Bethesda, MD). All cell lines were grown in regular RPMI medium and 5% fetal calf serum in routine culture.

Plasmid Construction. A 2-kb genomic DNA fragment from the K-ras protooncogene was subcloned into an Apr-1-neo vector in both sense and antisense orientation. A 2-kb EcoRI/PstI fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences) and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with EcoRI and blunt end ligation was performed to obtain H460a or H322a cells were electroporated with 10 µg of Apr-1-neo-AS or Apr-1-neo-S plasmid DNA. Forty-eight h after transfection G418 was added into the medium at a concentration of 300 µg/ml for H460a and 200 µg/ml for H322a. Individual colonies were picked up and grown in culture for further analysis.

Southern Blot Analysis. High molecular weight DNA was isolated, digested with EcoRI (Boehringer-Mannheim) (20 µg), electrophoresed in 0.8% agarose gel, transferred onto a Gene Screen membrane (NEN), and hybridized with a 32P-nick-translated 2-kilobase genomic K-ras DNA probe.

Measurement of RNA Expression. Total cellular RNA was isolated from the cell lines (18). Twenty µg of total RNA was size fractionated in 4-morpholinopropanesulfonic acid/formaldehyde gel, transferred onto a Gene Screen membrane, and processed for hybridization with riboprobes. A 302-base pair genomic DNA of the K-ras gene was amplified by PCR spanning the third exon and intron sequences and was subcloned into a Bluescript vector. In vitro S and AS RNA probes were synthesized using either a T7 or T3 promoter.

Polymerase Chain Reaction. Polymerase chain reactions were performed as previously described using TagI DNA polymerase (16). Oligonucleotide primers corresponding to the 5' and 3' regions of codons 12 and 61 of human K-ras, H-ras, and N-ras genes were synthesized. Two µg of genomic DNA was subjected to 35 cycles of amplification. DNA sequences of oligonucleotide primers used for PCR amplification are listed in Table 1.

Slot Blot Oligonucleotide Hybridization. PCR-amplified DNA samples (12.5, 25, and 50 ng) were blotted onto a Gene Screen membrane using a slot blot apparatus (Schleicher and Schuell). The filters were prehybridized and hybridized at 55°C in 6× standard saline citrate, 5× Denhardt's solution, and 100 µg/ml of salmon sperm DNA for 2 h. Filters were washed twice in 6× SSPE at room temperature and once for 30 min at 58°C. Finally, blots were washed for 5 min at 64°C. The filters were exposed to X-ray film for 12–24 h at −80°C.

Direct Sequencing of PCR-amplified DNAs. PCR DNA corresponding to the second exon was purified in 8% polyacrylamide gel. A single DNA band was excised and purified DNA was used for asymmetric amplification in 100 µl of PCR reaction mixture. One (KA 61) amplifier was added to this mixture. After 20 cycles, single-stranded DNA was purified through gene clean (Bio 101) and DNA was eluted in 15 µl of water. Four µl of DNA was mixed with 4 µl of 10× TaqI buffer and 1 µl (10 pmol) of a second amplifier (KB 61) was used as a sequencing primer. DNA was sequenced using a Sequenase kit.

RNA PCR Analysis. cDNA synthesis was carried out in a total volume of 20 µl containing 5 µg of total RNA and oligo(dT) as a primer (17). A portion of the cDNA corresponding to the first and second exons was amplified to monitor the level of endogenous K-ras mRNA (Fig. 24) using KA12 and KB61 amplimers. Denaturation, annealing, and extension were done at 92°C for 1 min, 51°C for 1 min, and 74°C for 1 min, respectively. However, annealing temperatures for N-ras and H-ras were 44°C and 42°C, respectively. In addition, two amplimers were also used in the same reaction mixture to amplify a 118-base pair fragment of the p53 gene as an internal control. PCR products were either transferred onto a membrane and hybridized with 32P-labeled cDNA probe or, alternatively, they were directly labeled during the last cycle of amplification by adding 1 µCi of [32P]dCTP. The labeled PCR products were loaded on an 8% nondenaturing polyacrylamide gel. The gel was photographed after ethidium bromide staining, dried, and exposed to X-ray film overnight at −80°C.

Western Blot Analysis of ras Protein. Protein extracts were prepared by lysing cells in TBS (10 mM Tris, pH 7.5-100 mM NaCl-1 mM...
The extracts were cleaned by centrifugation at 10,000 x g for 1 h. The protein concentration of the supernatant was calculated spectrophoto-

phenylmethylsulfonyl fluoride-1% Nonidet P-40-1% deoxycholate).

A2). Mutations were confirmed by a direct PCR DNA-sequenc-

was examined by s.c. inoculation of IO5 (Fig. 3Ä)and IO6 cells in nul

nu mice. Each cell line was injected into 5 animals. Tumors were

examined by I25l-labeled goat anti-mouse second antibody.

Results and Discussion

Segments of the K-ras gene containing first and second exons were amplified from a number of NSCLC cell line DNAs by polymerase chain reaction (16) and subsequently hybridized with a set of 32P-labeled oligonucleotide probes (Fig. 1, A7 and A2). Mutations were confirmed by a direct PCR DNA-sequencing method. A homozygous mutation at codon 61 was detected in the NCI-H460A large cell undifferentiated NSCLC cell line with a normal glutamine residue (CAA) substituted by histidine (CAT). This cell line is highly tumorigenic in nude mice. A recombinant plasmid clone was constructed using a wild-
type 2-kilobase K-ras genomic DNA segment carrying second and third exons together with flanking intron sequences sub-
cloned into an Apr-1-neo expression vector (18) in the AS

orientation. S plasmid constructs were used as a control (Fig. 1B). AS or S K-ras RNA synthesis was accomplished by transfecting H460a cells, a cloned derivative of the NCI-H460A cell line, with Apr-1-neo-AS or Apr-1-neo-S constructs by electro-

poration. The β-actin promoter of the vector was constitutively capable of directing the synthesis of RNA from the inserted DNA. The Apr-1-neo vector offered suitable G418 marker gene expression for selection of the transfectants. Individual G418-

resistant colonies were selected and grown in culture for further analysis. Stable integration of the plasmid DNA in the transfectants was examined by Southern hybridization with a 2-
kilobase DNA insert from the original plasmid clone as a probe (Fig. 1C). The Southern blot analysis showed a single 3-kilobase

DNA insert corresponding to the third exon and part of the intron sequences (Fig. 1D). Interestingly, the clones carrying

the Apr-1-neo-S vector show one RNA band at about 1.5

tilobases, but the cells carrying the S construct show two RNA species. The reason for this is unknown, but the possibility exists that the RNA synthesized from the genomic DNA under control of the β-actin promoter could be processed in vivo. However, no corresponding hybridization band was detected in H460a cells, which indicated that a significantly higher level of K-ras RNA was synthesized under the β-actin promoter.

We next analyzed the p21 protein level in these transfectants by Western blot analysis (Fig. 1, E and F). A K-ras-specific p21 monoclonal antibody (Oncogene Science) was used to determine the level of K-ras protein in transfectants, parental H460a cells, and Calu-1 cells, which have a high level of K-ras gene expression (Fig. 1E). Western blot analysis showed a 95% reduction in K-ras p21 protein synthesis in the clones expressing the AS RNA, while parental cells, S K-ras clones, and Calu-1 cells showed a significant level of K-ras p21 protein. These results indicate that AS RNA can effectively block the synthesis of K-ras-specific protein. Since members of the ras gene family share a great deal of sequence homology and code for a similar p21 ras protein, we examined the total ras protein product in these clones using a pan ras monoclonal antibody (New England Nuclear) to determine whether a reduced level of K-ras protein reflects any change in H-ras and N-ras p21 protein synthesis (Fig. 1F). Western blot analysis revealed only a slight decrease in overall ras protein level in all clones containing Apr-1-neo-

AS as compared to 460a parental cells.

The effect of AS RNA on the specific production of mature endogenous K-ras mRNA was analyzed by cDNA PCR (Fig. 2). cDNA synthesized from the total RNA (18) was subjected to PCR amplification using amplifiers corresponding to the 5’ end of the first exon and the 3’ end of the second exon (Fig. 2A). Because the AS RNA was generated only from a second and third exon of the K-ras gene, PCR-amplified cDNA represented the level of endogenous K-ras mRNA. A 246-base pair amplified DNA fragment was labeled by [32P]dCTP and subse-

quently analyzed by polyacrylamide gel electrophoresis. In addition, a 118-base pair segment of endogenous p53 cDNA was amplified in the same reaction mixture using p53-specific amplimers to serve as an internal control for the PCR.

Results showed that H460a cells, clones expressing S RNA, and the Calu-1 cell line expressed K-ras mRNA, as evidenced by the presence of a high level of amplification of the 246-base pair cDNA product (Fig. 2B). H460a clones expressing AS RNA showed very little amplification, and cellular K-ras mRNA synthesis appeared to be completely inhibited (Fig. 2B, lanes 5 and 6). In contrast, the endogenous p53 expression remained unaffected. This prompted us to investigate the level of expression for other ras genes in these clones. We used the same cDNA PCR methodology to analyze the N-ras and H-ras mRNA level using N-ras- and H-ras-specific oligonucleotides as amplimers. A steady state level of H-ras and N-ras gene expression was observed, but no obvious change in either Apr-

1-neo-AS or Apr-1-neo-S transfectants was noticed (Fig. 2, C and D). The p53 gene expression serving as a control in these experiments remained unaffected. Thus, inhibition of K-ras expression by our AS RNA construct is specific.

H460a clones expressing AS K-ras RNA continued to grow in culture. However, H460a Apr-1-neo-AS transfectants showed a 3-fold reduction in growth, compared to the H460a Apr-1-neo-S transfectants and the parental H460a cells (Fig. 3a). The H322 NSCLC line has wild-type ras family genes. H322 Apr-1-neo-AS and Apr-1-neo-S transfectants had identical growth characteristics, indicating that inhibition of wild-

<table>
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<tr>
<th>Primers</th>
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<tr>
<td>KA61</td>
<td>5’ TTC CTA CAG GAA GCA AGT AGT A 3’</td>
<td>K-ras 2nd exon</td>
</tr>
<tr>
<td>KB61</td>
<td>5’ ACA CAA AGG ACC DCC CCA 3’</td>
<td>K-ras 1st and 2nd exon</td>
</tr>
<tr>
<td>KA12</td>
<td>5’ GAC TGA ATA TAA SCT TGT GG 3’</td>
<td>K-ras 1st and 2nd exon</td>
</tr>
<tr>
<td>KB61</td>
<td>5’ ACA CAA AGG ACC DCC CCA 3’</td>
<td>H-ras 1st and 2nd exon</td>
</tr>
<tr>
<td>HA12</td>
<td>5’ GAC GGA ATA TAA GCT GGT GG 3’</td>
<td>N-ras 1st and 2nd exon</td>
</tr>
<tr>
<td>HB61</td>
<td>5’ CGC ATG TAC TGG TCG CCA AT 3’</td>
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<td>NB61</td>
<td>5’ ATA CAC AGA GGA AGC CTT CG 3’</td>
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Fig. 1. A, the second exon of the K-ras gene was amplified from genomic DNA of H522, H322, Calu-1, H226, H460a, and human placenta by PCR, blotted onto a Gene Screen membranes, and hybridized with 32P-end-labeled oligonucleotide probes. A1, presence of wild-type glutamine residue (CAA) at 61 codon in five cell lines except H460a. The same blot was reprobed with a histidine-specific mutated oligo probe (CAT) and only the H460a cell line PCR DNA hybridized (A2). The mutation was confirmed by direct PCR DNA sequencing. Wild-type K-ras 61 codon sequence in human placenta (A3) was compared with the H460a cell line (A4). In B, a 2-kilobase genomic DNA from the K-ras oncogene was subcloned into an Apr-1-neo vector in both sense and antisense orientation. A 2-kilobase EcoRI/PstI fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences) and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with BamHI and blunt end ligation was performed to obtain the Apr-1-neo-S or Apr-1-neo-A constructs. C, a Southern blot analysis of the K-ras oncogene in H460a and H460a transfectants. Blots were probed with a 32P-nick-translated 2-kilobase EcoRI/PstI insert DNA. Lane 1, H460a; lanes 2 and 3, H460a transfected with Apr-1-neo-S Cl#1 and C2#1; lanes 4 and 5, H460a cells transfected with Apr-1-neo-AS, C3#2, and C2#32, respectively. D, a Northern blot analysis of sense and antisense K-ras RNA. Lane 1, H460a; lanes 2 and 3, Apr-1-neo-S transfecants; lanes 4 and 5, Apr-1-neo-AS transfected clones. E and F, Western blot analysis of K-ras-specific p21-protein (JE) and total ras protein (IF) was done using either pan ras or K-ras-specific monoclonal antibodies. Lane 1, Calu-1 control cell line overexpressing K-ras-specific protein; lane 2, H460a; lane 3, H460a Apr-1-neo-S; lanes 4 and 5, H460a Apr-1-neo-AS.

K-ras EXPRESSION AND TUMORIGENICITY INHIBITION

Inoculation of H460a cells at both doses led to the formation of tumors in 15 days in all mice (3–5 mice/group in 3 separate experiments). No tumor developed in mice treated with 10^5 cells for both clones of H460a AS cells during 120 days of observation in a total of 10 mice, whereas all mice receiving H460a cells developed tumors (data not shown). When the inoculum was increased to 10^6 cells, tumors grew in all mice, but the tumors in mice receiving AS clones grew at a slower
rate than H460a cells or the S control (Fig. 3B). Tumors were excised and analyzed for K-ras expression by cDNA-PCR. K-ras expression was not detected in tumors arising from injection of AS clones but was present in S clones and H460a tumors.

The above experiments indicate that, in H460a cells engineered to synthesize AS K-ras RNA, the levels of K-ras mRNA and K-ras p21 protein are effectively down-regulated. Reduction in the expression of K-ras mutated gene reproducibly reduced the rate of tumor growth in nu/nu mice. Our studies show that a construct can be made that distinguishes among members of...
expression. In our model inhibition of activated K-ras reduced (19, 20). Our data indicate that AS RNA generated from the sectional diameters. Palpable tumors were first detected on day 15. Point, mean: diameters of the external tumor were measured without knowledge of the cell carrying Apr-I-nco-AS showed growth inhibition (h). H460a cells (10⁶) were injected s.c. into the left flanks of female Balb/c nu/nu mice. Cross-sectional intervals. Growth curves for H460a and H460a transfectants for absence of expression by one member of this family so that functions essential for maintenance of cell viability are preserved. However, tumorigenicity was maintained in the absence of activated K-ras expression, although the rate of tumor growth was diminished. We hypothesize that, in human NSCLC, ras mutations confer a growth advantage to the malignant cell.

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References
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