Growth Suppression Mediated by Transfection of p53 in Hut292DM Human Lung Cancer Cells Expressing Endogenous Wild-Type p53 Protein

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Abstract

This study was undertaken to analyze the effect of wild-type p53 transfection on the growth potential of a human lung cancer cell line Hut292DM expressing endogenous wild-type p53. Transfection efficiencies obtained with either the wild-type or a mutant p53 complementary DNA revealed a significant decrease in the number of colonies obtained with the wild-type p53 as compared to the mutant p53 complementary DNA (27%) or control vector DNA only (20%), suggesting that wild-type p53 inhibited the growth of Hut292DM cells. A series of wild-type and mutant p53 transfection clones were then analyzed for the presence and expression of the exogenous p53 gene. Polymerase chain reaction amplification revealed that 98% of mutant p53 transfection clones analyzed contained the exogenous p53 gene as opposed to 47% for wild-type p53 clones. The majority of mutant p53 clones expressed higher levels of exogenous p53 mRNA and protein as analyzed by Northern and Western blots, respectively. In contrast, all wild-type p53 clones analyzed failed to express exogenous p53 mRNA transcript or protein of a normal size. Aberrant size p53 mRNA was detected in two wild-type p53 clones (X333.W2 and W18), and Western blot analysis revealed that these clones expressed truncated p53 proteins (Mf, 45,000 and 33,000 respectively). No difference in proliferation rates in vitro or in tumorigenic potential in nude mice were observed between mutant p53 clones or control cell lines. In contrast, a wild-type p53 clone (X333.W2) exhibited a significantly reduced tumorigenic potential in nude mice, whereas its in vitro proliferation rate was comparable to parental Hut292DM cells. The data indicate that exogenous expression of wild-type p53 is in competition with Hut292DM lung cancer cell proliferation in vitro and suggest that p53-mediated growth control in vitro and in vivo may be dissociated and exerted by separate domains of the p53 protein.

Introduction

Since its discovery approximately a decade ago, the p53 tumor suppressor gene has attracted a great deal of attention in cancer research, partly due to its capacity to prevent uncontrolled growth of cancer cells and due to the realization that most types of human cancers studied, including lung, display expression of the p53 gene (1). The molecular mechanisms by which p53 may regulate cell cycle progression and therefore prevent tumor cell growth are poorly resolved at the present time. A variety of human tumor cells have now been shown to be growth arrested when transfected with the p53 tumor suppressor gene (2–5), and the cell cycle progression of these recipient cells is blocked in the G1/S transition phase (3, 6). However, this effect is seen only when the cancer cells contain mutated p53 or are null for expression. To date, reports of the introduction of wild-type p53 cDNA2 into tumor cells containing endogenous wild-type p53 have shown that there is no effect on in vitro growth (2, 4).

The product of the p53 gene is a 393-amino-acid-long protein, primarily localized in the cell nucleus, where it may interact with genomic DNA sequences to regulate the cell cycle in two ways. The carboxy-terminal portion of the protein is associated with DNA binding, and a consensus DNA sequence which binds to p53 has now been defined that shares homology with DNA origins of replication (7, 8). The amino-terminal domain functions as a transcriptional modulator, possibly involved in the repression of nuclear oncogene transcription (9).

In addition, p53 may control cell growth by binding to, and interacting with, nuclear proteins critically involved in cell cycle regulation such as the cyclin-dependent cdc2 kinase (10) or the murine double minute 2 oncogene product (11). Recent studies have shown that many cancer cells no longer express a normal p53 protein, the result of both alleles being inactivated by a combination of single allele deletion and point mutation of the remaining allele (12, 13). However, a significant proportion of human cancers arise in which no detectable alteration in p53 expression is observed, seemingly precluding any role of the p53 tumor suppressor gene in the natural development of these tumors. The present study was undertaken to determine whether human lung cancer cell growth could be influenced by transfection of the p53 gene. To our knowledge, we are the first to report growth suppression induced by high level expression of exogenous wild-type p53 in lung cancer cells expressing normal endogenous p53 protein.

Materials and Methods

Transfection. Human lung cancer Hut292DM cells were kindly provided by C. Harris (14) and were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum. Transfections were performed by the polybrene-dimethyl sulfoxide technique as described (15) using plasmid constructs obtained from B. Vogelstein: pCMVneoBam, a eukaryotic expression vector containing the cytomegalovirus constitutive promoter and the neomycin resistance gene under control of the simian virus 40 promoter; pC53SN3, derived from pCMVneo by insertion of a full-length human wild-type p53 cDNA under control of the cytomegalovirus promoter; and pC53SCX3, which contains a single point mutation in the p53 cDNA at codon 143 (substitution of alanine to valine). Hut292DM cells (2 × 10⁶) were transfected with 20 μg of plasmid DNA, and following 3–4 weeks of selection in medium containing 1100 μg/ml geneticin, colonies were either stained with 5% Giemsa to assess transfection efficiency or cloned and expanded for further analysis.

Molecular Analysis of p53 Expression. Standard nucleic acid procedures were performed as described (15). PCR analysis was performed

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using DNA extracts obtained by the rapid detergent method described (4). The two primers used for p53 cDNA amplification (p53.1: 5'-CAC GAC GGT GAC CTT CCC TG-3'; p53.2: 5'-GTC CTG GGT GCT TCT GAC GCA CAC-3') are complementary to 5' and 3' flanking sequences of the p53 coding region, respectively, and yield a 1.2-kilobase PCR product. Southern and Northern blot analyses were performed using genomic DNA extracts digested with BamHI or total cytoplasmic RNA extracted as described (16). Nucleic acids were separated on 1% agarose gels (DNA) or 1.2% agarose-formaldehyde denaturing gels (RNA) and transferred to nitrocellulose membranes. All hybridizations were performed using a 1.8-kilobase full-length p53 cDNA probe derived from pC53SN (following BamHI digestion) and radiolabeled by the random primer method. Protein extraction and Western blot analysis were performed as described (17). Protein extracts were isolated from 50% confluent cells using RIPA buffer for cell lysis, and the protein concentration was determined by a modified Lowry colorimetric assay (DC protein assay kit; BioRad, Richmond, CA). Equal amounts of protein extracts (100 μg) were denatured (5 min at 100°C in presence of 0.1 M dithiothreitol), separated through 10% polyacrylamide-sodium dodecyl sulfate gels, and transferred to nitrocellulose membranes. Immunoblotting was performed using the monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, NY) diluted 1:500 as the primary antibody and a horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad) diluted 1:2000 as the secondary antibody. Detection of p53 antigen was performed using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and following the manufacturer's recommendations.

Cell Growth Analysis and Tumorigenicity Assay. Cells (1.5 × 10⁴) were plated in 35-mm tissue culture wells, and at various time points cells from duplicate wells were counted. Culture medium was changed 3 times each week. Tumorigenicity was assessed by inoculating 5 × 10⁵ cells at 6 sites into 4-6-week-old athymic nude mice (one inoculation per animal). Tumors were measured with linear calipers at regular intervals to compare growth properties between the different cell lines.

Results and Discussion

Three separate transfection experiments were performed in an attempt to express exogenous p53 cDNA (wild-type and mutant versions) in Hut292DM human lung cancer cells. Transfection efficiencies (see Table 1) from all three experiments indicate a significant decrease in the number of geneticin-resistant colonies obtained with the wild-type p53 cDNA as compared to the vector construct alone. In contrast, the mutant p53 cDNA did not appear to affect the transfection efficiency. These results suggested that expression of exogenous wild-type p53 inhibits Hut292DM cell growth or that high levels of expression are incompatible with sustained proliferation of these cells. A detailed molecular analysis of p53 expression in a series of transfection clones was then undertaken to gain more insight into the possible role of p53 in the control of Hut292DM tumor cell growth. PCR analysis of p53 cDNA was performed on a series of mutant and wild-type p53 transfection clones derived from two separate experiments (X813 and X833). The majority of X833 mutant p53 clones analyzed displayed a 1.2-kilobase PCR amplification product which contains the entire p53 coding region (16 of 18, or 89%) whereas only 47% (8 of 17) of the wild-type p53 transfection clones displayed a p53 cDNA-related PCR product (Fig. 1A). Interestingly, one of these clones (X833.W2) yielded an unusually large PCR product of 1.8 kilobases which indicates a probable rearrangement of the p53 coding region either by insertion of foreign sequences or by partial duplication of the introduced p53 cDNA. Southern blot analysis of PCR-positive clones confirmed the presence of exogenous p53 cDNA sequences in all X833 mutant p53 clones analyzed (8 of 8) and in most (7 of 9) wild-type p53 clones (data not shown).

However, only a few of the wild-type clones (3 of 9, or 33%) displayed the expected 1.8-kilobase hybridization signal, the other clones being associated with larger-size hybridization signals corresponding to plasmid DNA rearrangement. In contrast, nearly all mutant clones (6 of 7, or 86%) displayed the expected 1.8-kilobase band, which suggests that the cDNA has not been rearranged upon integration. These results not only confirm the PCR analysis carried out on the mutant p53 transfecants but also show a further decrease in the number of clones harboring a normal copy of wild-type p53 cDNA.

Northern blot analysis of these transfection clones was then performed to examine p53 mRNA expression (Fig. 1B). All transfection clones analyzed, as well as parental Hut292DM cells, express a 2.8-kilobase band which corresponds to endogenous p53 mRNA. This band appears to be of comparable intensity for all clones analyzed and therefore indicates equal RNA loading of all samples. The exogenous p53 mRNA can be differentiated in this analysis from endogenous mRNA due to its smaller size of 2.65 kilobases. The majority (5 of 8, or 63%) of the mutant p53 clones analyzed expressed very high levels of the 2.65-kilobase exogenous mRNA species. In contrast, no normal-size transcript characteristic of endogenous p53 was detected in any of the wild-type p53 clones analyzed. The same analysis performed on a series of separate transfection clones (experiment X813) was also performed and resulted in similar findings. Briefly, PCR analysis for the presence of p53 cDNA was positive for the majority of mutant X813 transfection clones analyzed (7 of 12, or 58%) whereas only two wild-type X813 clones were positive in this assay (2 of 12, or 17%). Northern blot analysis showed high levels of expression of the exogenous p53 transcript in most of the X813 mutant p53 transfection clones analyzed, whereas the X813 wild-type p53 clones positive by PCR analysis failed to express the exogenous transcript.

Two of the wild-type p53 transfection clones (X833.W2 and W18), however, did express high levels of aberrant p53 mRNA transcripts, a finding which prompted us to analyze whether these transcripts might be translated. Fig. 1C shows the expression of p53 protein by clones previously analyzed for p53 mRNA expression. As with the Northern blot analysis, p53 antigen was easily detected in most mutant p53 clones analyzed (7 of 8, or 88%), which is in agreement with the prolonged half-life of mutant forms of p53 compared to that of wild-type. In contrast, a very faint band corresponding to endogenous p53 protein was detected in parental Hut292DM cells as well as in all of the wild-type clones. No increase in the expression level of this M₇, 53,000 band was observed in the wild-type p53 clones, which correlates with the absence of normal-size exogenous transcript detected by Northern analysis. Several wild-type clones did, however, express high levels of truncated forms of

Table 1. Transfection efficiencies of wild-type and mutant p53 expression vectors in Hut292DM cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pC53SN (wild-type)</th>
<th>pC53SCX3 (mutant)</th>
<th>pCMVNeoBam (vector)</th>
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<tr>
<td>X813</td>
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<td>600</td>
<td>720</td>
</tr>
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<td>X832</td>
<td>55</td>
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GROWTH SUPPRESSION MEDIATED BY p53 IN HUMAN LUNG CANCER CELLS

1.2 - 1.8 -

WILD-TYPE p53 CLONES

MUTANT p53 CLONES

1.2 -

WILD-TYPE

MUTANT

2.8 - 2.65 -

Fig. 1. Analysis of exogenous p53 expression in a series of wild-type and mutant p53 transfection clones derived from Hut292DM cells (transfection experiment: X833). A, PCR amplification of a 1.2-kilobase fragment corresponding to the full-length coding region of p53 cDNA. Top, wild-type p53 transfection clones; bottom, mutant p53 clones. B, Northern blot analysis of wild-type (left) and mutant p53 (right) transfection clones as well as control parental Hut292DM cells (Lane Hut). Endogenous p53 mRNA migrates at a position corresponding to 2.8 kilobases and may be differentiated from exogenous p53 mRNA (2.65 kilobases). C, Western blot analysis of p53 antigen using the same cells as in B. The position of migration of the normal p53 is indicated.

p53 protein. These truncated p53 variants appear to be related to the aberrant mRNA transcripts expressed by these clones. In particular, clones X833.W2 and W18, which were associated with a larger and smaller size mRNA transcript, respectively, both appear to synthesize, respectively, truncated Mr. ~45,000 and ~33,000 p53 proteins. Two additional bands of higher molecular weight (approximately 55,000 and 75,000) were detected in all samples analyzed; these probably represent common epitopes shared by proteins unrelated to p53 but recognized by the monoclonal antibody used in this assay.

Selected clones were further analyzed for their growth properties in vitro (Fig. 2). The growth rates (e.g., doubling times) and saturation densities of various mutant p53 clones did not vary significantly from that of parental Hut292DM cells. Furthermore, wild-type p53-transfected cells shown to express high levels of truncated p53 protein exhibited similar in vitro growth properties as parental Hut292DM cells or cells transfected with the control vector only (data not shown). The results, however, were different when these transfected clones were assayed in vitro for their tumorigenic potential (Fig. 3). A particular wild-type p53 transfection clone (X833.W2) consistently exhibited a reduced tumorigenic potential compared to parental or vector transfected cells. Interestingly, X833.W2 cells were shown by Western blot analysis to express what appears to be a mildly truncated form of the p53 protein with a molecular weight of approximately 45,000. Moreover, all other forms of truncated p53 protein of smaller size did not appear to affect the tumorigenic potential of Hut292DM transfection clones. Various mutant p53 transfection clones, tested under identical experimental conditions, displayed a tumorigenic potential similar to that of parental or vector transfected Hut292DM cells (data not shown).

The results of the molecular analysis of p53 expression in wild-type and mutant p53 transfection clones suggest that growth suppression is mediated by high expression levels of wild-type p53 in this human lung cancer cell line. Indeed, the
clonal analysis, repeated twice on two separate transfection experiments, consistently failed to identify any wild-type transfection clones expressing a normal exogenous p53 protein, whereas exogenous mutant p53 appeared to be stably expressed at high levels in nearly all clones analyzed. We conclude from these results that expression of exogenous wild-type p53 is incompatible with the sustained proliferation of Hut292DM cells, a finding consistent with the transfection efficiencies obtained with the wild-type and mutant cDNA constructs (see Table 1). These results also demonstrate that a single point mutation at codon 143 (a transition from an alanine to a valine residue) results in the apparent loss of function of p53 with respect to its growth-inhibitory properties for Hut292DM cells, since no difference in transfection efficiency was noted using either the mutant p53 cDNA or the control vector. Several studies involving human colon, breast, and lung cancer cells (2, 4, 5) have also shown that restoration of normal p53 protein expression in otherwise defective cell lines (either null for p53 expression or carrying a p53 mutation) severely affected the growth capacity of these model cell lines. This indicates that the loss of function resulting from p53 alterations may represent a critical step in human carcinogenesis and could lead to some form of gene therapy based on the restoration of expression of p53 in defective cell lines. However, previous efforts to influence the growth potential of tumor cells expressing endogenous wild-type p53 by transfection of the p53 gene were not successful (2, 4), although the reason for this failure is poorly understood. We now demonstrate that expression of exogenous wild-type p53 may lead to the growth arrest of highly proliferative tumor cells constitutively expressing endogenous wild-type p53. The status of the p53 gene in this cell line was analyzed previously by full-length sequencing of the entire coding region (14). Hut292DM cells do not contain a p53 mutation, consistent with our present Western blot analysis showing the expression of trace amounts of p53 protein in these cells. At present, we may only speculate as to how exogenous expression of p53 results in growth suppression of Hut292DM cells, perhaps by overwhelming the normal regulatory mechanism of p53 function. To date, possible mechanisms include p53 phosphorylation or interaction with another protein (18). An alternative explanation for the capability of additional exogenous wild-type p53 to inhibit cell growth may reside in the very short half-life associated with wild-type p53 protein as compared with the prolonged half-life of mutant forms of the protein. It may be argued that specific proteases are involved in the degradation and elimination of p53 function throughout the cell cycle and that when a critical level of p53 protein synthesis is reached these proteolytic events are no longer able to control overexpression of wild-type p53. Furthermore, a negative feedback mechanism for p53 expression has recently been described whereby p53 represses the transcriptional activity of the p53 natural promoter (19). Evidently, this negative regulatory mechanism was not effective in our transfection studies, which used a cytomegalovirus heterologous promoter for exogenous p53 expression. We do not necessarily favor a mere toxic effect elicited by high levels of wild-type p53 expression, as previous studies using identical expression vectors have shown that certain tumor cells tolerate such levels of expression without any observed effect (2, 4). Therefore, our present work extends the scope of the potential effectiveness of wild-type p53 to control tumor growth to recipient cells that contain no apparent defect in endogenous wild-type p53 expression. As our knowledge of p53 regulatory mechanisms increases it will be of interest to compare our results with those of other model systems, which may help understand why Hut292DM cells are sensitive to exogenous wild-type p53 expression.

Our data also indicate that although expression of exogenous p53 is incompatible with Hut292DM cell growth in vitro, we were able to isolate stable transfection clones expressing various truncated forms of p53 protein. Cells expressing high levels of truncated p53 protein were not, however, affected in their in vitro growth properties. This may indicate that a critical domain of the molecule which mediates tumor cell growth control in vitro has been deleted from the final translation product, possibly as a result of gene rearrangement or point mutation leading to a stop codon. The lack of any apparent inhibition of
growth $in \textit{vitro}$ displayed by cells expressing various truncated p53 protein was in sharp contrast to the tumorigenic potential of X833.W2 cells, which synthesize a high level of a Mr 45,000 p53 truncated protein and consistently displayed growth inhibition $in \textit{vivo}$. Although our observations to date are based on a single clonal event, the results suggest that growth control mechanisms mediated by p53 differ $in \textit{vitro}$ from $in \textit{vivo}$ situations and that separate domains of the p53 molecule may be responsible for these effects. We are now in the process of sequencing these altered forms of p53 in order to obtain a better definition of the domains possibly involved in growth regulation observed $in \textit{vitro}$ and $in \textit{vivo}$. Although we are only beginning to understand the mechanisms by which the p53 tumor suppressor gene may control abnormal proliferation of cancer cells, this study provides original evidence that a human lung cancer cell line expressing endogenous wild-type p53 can be growth inhibited by high levels of p53 expression. These results should also provide useful information on the domain-function organization of the p53 protein.

References

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