Wild-type but not Mutant p53 Suppresses the Growth of Human Lung Cancer Cells Bearing Multiple Genetic Lesions

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Abstract

Accumulating evidence indicates that lung cancer arises due to multiple genetic changes in both dominant oncogenes, such as ras, and tumor suppressor genes, such as p53. In this report we examined whether the wild-type p53 gene is able to suppress in vitro and/or in vivo cellular growth of lung cancer cell lines which carry multiple genetic abnormalities. Introduction of a wild-type p53 complementary DNA expression vector into lung cancer cell lines carrying either a homozygous deletion (NCI-H358) or a missense mutation (NCI-H23) in the p53 gene greatly suppressed tumor cell growth. In contrast, p53 expression vectors bearing lung cancer derived mutations affecting single amino acids had lost this growth suppressing ability.

Introduction

The p53 gene is one of the most common targets for genetic abnormalities in human tumors (1). Previously we have reported that p53 mutations occur in all histological types of lung cancer (2, 3) at frequencies of ~75% in small cell lung cancer (SCLC), ~50% in NSCLC (4, 5), and ~50% in NSCLC (6, 7). The characteristic predominance of G to T transversions in p53 and ras mutations in lung cancer and the molecular epidemiological evidence showing a close association between smoking and p53 mutations in NSCLC have suggested that the p53 gene is a good candidate for molecular targets of genetic damages caused by cigarette smoke (8).

Materials and Methods

Construction of wt-p53 and Mutant p53 Expression Plasmids. Mutant p53 cDNAs (MT1 and MH1) were isolated from a NSCLC tumor (T104, deletion of asparagine at codon 239) and a SCLC cell line (NCI-H1436, histidine to glutamine substitution at codon 179) by the cDNA/PCR method using primers just outside the p53 open reading frame as described previously (9). They were cloned into the EcoRI site of pcDNAI (Invitrogen, San Diego, CA) and transferred to the HindIII-XbaI site of pRC/CMV (Invitrogen) which contains the cytomegalovirus promoter/enhancer for expression and a neomycin resistance gene allowing G418 selection. The inserts of both mutant clones, pCT1R (T104) and pCH1R8 (H1436), were sequenced entirely and shown to have only the mutations expected from our previous study (2). Normal p53 clones in both sense (pcNXR5) and antisense (pcNXRAS) orientations were prepared in the same vector using the XbaI-XbaI fragment of pH53c1 (containing 5′ and 3′ untranslated regions ~570 base pairs longer than those of the mutants) (9). Schematic diagrams of these constructs are shown in Fig. 1.

Transfection of wt-p53 and Mutant p53 Expression Plasmids. Cells (1 x 10⁶) of NCI-H358 (lung bronchioloalveolar carcinoma) or NCI-H23 (lung adenocarcinoma) were transfected with 10 µg of various CscI purified plasmid DNAs using 50 µg of Lipofectin according to the manufacturer's instructions (Bethesda Research Laboratories, Bethesda, MD). NCI-H358 and NCI-H23 have been shown previously to contain a homozygous deletion and a missense mutation (methionine to isoleucine at codon 246) in the p53 gene, respectively (2). In addition to p53 abnormalities, both cell lines carry K-ras mutations at codon 12 (10) and retinoblastoma abnormalities (11), while NCI-H358 and NCI-H23 have a chromosome 3p deletion (del(3)(p14p23)) (12) and amplified c-myc (8), respectively. Following a 24-h incubation, cells were washed and fed with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfected cells were split the following day and selected with 500 µg/ml of G418 (GIBCO, Bethesda, MD). At least two independent transfections were done in duplicate each using different plasmid preparations. After 2–3 weeks of G418 selection, plates were scored for the number of macroscopic G418 resistant colonies and individual colonies were picked and expanded for further analyses. Northern blot and RNAse protection analyses were performed as described previously using pH53c1 and p53PA as probes, respectively (2). Assays for in vitro growth characteristics were performed in triplicate in two independent experiments as described previously (13). Tumorigenicity was tested by injecting 2 x 10⁶ cells of control and test clones into each side of the back of SCID mice (at least three mice per clone) and tumor formation and tumor size were measured at 3 weeks postinjection.
GROWTH SUPPRESSION OF LUNG CANCER CELLS BY WILD-TYPE p53

Results and Discussion

We first introduced normal or mutant p53 cDNAs into a lung cancer cell line (NCI-H358) with a homozygous deletion of p53 which expresses no p53 mRNA or protein. cDNA constructs and representative dishes stained with crystal violet are shown in Fig. 1. As expected, no appreciable difference in the number of G418 resistant colonies was observed between vector alone and antisense wt-p53 (normal antisense) transfections. The average numbers of colonies per μg plasmid DNA for vector control and normal antisense transfections were 242 and 247, respectively. In contrast to these controls, transfection with sense wt-p53 (normal sense, NS) yielded a markedly reduced number of colonies (22 colonies/μg DNA) whereas two mutant constructs (MT1 and MH1) had virtually no effect on the number of colonies (235 and 255 colonies/μg DNA, respectively).

We next examined if the colonies surviving G418 selection expressed p53 transcripts. Forty-one individual clones were isolated from the normal sense wt-p53 transfections and 37 were successfully expanded to allow Northern blot analysis. However, only one clone (NS-32) was found to express p53 transcripts (Fig. 2). Although p53 was expressed in the NS-32 clone, the transcript was smaller (~2.0 kilobases) than expected (~2.7 kilobases) (Fig. 2) due to an alteration within the open reading frame as determined by cDNA/PCR analysis (data not shown). Thus, we have detected no continuously replicating G418 resistant colonies retaining an intact wt-p53 gene. These results suggest that expression of exogenous wt-p53 is incompatible with tumor cell growth of this lung cancer cell line bearing a p53 homozygous deletion. In contrast, transfections with mutant p53 cDNAs yielded clonal lines in which stable expression of both mutant mRNAs and proteins were readily detectable by Northern blot analysis (Fig. 2) and immunostaining with a p53 monoclonal antibody (data not shown), respectively. The clones expressing exogenous mutant p53 showed similar growth characteristics in vitro as well as in vivo indicating that the subtle mutations identified in lung cancer abolish the wt-p53 suppressive effects on tumor cell growth (data shown). These results are consistent with previous studies using other tumor types in which transfection of wt-p53 cDNA led to severe retardation of cell growth (14–19).

We next examined whether introduction of wt-p53 into lung cancer cells expressing mutant p53 is able to yield growth suppression similar to that observed in NCI-H358 expressing no p53. Transfection of wt-p53 was performed using NCI-H23 cells which express high levels of mutant p53 and yielded no significant decrease in the number of G418 resistant colonies (~20 colonies/μg DNA in both wt and vector controls). However, when 26 individual wt-p53 G418 resistant colonies were picked, only 6 could be expanded in contrast to vector controls. Further analysis by RNase protection assay suggested that colony NS-26B stably expresses wt-p53 (Fig. 3). Of note, expression of wt-p53 mRNA was severalfold lower than that of the endogenous mutant p53 based on the adjustment of the number of UTP residues in the corresponding fragments. We confirmed the integrity of the exogenously introduced wt-p53 in NS-26B by sequencing the entire open reading frame of cDNAs prepared by cDNA/PCR using primers complementary to the vector sequence. Although the saturation density of NS-26B was slightly lower than that of the controls (2 × 10^9 versus 3 × 10^9/60-mm dish), NS-26B exhibited the morphology and a growth rate similar to those of control clones in which the expression vector alone was introduced, (Fig. 4). However, neoplastic phenotypes both in vitro and in vivo were significantly altered; i.e., an 85% reduction was observed in colony forming efficiency in soft agar (a mean of 18 colonies/10^5 cells seeded in NS-26B when compared to a mean of 113 colonies/10^5 cells in control clones). Furthermore, tumor formation of NS-26B in SCID mice was completely suppressed in two independent experiments (0 of 6 mice with NS-26B versus 6 of 6 mice with VC-1B). As a more stringent test of wt-p53 ability to suppress the in vivo growth of NCI-H23 cells, pools of NCI-H23 G418 resistant wt-p53 transfecants in which low levels of wt-p53 expression were detectable by the RNase protection assay also were injected into SCID mice. These pools showed tumorigenicity similar in latency and size to those of vector controls. However, when these tumors were harvested and assayed for expression of wt-p53 mRNA, wt-p53 expression was below detectable level by the RNase protection assay in those tumors,
GROWTH SUPPRESSION OF LUNG CANCER CELLS BY WILD-TYPE p53

Fig. 2. Northern blot analysis of transfectants of the NCI-H358 lung cancer cell line. Ten µg of total RNA from controls and expanded individual normal (NS) or mutant (MT1 and MH1) clones were loaded in each lane and hybridized with the human p53 cDNA probe (php53cl). Ethidium bromide staining of the gel showed that the amount of RNA loaded per lane was approximately equal (data not shown). Arrowheads, expected mRNA sizes for the introduced wt-p53 and mutant p53 (2.1 and 2.1 kilobases, respectively).

Fig. 3. RNase protection analysis of transfectants of the NCI-H23 lung cancer cell line. H23, parental cell line of the transfection; H358, negative control with the homozygous deletion; NS-26B, -12B, -11B, and -27B, wt-p53 transfected clones; VC-4B, transfactant introduced with expression plasmid alone; YRNA, yeast tRNA; M.M., molecular markers in base pairs (bp). Schematic diagram of p53 mRNA and location of antisense RNA probe (p53PA) as well as the missense mutation in NCI-H23 are shown below. The endogenous p53 mRNAs in the transfectants produce both 391- and 303-base pair fragments whereas mRNAs transcribed from the exogenous p53 sequence protect a 694 base pair fragment.

Fig. 4. In vitro growth characteristics of wt-p53 expressing clone (NS-26B) in comparison with those of a representative control clone (VC-1B). (A) NS-26B shows similar growth rate and slightly lower saturation density when compared to those of VC-1B (B) NS-26B and VC-1B clones exhibit similar morphology in culture. Numbers in parentheses, tumorigenicity of each clone in SCID mice.

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References


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