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Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β_1

(carcinogenesis/tumor suppressor gene/heat shock protein)

BRENDA I. GERWIN*, ELISA SPILLARE*, KATHLEEN FORRESTER*, TERESA A. LEHMAN*, JENNIFER KISPERT*, JUDITH A. WELSH*, ANDREA M. A. PFEIFER†, JOHN F. LECHNER*‡, SUZANNE J. BAKER§, BERT VOGELSTEIN§, AND CURTIS C. HARRIS*¶

*Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; †Genetic Toxicology Section, Nestec, Vevey, Switzerland; and §The Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, MD 21213

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ABSTRACT **Loss of normal functions and gain of oncogenic functions when the p53 tumor suppressor gene is mutated are considered critical events in the development of the majority of human cancers. Human bronchial epithelial cells (BEAS-2B) provide an *in vitro* model system to study growth, differentiation, and neoplastic transformation of progenitor cells of lung carcinoma. When wild-type (WT) or mutant (MT; codon 143$^{Val\text{-}Ala}$) human p53 cDNA was transfected into nontumorigenic BEAS-2B cells, we observed that (*i*) transfected WT p53 suppresses and MT p53 enhances the colony-forming efficiency of these cells, (*ii*) MT p53 increases resistance to transforming growth factor β_1, and (*iii*) clones of MT p53 transfected BEAS-2B cells are tumorigenic when inoculated into athymic nude mice. These results are consistent with the hypothesis that certain mutations in p53 may function in multistage lung carcinogenesis by reducing the responsiveness of bronchial epithelial cells to negative growth factors.**

Mutation of the p53 tumor suppressor gene has been shown to be a frequent event in many types of human cancer (1). Studies of the actions of p53 that contribute to growth regulation, including those showing that the wild-type (WT) gene product is a nuclear phosphoprotein that blocks tumor cell proliferation through arrest at the G_1/S boundary, have been recently reviewed (2, 3). In contrast, although not all mutations in p53 have identical activities (4), most mutations provide cells with a growth advantage (2, 3). Recent studies show that different mutations in the p53 gene lead to proteins that vary in their DNA-binding specificity and affinity (4–7) and activity in transcriptional transactivation assays (8–10). One study of transactivation in different human lung tumor cell lines indicates that the cell line, as well as the kind of mutation, has a major influence on the degree of transactivation (58). Therefore, both the cell and the precise mutation will play a critical role in determining the consequences for a specific cell type.

Normal human bronchial epithelial (NHBE) cells represent the progenitor cells for human bronchogenic carcinoma. This tumor results from exposure to such exogenous genotoxic carcinogens as tobacco smoke (11) and radon (12). To study the pathogenesis of this disease, we have utilized the simian virus 40 (SV40) large T "immortalized" cell line BEAS-2B derived from NHBE cells (13). BEAS-2B cells produce SV40 T antigen, are nontumorigenic, and respond to treatment with serum or transforming growth factor β_1 (TGF-β_1) by ceasing cell division and initiating terminal squamous differentiation (14, 15). Using this *in vitro* model system, we have examined growth regulation and tumorigenicity of BEAS-2B cells expressing either WT-p53 or the codon 143^{Ala} mutant MT-p53 gene contained in a vector driven by the cytomegalovirus promoter (16). We have found that, despite measurable growth-suppressive effects from exogenous WT-p53 gene expression in BEAS-2B cells and unlike Rat-1 cells and human tumor cell lines (16–21), BEAS-2B cells can sustain exogenous WT-p53 expression for >120 population doublings (PDs). In contrast, growth-stimulatory effects of expression of the MT-p53 gene are readily detectable and clonal derivatives expressing this mutant at high levels undergo tumorigenic conversion.

MATERIALS AND METHODS

Transfection and Cloning. BEAS-2B cells (passage 67) were transfected by strontium phosphate precipitation (22) with 10 μg of DNA from pC53-SN, pC53-CX3, or CMV-neo (16). After 14 days of selection in LHC-9 medium with 125 μg/ml of G418 (Geneticin, GIBCO/BRL) and 3% chemically denatured serum (Upstate BioTechnologies, Lake Placid, NY) (23), mass cultures were established and expanded. Mass cultures of BEAS-2B transfected with pC53-CX3 (2B-MT-p53) or pC53-SN (2B-WT-p53) (PDs 143 and 59, respectively) were plated at 1×10^3 cells per 10-cm fibronectin/collagen-coated dish. After 7–8 days when colonies contained ≈100 cells, colonies were isolated in cloning cylinders and plated for expansion.

Colony-Forming Efficiency (CFE) and Growth Rate. Cells were plated in triplicate at 1×10^3 cells per 60-mm fibronectin/collagen-coated tissue culture dish in LHC-9 medium or, for TGF-β_1 experiments, in LHC-8 medium (24) containing the concentrations of TGF-β_1 shown in Fig. 4 (human TGFβ_1, R & D Systems, Minneapolis). After 8–10 days, formalin-fixed and crystal violet-stained dishes (16 dishes per cell line) were counted four times using an Autocount image analyzer (Dynatech) to determine colonies (≥15 cells per dish). Growth rates were determined by plating cells as above and fixing and staining three dishes per day at 24-hr intervals for 8 days. The number of cells per colony was determined using a R&M Bioquant image analysis system (30 colonies per dish; 90 colonies). PDs (mean ± SD) are $\log_2$(cells per colony).

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Abbreviations: TGF-β_1, transforming growth factor β_1; PD, population doubling; CFE, colony-forming efficiency; WT, wild-type; MT, mutant; NHBE, normal human bronchial epithelial; SV40, simian virus 40; T antigen, large T antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp, heat shock protein.

‡Present address: Lovelace, Inc., Albuquerque, NM 87185.
¶To whom reprint requests should be addressed at: National Cancer Institute, Building 37, Room 2C05, Bethesda, MD 20892.

Tumorigenicity. Tumorigenicity was defined as the ability to induce tumor formation ($\geq$125 mm^3) upon inoculation of 5 $\times$ 10^6 cells s.c. into athymic nude mice irradiated with 350 rads (1 rad = 0.01 Gy) 24 hr prior to inoculation.

Immunoprecipitation. ^{35}S-labeled cell lysates were prepared and immunoprecipitation analysis was performed as described (25). Briefly, 2 $\times$ 10^7 cpm of acid-precipitable lysate were immunoprecipitated with 50 μl of pAb421 (anti-p53, Oncogene Sciences, Mineola, NY), 1 μl of anti-heat shock protein 70 (hsp70) peptide serum (26), or 50 μl of pAb416 (anti-SV40-T, Oncogene Sciences). The immune complexes were collected by addition of Pansorbin cells (Calbiochem) and analyzed as described (25).

RNase Protection Assays. To measure endogenous and exogenous p53 mRNA, a ^{32}P-labeled antisense RNA probe was synthesized from a riboprobe vector containing a region of the 3′ untranslated sequence of human p53 from nucleotide (nt) 1750 to 2138 (16) in the Bluescript SKII(+) (Stratagene) vector. Endogenous mRNA from human cells protect all of this sequence, whereas the exogenous p53 cDNAs terminate at nt 1917. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe was constructed by subcloning rat GAPDH cDNA from nt 167 to 632 (27) into Bluescript SKII(+). Human total cellular RNA generates several protected fragments from the rat antisense probe due to cleavage at nonhomologous positions. These protected fragments have been shown by densitometry to accurately represent the amount of RNA loaded in probe excess (data not shown).

For protection assays, riboprobes were synthesized with the T7 (p53) or T3 (GAPDH) polymerase *in vitro* transcription system (Promega) using 50 μCi (p53) or 12.5 μCi (GAPDH) of [α-^{32}P]CTP (Amersham, 400 Ci/mmol; 1 Ci = 37 GBq) and riboprobes purified by electrophoresis in 5% denaturing PAGE. Bands were excised and extracted in 2 M NH_4OAc/0.1% SDS overnight at 37°C. Eluted probes were ethanol precipitated and dissolved in hybridization buffer (85% formamide/0.4 M NaCl/1 mM EDTA/40 mM Pipes, pH 6.7).

Total cellular RNA prepared by acid-guanidinium extraction was hybridized to the p53 (10^5 cpm) and GAPDH (10^5 cpm) riboprobes for 12–18 hr at 58°C. RNase A digestions were performed for 1 hr at 30°C by adding 300 μl of buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.3 M NaCl) containing 35 μg of RNase A per ml (Sigma, type IIIA) and were terminated by adding 15 μl of proteinase K (10 mg/ml) and 15 μl of 10% SDS and incubating for 15 min at 37°C. Reaction products were extracted with phenol/chloroform and ethanol precipitated. Fragments were separated by electrophoresis on 5% denaturing PAGE for 2–3 hr at 250 V. Gels were exposed to XAR5 (Kodak) film at −70°C.

Results were analyzed on a Molecular Dynamics laser densitometer. In-lane backgrounds were individually subtracted from each positive band and signals for endogenous and exogenous p53 protection were normalized for the GAPDH internal control. Since no absolute quantitative standards were available, units were adjusted to the value obtained at PD 53. In general, the level of endogenous expression was two to three times higher than exogenous WT-p53 expression at the earliest passage measured.

RESULTS

Expression of Exogenous WT-p53 or MT-p53 in Transfected Mass Cultures. BEAS-2B cells at 25 or 83 PDs, after transfection with WT- (2B-WT-p53) or MT-p53 (2B-MT-p53) and subsequent G418 selection, were tested for expression levels of endogenous and exogenous p53 by an RNase protection assay. The WT- and MT-p53 exogenous genes are expressed in transfected BEAS-2B cells for at least 83 PDs (Fig. 1). Direct DNA sequencing of the exogenous WT-p53 at 83 PDs reveals WT sequence, ruling out the possibility of a dominant MT clone arising in the mass culture (data not shown). Expression of the exogenous MT-p53 was retained during culture expansion at all stages tested up to 190 PDs. Direct DNA sequencing of all 11 exons of the BEAS-2B endogenous p53 gene demonstrated that it, like the WT-cDNA construct, contained the codon 72 polymorphism and, in addition, a C $\rightarrow$ T transition at codon 47 resulting in the substitution of proline by serine (data not shown). This mutation was also found in normal esophageal tissue from the donor of the original bronchus (data not shown) and therefore most likely represents a germ-line polymorphism in this individual.

The consequences of the codon 47 alteration as well as the exogenous p53 expression for protein production and binding to T antigen and hsp70 were explored by immunoprecipitation (Fig. 1*B*). The BEAS-2B control cells contain detectable p53 protein, as would be expected for T-antigen-expressing cells (28–32). Furthermore, this protein is coimmunoprecipitated with T antigen though there is no detectable hsp70–p53 complex in BEAS-2B or 2B-WT-p53 cells, indicating that the endogenous p53 as well as exogenous WT-p53 is WT with respect to the conformational parameters required for these protein-binding properties (29, 33–38). However, in 2B-MT-p53 cells, where the 143^{Ala} p53 protein is expressed, a hsp70–p53 complex can be detected as well as an increment in the p53 protein that is complexed to T antigen. These results demonstrate that the expected complexes are present and suggest that the 143^{Ala} p53 may itself have some ability to form complexes with T antigen or may aggregate with the endogenous WT-p53 protein in such complexes.

Effect of Exogenous p53 Expression on Growth of BEAS-2B Cells. The isolation of BEAS-2B cell lines overexpressing either WT- or MT-p53 allowed us to determine whether these genes would have measurable effects on the CFE and/or growth rate of the parental BEAS-2B cell line. Fig. 2 demonstrates depression of the CFE of BEAS-2B cells in response to overexpression of WT-p53. Whereas the CFE

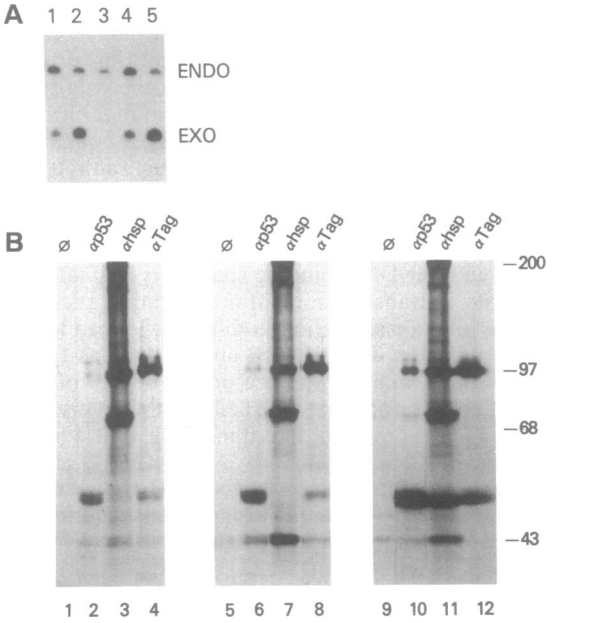

FIG. 1. Measurement of p53 RNA and protein in 2B-WT-p53 and 2B-MT-p53 mass cultures. (*A*) Expression of endogenous (ENDO) and either WT or MT exogenous (EXO) p53. RNase protection experiments were performed as described in the text. Lanes 1 and 2, 2B-WT-p53 and 2B-MT-p53 at PD 35 posttransfection; lane 3, BEAS-2B control; lanes 4 and 5, 2B-WT-p53 and 2B-MT-p53 at PD 83 posttransfection. (*B*) Immunoprecipitation analysis of p53 protein. Cell lysates were prepared and analyzed as described in the text. Lanes 1–4, BEAS-2B cells; lanes 5–8, 2B-WT-p53 cells; lanes 9–12, 2B-MT-p53 cells. ø, No antibody.

value (mean ± SD) for the control BEAS-2B cells is 18% ± 4%, the value for 2B-WT-p53 is 9% ± 1% and that for 2B-MT-p53 is 26% ± 2%. These differences are significant ($P < 0.0005$; Student t test). In contrast, whereas the doubling rate of the 2B-MT-p53 cells is greater than that of the 2B-WT-p53 cells, the growth rate of clonogenic cells is less significantly affected than their numbers as measured in the CFE assay (data not shown).

Loss of Exogenous WT-p53 Expression with Increasing PDs. The negative growth effects of WT-p53 expression (Fig. 2), and the decreased RNA expression of exogenous WT-p53 as compared to MT-p53 (Fig. 1), suggest that the 2B-WT-p53 mass culture might lose exogenous WT-p53 expression as a function of culture expansion. To test this hypothesis, steady-state levels of endogenous and exogenous p53 mRNA were determined in 2B-WT-p53 cultures as a function of the number of PDs either with or without the inclusion of G418 in the growth medium. To quantitate the levels of endogenous and exogenous gene expression relative to an internal standard, a riboprobe for GAPDH was included (see *Materials and Methods*). There is a gradual selective loss in the expression of exogenous WT-p53 (Fig. 3). Surprisingly, the time course of this loss was not affected by G418. The steady-state expression level of the endogenous gene is always higher than that of the exogenous gene and expression of the endogenous p53 increases as the expression of the exogenous WT-p53 decreases. This observation suggests some regulatory control by WT-p53 protein on p53 gene expression.

Effect of MT-p53 or WT-p53 Overexpression on the TGF-β_1 Responsiveness of BEAS-2B Cells. The ability of TGF-β_1 to modulate growth of the BEAS-2B, 2B-WT-p53, and 2B-MT-p53 cells was compared by measuring the effect of increasing doses of TGF-β_1 on the CFE of these cell lines (Fig. 4*A*). All three cultures show a decrease in CFE but the 2B-MT-p53 cells are less inhibited. The similarity in the dose of TGF-β_1 required for 50% decrease in CFE for all three cell lines suggests the existence of some subpopulations with comparable responsiveness and others that might be totally resistant. This possibility was explored by isolating subclones from the 2B-WT-p53 and 2B-MT-p53 cultures at PD 59 or PD 143, respectively. These clonal cultures were expanded and tested for TGF-β_1 responsiveness. The results shown in Fig. 4*B* indicate that the original cultures did indeed contain subpopulations with varying TGF-β_1 responsiveness. There is also a significant range of responsiveness in 2B-WT-p53 and 2B-MT-p53 subclones. Measurement of exogenous p53 mRNA steady-state levels indicated that there was not a quantitative relationship between this parameter and responsiveness to TGF-β_1 (data not shown). However, the data clearly indicate that the 2B-MT-p53 subclones are all less responsive to TGF-β_1 than the 2B-WT subclones.

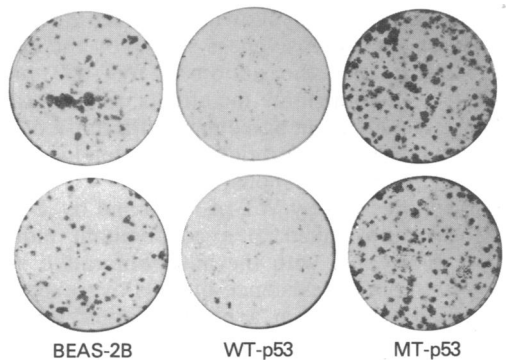

FIG. 2. CFE of BEAS-2B cultures overexpressing WT- or MT-p53. Dishes were seeded with 1×10^3 cells and were grown for 8 days. CFE values were calculated as described.

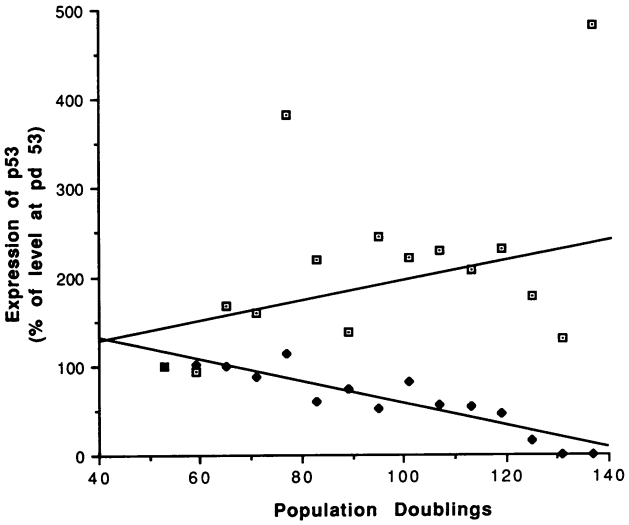

FIG. 3. Loss of expression of exogenous p53 mRNA in the 2B-WT-p53 mass culture: changes in steady-state mRNA expression levels for exogenous and endogenous p53 as a function of culture expansion. RNase protection data from two independent experiments were quantitated by laser densitometry and normalized to the mRNA levels measured at the outset of the experiment (PD 53). These assays incorporated a GAPDH riboprobe for quantitation. Expression measured from the endogenous p53 gene was severalfold higher than that measured from the exogenous p53 and, at PD 35, was indistinguishable from that of the untransfected control. ■, Exogenous p53/GAPDH; ⊡, endogenous p53/GAPDH.

Induction of Tumorigenicity by MT-p53. Subclones of 2B-MT-p53 were tested for tumorigenicity in athymic nude mice. The results (Fig. 5) show that three of four clones generated progressively growing cystic tumors. The histopathology indicates a cuboidal monolayer lining the cystic tumors with local areas of invasion (data not shown). Cell lines derived from these tumors had a decreased latency and increased probability of tumor formation when inoculated into athymic nude mice (Fig. 5). In control experiments, the parental culture, a vector control cell line, and three 2B-WT-p53 subclones did not induce tumors under the same conditions.

DISCUSSION

The effects of overexpression of WT- and MT-p53 have been examined in a model system for lung carcinogenesis. Although there is a steady decline in the exogenous WT-p53 level as a function of PD, this gene is expressed at significant levels for at least 120 PDs (Fig. 3). Thus, BEAS-2B cells can sustain the expression of exogenous WT-p53 as might be expected for a cell line that has not undergone tumorigenic conversion (16, 18, 39). Since WT- and MT-p53 genes are expressed from the same vector, the fact that the steady-state mRNA level of the exogenous WT-p53 gene is much lower than that of the MT-p53 gene suggests control of this expression. This might operate through selection of cells that are low-level expressors of the gene or have lost exogenous WT-p53 expression. Alternatively, the cells may possess the capacity to selectively regulate the expression of WT-p53, even from the cytomegalovirus promoter.

The loss of WT-p53 but not MT-p53 expression from these cells is, in itself, evidence for a negative contribution of WT-p53 gene expression to growth potential, even in the presence of SV40 T antigen. In addition, measurements of CFE and PD time show growth suppression in cells overexpressing WT-p53 and stimulation in cultures expressing the exogenous MT-p53 gene. Thus, in the BEAS-2B cell line, as in SV40 T antigen-transformed hamster cells (39), the pres-

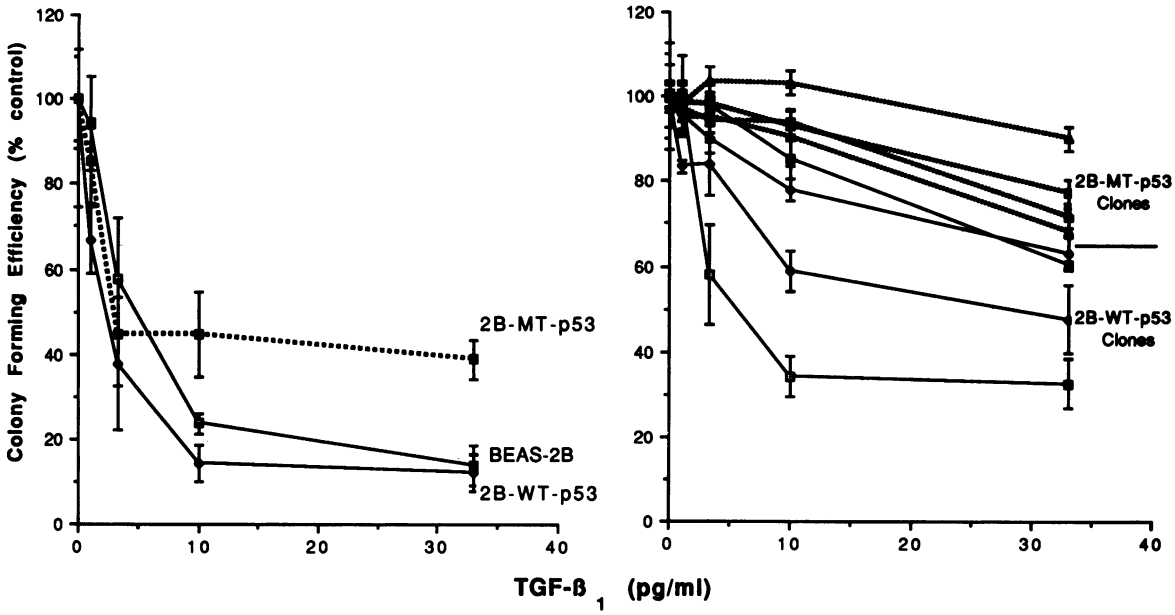

FIG. 4. Responsiveness of cultures to TGF-β_1. (*A*) Sensitivity of BEAS-2B mass cultures to TGF-β_1. The graph shows the inhibition of the CFE of the three cell lines as a function of TGF-β_1 concentration. CFE experiments were performed as described in the text. (*B*) Sensitivity of clonal derivatives of the 2B-MT-p53 or 2B-WT-p53 mass cultures to TGF-β_1. Data like that in *A* are shown for eight clonal cultures; four were subclones of 2B-MT-p53 (broken lines) and four were subclones of 2B-WT-p53 (solid lines).

ence of T antigen may diminish the growth suppressive effects of WT-p53 but it does not negate them. In this regard, only a small percentage of the total p53 available to the anti-p53 antibody (Ab421) was precipitated as part of a T antigen complex in cells sampled at logarithmic phase growth (Fig. 1*B*). Preliminary evidence indicates that expression from the exogenous gene in 2B-WT-p53 subclones is a logarithm lower than in 2B-MT-p53 subclones (data not shown). The possible interaction between endogenous and exogenous WT expression suggests the possibility of down-regulation of the endogenous gene by its product, perhaps through binding to a negative regulatory region such as that mapped upstream of promoter p53P1 (40). This transfected

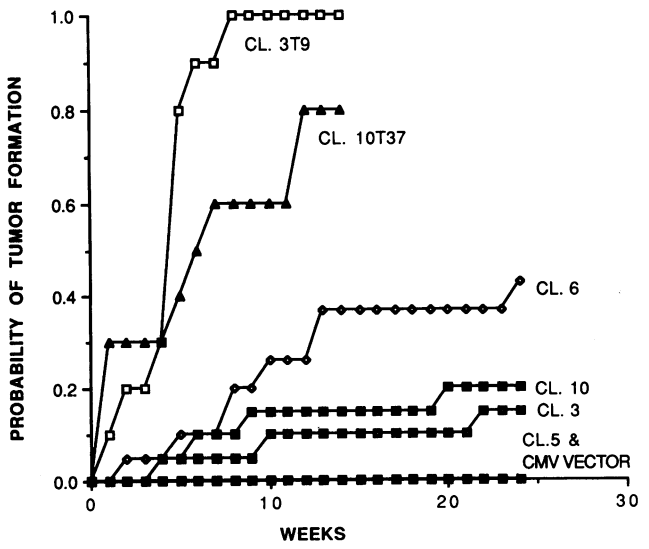

FIG. 5. Tumorigenicity of 2B-MT-p53 subclones. Each curve presents the probability of tumor formation as a function of time after inoculation for subclones of 2B-MT-p53 as well as for explant cell lines derived from clone 3 (3T9) or clone 10 (10T37) tumors. Four of 21 tumors from 2B-MT-p53 subclones regressed. Growing tumors reached a mean size of 1157 mm^3 and a maximum size of 7140 mm^3 at 48 weeks. CMV, cytomegalovirus.

BEAS-2B cell system should allow critical testing of this hypothesis utilizing p53 promoter–reporter gene constructs (40).

Previous experiments have shown that TGF-β_1 is growth inhibitory for most normal epithelial cell types (41, 42). In NHBE cells, TGF-β_1 growth inhibition is correlated with the initiation of terminal squamous differentiation (43). In contrast, lung carcinoma cells are largely insensitive to induction of differentiation or negative regulation by TGF-β_1 and serum (44, 45). These observations are consistent with the suggestion that loss of responsiveness to TGF-β_1 provides a selective advantage leading to clonal expansion and are supported by the observations (46) that human colonic adenoma but not colon cancer lines are inhibited by TGF-β. In the BEAS-2B model of lung carcinogenesis, it has been shown that nontumorigenic BEAS-2B cells are growth inhibited and induced to undergo terminal squamous differentiation by TGF-β_1 (43), whereas cells that have undergone tumorigenic conversion after oncogene transfection acquired resistance to TGF-β_1 growth inhibition (47). The present study was designed to test the ability of MT-p53 to confer resistance to growth inhibition by TGF-β_1 and to induce tumorigenicity. Expression of exogenous WT- as well as MT-p53 in BEAS-2B cells provides the opportunity to evaluate the influence of p53 protein on growth inhibition of this cell line by TGF-β_1. The decreased responsiveness to TGF-β_1 shown by the 2B-MT-p53 culture and the increased responsiveness shown by the 2B-WT-p53 culture suggest that the action of WT-p53 protein in BEAS-2B cells can facilitate the effects of TGF-β_1 on these cells. These results are in agreement with those of Wyllie *et al.* (48), who observed a correlation between loss of WT p53 function and TGF-β unresponsiveness.

Growth suppression by WT-p53 (49) and by TGF-β_1 (50) has recently been linked to a concomitant induction of apoptosis, in keeping with the antiproliferative synergism noted in this report. Resistance of cells overexpressing the MT-p53 could result from a positive interference by the mutant protein with TGF-β_1 inhibition. For example, mutations in p53 could alter nucleic acid binding specificity (7) or affinity (5), modifying the gene regulatory functions of the protein. Alternatively, a blockage of the effects of WT-p53 through formation of oligomeric complexes (3) could account

for these effects. A combination of these interpretations would hold if cotranslation of WT-p53 and MT-p53 in this cell line from the endogenous and exogenous mRNAs resulted in a shift of the WT protein into a MT conformation, as has been reported for *in vitro* translation systems (51).

Although a wide range of responsiveness to TGF-β_1 exists among the subclones of both transfected mass cultures (Fig. 4*B*), the observation that all clones expressing exogenous MT-p53 are more resistant to TGF-β_1 than all clones expressing the exogenous WT-p53 gene strongly suggests that the cascade of effects induced by the presence of these exogenous proteins intersects with changes induced by TGF-β_1. Increased expression of WT-p53 appears to potentiate these changes, whereas expression of 143^{Ala} MT-p53 interferes with their induction. Interestingly, TGF-β_1 induces a decrease in c-*myc* expression that correlates with growth inhibition and this effect is blocked by expression of HPV-16 E7, adenovirus 5 E1A, or SV40 T (52–54). Since the p53 promoter region contains a *myc*/MyoD consensus binding site (55), a shift in the various binding interactions of the *myc* protein may constitute an area of intersection between p53 and TGF-β_1 in control of cellular growth.

Loss of growth regulation and/or inducibility for differentiation can correlate with a gain in tumorigenic potency (46, 56). Therefore, the acquisition of resistance to TGF-β_1 by 2B-MT-p53 clones suggested that they had undergone progression in the pathway to tumorigenic conversion. The observation reported here that 2B-MT-p53 clones from the transfected BEAS-2B culture are tumorigenic provides strong evidence for a positive contribution of at least this MT-p53 protein to tumorigenicity. In this instance, the MT p53 protein contains the tumor-derived 143^{Ala} mutation. It is expected that different mutations will produce different consequences, as has been shown in other systems (3, 57). The positive acquisition of tumorigenicity in this case suggests the usefulness of BEAS-2B cells for evaluating positive oncogenic contributions of various p53 mutations. The decreased latency and increased tumorigenic potential of tumor explant lines (Fig. 5) suggest that progression has occurred. It will be of interest to evaluate whether this results from acquisition of other identifiable changes commonly associated with lung cancer (11).

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