Involvement of the Tumor Suppressor Gene p53 in Tumor Necrosis Factor-induced Differentiation of the Leukemic Cell Line K562

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Abstract
The cDNA of the human wild-type p53 tumor suppressor gene was constitutively overexpressed in the leukemic cell line K562 (which lacks detectable amounts of p53 protein) in order to investigate the consequences for growth and differentiation. Several stable clones were established by transfection of the expression vector pc53S/N3. Expression of p53 protein was characterized by biosynthetic labeling and immunoprecipitation with the monoclonal antibodies pAb 1801 (reacting with wild-type and mutant human p53), pAb 240 (reacting with mutant human p53) and pAb 1620 (reacting with wild-type human p53). All clones which were 1801+, 240−, 1620− or 1801+, 240−, 1620+ were defined as "wild-type-like p53-expressing" clones. Our results show that expression of p53 protein is compatible with continuous proliferation of K562 cells. The growth characteristics of wild-type-like p53-expressing clones did not differ from that of control clones. However, the former were more sensitive than p53-negative control clones to growth inhibition by tumor necrosis factor (TNF), a cytokine with a potential role in growth and differentiation of myeloid leukemic cells. In addition, a 2- to 4-fold increase of the amount of hemoglobin, a marker of erythroid differentiation, was observed when wild-type-like p53 protein-expressing clones were incubated with TNF. This suggests that differentiation is the mechanism responsible for the increased TNF sensitivity of these clones. Our results support a role for p53 in mediating growth inhibitory and differentiation inducing signals by TNF.

Introduction
Several lines of evidence indicate that normal function of the tumor suppressor gene p53 is important for maintaining the benign phenotype of mammalian cells. For instance, functional inactivation of p53 (by a variety of mechanisms) is probably of importance for the development of several cancers (1–7). Moreover, overexpression of wild-type p53 protein in various cell lines often leads to reversion of the malignant phenotype (8–11).

The exact biological function of p53 is unclear but it seems to be closely related to cell cycle control. G1 arrest followed by certain kinds of DNA damage has been shown to depend upon a normal regulation of wild-type p53 expression (12–14). This allows DNA repair mechanisms to operate before continued progression into the cell cycle occurs. A mechanism by which wild-type p53 can induce G1 arrest has recently been clarified (15, 16). Wild-type p53 also seems to be important for the induction of apoptosis. For example, overexpression of wild-type p53 induces apoptosis in the myeloid cell-line M1 lacking endogenous p53 (17, 18). G1 arrest and induction of apoptosis may provide important defense mechanisms against survival and accumulation of genetically altered cells.

Besides playing an important role in controlling proliferation and apoptosis, p53 is probably involved in the regulation of differentiation. Some evidence supporting this notion exists. For example, reintroduction of wild-type p53 in a pre B-cell line leads to partial differentiation (19). Similarly, overexpression of wild-type p53 protein in leukemic K562 cells, HL-60 cells, or Friend virus-transformed erythroleukemic cells induces signs of differentiation (20–22). Yet another example is the appearance of differentiation markers in squamous carcinoma cells upon overexpression of wild-type p53 (23). On the other hand, transgenic mice lacking the p53 gene display an apparently normal embryonic development, thus questioning an important role for p53 in the differentiation process (24).

In leukemia, functional inactivation of p53 does not seem to be a general phenomenon (25–30). However, some immortalized leukemic cell lines (such as the human myeloid leukemic cell line K562) have lost the expression of p53, presumably as a step in the process of immortalization (31, 32). In vitro, it is possible to induce differentiation of leukemic cell lines with various agents. Included among such agents are, for example, sodium butyrate, (all-trans) retinoic acid, and cytokines such as TNF3 (33–35). The sensitivity for such agents varies among different cell lines. For instance, TNF can induce differentiation in the leukemic cell line HL-60 but not in the myeloid leukemic cell line K562 (35).

Would it be possible to restore genetic programs of differentiation by reintroducing p53 in a leukemic cell line lacking p53 such as K562? To answer this question, we decided to artificially express p53 in K562 cells by transfection of wild-type p53 cDNA.

Transient overexpression of wild-type p53 in several tumor-derived cell lines leads to a dramatic inhibition of growth (8–11). We found, however, that constitutive overexpression of wild-type p53 in K562 cells is compatible with continuous proliferation. Moreover, overexpression of wild-type p53 in K562 cells resulted in increased sensitivity.

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1 The abbreviations used are: TNF, tumor necrosis factor; CMV, cytomegalovirus; FBS, fetal bovine serum.
to TNF-induced growth inhibition. This growth inhibition appeared to depend, at least partly, on increased disposition towards differentiation.

Results
Transfection of pc53SN3 and SN3 and Determination of Genomic Integration of Transfected DNA by PCR. By electroporation, cells were transfected with plasmid DNA as described in "Materials and Methods." Equal numbers of wells with electroporated and untreated (negative control) cells were seeded with one thousand cells each. After 3 to 5 weeks of culture with geneticin, no viable cells were observed in the negative control wells. Seventy clones arose from one million cells electroporated with the pc53SN3 plasmid. Twelve of these pc53-transfected clones were randomly picked, of which eight had integrated exogenous p53 as judged by PCR (data not shown). These eight clones were further expanded to mass cultures and designated K562/pc53SN3/A4, A11, A19, A29, A30, A50, A54, and A55, respectively. With the intention of obtaining control clones, one million cells were also electroporated with the SN3 plasmid in exactly the same way. Because of the lack of p53 cDNA, these clones were called mock transfectants. From these cells, three hundred clones arose, which was four times more than from the cells transfected with pc53SN3, thus suggesting a selection pressure against the integration of p53 cDNA into the genome. Six of these mock-transfected clones were randomly picked, expanded to mass cultures, and called K562/SN3/M1, M2, M3, M4, M5, and M6, respectively.

Characterization of the Exogenous p53 Protein by Biosynthetic Labeling and Immunoprecipitation and Characterization of in Vitro Translated Wild-type p53 by Immunoprecipitation. p53 is frequently mutated in human tumors. In many cases, a specific mutation gives rise to a protein with an altered conformation, thus exposing other epitopes and changing the immunoreactivity with different p53-specific antibodies. Several monoclonal p53-specific antibodies have been raised, some of which are specific for wild-type p53, and others are specific for mutant forms of p53. Mutation of p53 often seems to be an important step in the evolution of many tumors (1, 2). Because of this fact and the observation that there seemed to be a
Table 1. p53-transfected clones A11, A19, A29, A30, A55 and the mocktransfected clone M1 and their respective reactivity with different p53-specific antibodies.

<table>
<thead>
<tr>
<th>Clone</th>
<th>pAb 1801</th>
<th>pAb 240</th>
<th>pAb 1620</th>
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<tr>
<td>M1</td>
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<tr>
<td>A19</td>
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The pAb 1801 reacts both with wild-type and mutant human p53. The pAb 240 reacts only with mutant human p53. The pAb 1620 reacts only with wild-type human p53 (although some mutant forms may react).

Fig. 2. In vitro translation of p53. RNA was transcribed in vitro from p53BSK− and translated in vitro with 125Icysteine as described in "Materials and Methods." The translation product was immunoprecipitated with indicated antibodies and subjected to SDS-PAGE and fluorography. The translation product prior to immunoprecipitation is also shown as a positive control. Molecular weight markers are indicated to the left (kilodaltons). p53 is indicated with an arrow to the right.

Fig. 3. Clonogenic growth of p53-transfected and mock-transfected clones. Cells were cultured in soft agar, and the number of colonies (>40 cells) was determined after 15 days as described in "Materials and Methods." Cells were plated in duplicate. The growth of the mock-transfected clones M1–M6 and of the p53-transfected clones A19, A11, A29, A30, and A55 is shown as the percentage of colonies relative to the number of seeded cells (clonogenicity). The mean values for each of the six mock transfectants M1–M6 and each of the p53 transfectants (all determined from three separate experiments) are shown. Bars, SEM. There was no statistically significant difference in clonogenicity between the group of wild-type-like p53-transfectants A11, A29, A30, and A55 and the group of mock transfectants M1–M6. Nor was there a significant difference between the mutant transfectant A19 and the group of mock transfectants.

as the transfected clones A11, A29, and A30. The reactivity of the specific antibody pAb 1620 is known to be weak compared to the other specific p53 antibodies (39, 40). Thus, the lack of reactivity with the wild-type-specific antibody pAb 1620 in the transfected clones does not seem to exclude the existence of wild-type p53 protein. The transfectants A11, A29, A30, and A55 were defined as "wild-type-like" and the transfectant A19 as "mutant."

The transfected clone K562/pc53SN3/A4 did not express any p53 protein, as judged by the lack of reactivity with any of the specific p53 antibodies (data not shown). Therefore, this clone was not used in the subsequent experiments. The expression of the p53 protein in the transfected clones K562/pc53SN3/A50 and A54 was not stable, since no reproducible pattern of immunoprecipitation was found on different occasions (data not shown). Thus, we were not able to characterize the quality of p53-expression in these clones. For this reason, they were excluded from further experiments.

Morphological Characteristics of Transfected Cells. Under certain circumstances, the expression of wild-type p53 in some cells is known to cause apoptosis or differentiation (17–23). For this reason, the p53 transfectants were examined for changes in morphology as compared to the parental cell line as described in "Materials and Methods." In general, the transfectants displayed a somewhat more heterogeneous picture with more mitoses, more vacuoles, more multinucleated cells, and more giant cells. There were, however, no obvious signs of a different phenotype in the transfectants as compared to the parental cell line. Thus, K562 cells seemed to tolerate p53 expression without obvious changes in morphology.

Growth Characteristics of Transfected Cells. Several investigators have demonstrated that transient expression of wild-type p53 is incompatible with continuous cell proliferation (9–11). In order to determine the effect of stable p53 gene expression on the clonogenic growth properties in soft agar, cells were plated as described in "Materials and Methods." After 15 days of culture, the number of colonies containing more than 40 cells was determined. In Fig. 3, the clonogenicity of the wild-type-like p53 transfectants and
that of the mock-transfectants and the mutant transfec tant K562/p53SN3/A19 is shown. The difference in clonogenicity between the group of wild-type-like p53 transfec tants and the group of mock transfectants was not statistically significant. Nor could we find any statistically significant difference between the mutant transfec tant K562/p53SN3/ A19 and the group of mock transfectants. This indicates that stable expression of wild-type-like p53 in K562 cells is compatible with cell proliferation. In order to determine the effect of p53 expression on the growth rate in suspension culture, cells were counted daily for 4 days. No differences in growth rate between the transfected clones and the mock-transfected clones could be observed (Fig. 4). Again, this indicates that stable expression of wild-type-like p53 is not inconsistent with continuous growth of K562 cells.

Effects of TNF, all-trans Retinoic Acid, and Sodium Butyrate on Growth Characteristics of Transfected Cells.

Previous work has shown that several agents such as TNF, all-trans retinoic acid, and sodium butyrate are capable of inducing differentiation in certain hematopoietic cells (33–35). We were interested in determining if the expression of p53 confers an increased sensitivity to the action of these agents. With the intention of determining this, soft agar cultures were made and plated with cells as described in "Materials and Methods" with different concentrations of TNF, all-trans retinoic acid, or sodium butyrate. As shown in Fig. 5A, rising concentrations of TNF led to dose-dependent reduction of clonogenic growth for all p53-transfected clones. However, the wild-type-like transfectants (K562/ pc53SN3/A11, A29, A30, and A55) were clearly more sensitive to the action of TNF than the mock transfectants or the mutant transfec tant K562/p53SN3/A19. In control experiments, the cells of the six mock transfec tants (K562/ SN3/ M1-M6) were plated with TNF in an identical way. As shown in Fig. 5B, the clonogenic growth of the mock transfec tants was also influenced by TNF in a dose-dependent manner. The influence of TNF was, however, clearly less pronounced than that seen in the experiments with the wild-type-like p53 transfec tants. For each concentration of TNF in the range 10^-12-10^-8 M, there was a statistically significant difference (P < 0.001) in TNF-induced inhibition of clonogenic growth between the group of wild-type-like p53 transfec tants and the group of mock transfectants. This indicates that expression of wild-type-like p53 may lead to an increased sensitivity to TNF. The sensitive clones were the ones expressing p53 protein not reacting with the mutant-specific antibody pAb 240 (240-negative (wild-type- like); A11, A29, A30, and A55), whereas the clone expressing a mutant form of p53 [240-positive (mutant); A19] showed no increased sensitivity to the action of TNF. When cells were exposed to all-trans retinoic acid or sodium butyrate, a dose-dependent inhibition of clonogenic growth was observed, but no difference in clonogenic growth between p53 transfec tants and mock transfectants could be observed (data not shown).

In order to determine the effect of TNF on proliferation in suspension culture, transfected clones were incubated with TNF at different concentrations, then counted daily for 4 days as described in "Materials and Methods." As shown in Fig. 6, the wild-type-like transfec tants K562/pc53SN3/A11, A29, A30, and A55 displayed a dose-dependent inhibition of growth when exposed to TNF. This effect was most pronounced for the transfec tant A55. No growth inhibition of the mutant transfec tant K562/pc53SN3/A19 or the mock

Fig. 4. Growth in suspension culture of p53-transfected and mock-transfected clones. Cells were grown in suspension culture and counted daily as described in "Materials and Methods." The growth of the p53-transfected clones A11, A19, A29, A30, and A55 and the mock-transfected clones M1, M2, and M4 is shown. Results are from one representative experiment.

Fig. 5. Effects of TNF on clonogenic growth of p53-transfected (A) and mock-transfected (B) clones. Cells were cultured in soft agar, and the number of colonies (>40 cells) was determined after 15 days as described in "Materials and Methods." Cells were plated in duplicate. Clonogenic growth is shown as the percentage of the number of colonies in control cultures without TNF. A, at different concentrations of TNF (10^-12-10^-8 M), the mean values for each of the p53 transfec tants A19, A11, A29, A30, and A55 (determined from three separate experiments) are shown. B, the mean values at different concentrations of TNF for each of the mock-transfectants M1-M6 (determined from three separate experiments) are shown. Bars, SEM. For each concentration of TNF used, there was a statistically significant difference (P < 0.001) in inhibition of clonogenic growth between the group of wild-type-like p53-transfected clones A11, A29, A30, and A55 and the group of mock transfec tants M1-M6.
transfectants K562/SN3/M1, M2, or M4 could be observed upon exposure to TNF (Fig. 6). Again, this indicates that expression of wild-type-like p53 leads to an increased sensitivity to the growth inhibitory action of TNF.

**Induction of Apoptosis in the Transfected Cells.** The increased sensitivity to TNF-induced inhibition of growth of the wild-type-like p53 transfectants shown above may depend on induction of apoptosis. Therefore, we were interested in determining if the differences in TNF sensitivity regarding clonogenic growth and growth rate in suspension culture between the wild-type-like transfected cells and control cells were due to induction of apoptosis. Cells were exposed to TNF, and the incidence of apoptosis was determined as described in "Materials and Methods." No significant difference in the incidence of apoptosis could be observed between p53-transfected control cells and p53-transfected cells incubated with TNF (data not shown). Thus, apoptosis did not seem to be the mechanism in cause for the increased TNF sensitivity.

**Hemoglobin Synthesis in the Transfected Cells.** If apoptosis does not seem to be responsible for the observed differences in TNF sensitivity between the wild-type-like p53 transfecteds and the mock transfecteds, then induction of differentiation could be the mechanism in cause. In order to determine the effect of p53 expression on the differentiation-associated hemoglobin synthesis, cell lysates were allowed to react with tetramethyl benzidine in the presence of hydrogen peroxide. An appreciation of the hemoglobin concentration can then be determined spectrophotometrically (38). As shown in Fig. 7, the wild-type-like transfecteds K562/pC53SN3/A11, A29, A30, and A55 seemed to have more hemoglobin than the mutant transfectant K562/pC53SN3/A19 or the mock transfecteds K562/SN3/M1, M2, or M4, thus suggesting partial induction of differentiation (as judged by the amount of hemoglobin) by wild-type-like p53. However, although there was a tendency for a difference in the amount of hemoglobin between the wild-type-like p53 transfecteds and the mock transfecteds or the mutant transfectant, it did not reach statistical significance. Upon incubation with TNF (0.1 nM) of the wild-type-like transfectants A11, A29, A30, and A55, but not of the mock transfecteds or the mutant transfectant A19, a 2- to 4-fold increase of the hemoglobin concentration could be observed (Fig. 7). The difference in increase was statistically significant (P < 0.01) between the group of wild-type-like p53 transfecteds and the group of mock transfecteds. The augmentation of the amount of hemoglobin was dose dependent in response to TNF in the range of 0.01 nM-0.1 nM (data not shown). This indicates that expression of wild-type-like p53 in K562 cells confers an increased sensitivity to induction of hemoglobin synthesis by TNF.
Discussion

We are interested in trying to investigate a role of p53 in the apoptotic and differentiation processes of leukemic cells. For this purpose, a cell system overexpressing wild-type p53 was created and assayed for differentiating and apoptotic potential. After having established stable p53 transfectants in K562 cells, a major problem we had to face was that of the quality of the expressed p53 protein. For several reasons, it is far from evident that the transfectants, although originally transfected with wild-type p53 cDNA, actually do express wild-type p53. Firstly, functional inactivation of p53 often seems to be an important step in the evolution of many tumors (1-7) and probably in the immortalization of some cell lines (32). Secondly, transient expression of exogenous wild-type p53 does not seem to be compatible with growth in many cases (9-11). Moreover, in our transfection experiments, over four times more clones arose from cells electroporated with the plasmid SN3 alone as compared to cells electroporated with p53 cDNA. These data suggest that there was a selection pressure against the expression of wild-type p53 of the transfected K562 cells. Thus, it is possible that the transfected cells, in order to survive, must have abrogated the effects of high levels of wild-type p53. One way for the cell to avoid the obvious inconvenience of expressing a protein with antiproliferative properties would be if most of the expressed p53 in the transfected cells actually is in a mutant form no longer capable of inhibiting growth. To characterize the p53 protein in the transfected clones, monoclonal antibodies with different specific reactivities were used to determine if the constitutively expressed p53 protein was actually in a wild-type or mutant form.

Our results show that one clone (A19) seemed to express a mutant form of p53 (reacting with the mutant-specific antibody 240), despite the fact that it had been originally transfected with wild-type p53 cDNA. The other transfected clones (A11, A29, A30, and A55) did not express mutant p53 as judged by the lack of immunoreactivity with this antibody. Judging from the reactivity with the wild-type-specific antibody pAb 1620, only one clone (A55) seemed to express small amounts of wild-type p53. The fact that the antibody pAb 1620 failed to detect p53 in all clones except A55 may indicate that p53 actually was in a mutant form in these clones. However, the lack of reactivity with pAb 1620 was identical for in vitro-translated wild-type p53, thus indicating that the specific antibody supposed to detect wild-type p53 in fact was not sensitive enough to detect this protein under these circumstances. Moreover, the reactivity of the specific wild-type antibody is known to be weak (39, 40), and it does not seem unreasonable to believe that the difficulties in precipitating wild-type p53 were due to insufficient sensitivity of the pAb 1620. Therefore, we believe that most of the clones (A11, A29, A30, and A55) indeed express wild-type p53 protein, and only one clone (A19) may have overcome possible growth inhibitory effects of wild-type p53 by expressing a mutant form of the protein.

Thus, overexpression of wild-type-like p53 in the K562 leukemic cell line does not seem to be incompatible with continuous cell proliferation. This is in contrast to previous findings where transient overexpression of wild-type p53 in tumor cell lines derived from colon cancer, osteosarcoma, and glioblastoma led to a dramatic inhibition of growth (8-10). However, in these cases, selection for stable clones constitutively expressing wild-type p53 was not made. Moreover, there does seem to be a selection pressure against clones expressing wild-type p53, as judged by the relative difficulties of obtaining clones transfected with wild-type p53 cDNA and the preferential expression of mutant p53 in one clone. The mechanism(s) for the ability of K562 cells to successfully cope with wild-type-like p53 is at present unclear.

Our results show that clones stably expressing wild-type-like p53 display an increased sensitivity to TNF-induced inhibition of growth. The clone expressing a mutant form of p53 (A19) did not seem to be more sensitive than the mock transfectants to the action of TNF. This was true for the clonogenic growth in soft agar as well as the growth rate in suspension culture. It seems reasonable to believe that the mutant clone A19 displays a relative insensitivity to TNF because most of the expressed p53 in this clone is in a mutant conformation.

It could be possible that the differences observed regarding TNF sensitivity were due to increased induction of necrosis or apoptosis by TNF. Overexpression of wild-type p53 can induce apoptosis in myeloid leukemic cells as well as in other cells (41, 42). In addition, induction of apoptosis in mice thymocytes upon ionizing radiation has been shown to depend on expression of wild-type p53 (43). TNF, too, is involved in the process of apoptosis. It is capable of inducing apoptosis in many kinds of tumor cells including several leukemic cells but not K562 cells (35, 44-48). Thus, both p53 and TNF may be involved in the induction of apoptosis. Our results show that TNF did not seem to induce apoptosis in the p53-transfected K562 cells, as judged by their morphological appearance. This suggests that induction of apoptosis is not the mechanism responsible for the reduced clonogenic growth or reduced growth rate in suspension culture of wild-type-like p53 transfectants when incubated with TNF. It is, however, difficult to completely rule out quantitative differences in a morphological assay. Moreover, probably only a minority of the cells are clonogenic (i.e., capable of giving rise to several generations of progeny). Theoretically, it is possible that TNF specifically induces apoptosis in clonogenic cells. This would be difficult to detect in a quantitative assay.

Another explanation for the observed differences regarding TNF-induced inhibition of growth would be if cells expressing wild-type-like p53 are more prone to induction of differentiation by TNF than cells expressing no p53 or a mutant form of p53. Some evidence for the involvement of p53 in the hematopoietic differentiation process exists. For example, overexpression of wild-type p53 in leukemic K562 cells, HL-60 cells, or Friend virus-transformed erythroleukemic cells leads to signs of differentiation in all three cases (20-22). Moreover, it is known that TNF could be involved in the induction of differentiation in myeloid leukemic cells (49-51). Our data suggested signs of partial differentiation in the clones expressing wild-type-like p53 as compared to the other clones. Although the difference in the amount of hemoglobin between wild-type-like p53 transfectants and mock transfectants did not reach statistical significance, others (20) have shown that wild-type p53 induces signs of erythroid differentiation in K562 cells.

What is even more interesting, a 2- to 4-fold increase of the amount of hemoglobin could be observed upon incubation with TNF. Thus, differentiation could be the mechanism in cause for the increased sensitivity to TNF of wild-type-like p53-transfected clones. It is possible that reintroduction of p53 restores parts of genetic programs designed for...
distinguishing geneticin transferred capacitance were host ratiation, of were from All-trans atmosphere supplemented Santa ence selection 240 reacts reads kindly mammalian cell. Cells/mI. Cell mass electroporation vectors were transfected with the restriction enzyme BamHI, followed by religation of the plasmid. Plasmid SN3 was used as a negative control (mock transfectant) in the experiments.

**Antibodies.** The monoclonal anti-p53 antibodies pAb 1801, 240, and 1620 were purchased from Oncogene Science (Uniondale, NY). The monoclonal antibody anti-ras (used as a negative control antibody) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pAb 1801 reacts both with wild-type and mutant human p53. The pAb 240 reacts only with mutant human p53. The pAb 1620 reacts only with wild-type human p53 (although some mutant forms may react).

**Cell Lines.** The human myeloid cell line K562 (36) was cultured in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS in a 5% CO₂ atmosphere at 37°C. Exponentially growing cells were used for all experiments.

**Tumor Necrosis Factor, all-trans Retinoic Acid, and Sodium Butyrate.** Recombinant TNF (produced by Genentech, Inc., South San Francisco, CA) was kindly supplied by Dr. G. Adolph (Ernst Boehringer Institut, Vienna, Austria). All-trans retinoic acid and sodium butyrate were purchased from Sigma Chemical Co., St. Louis, MO.

**Transfection Procedure.** Plasmids pc53SN3 and SN3 were linearized with HindIII in order to facilitate integration of the vectors into the genome of the transfected cells. Cells were harvested at exponential growth. All subsequent steps were performed at 4°C. Cells were washed once in ice-cold transfection buffer [21 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₃PO₄, and 6 mM glucose] and then suspended in transfection buffer at a concentration of 1.2 × 10⁷ cells/ml. The cell suspension (0.8 ml) was incubated with 16 µg of linearized plasmid on ice for 10 min, followed by electroporation. Electroporation was performed using the Bio-Rad gene-pulsor (Bio-Rad, Melville, NY) with a capacitance setting of 25 µF and two alternative voltage settings of 1500 and 1600 V, respectively. After electroporation, cells were again incubated on ice for 10 min, then transferred to fresh culture medium (RPMI + 10% FCS) at a concentration of 0.5 × 10⁶ cells/ml and incubated at 37°C. After 48-72 h, the electroporated cells as well as negative control cells (not electroporated) were distributed in 96-well plates at a number of 1000 cells/well, and genetin (Sigma) at a concentration of 1 mg/ml was added for the selection of stably transfected clones. After selection with genetin for 3-5 weeks, individual clones were expanded to mass cultures and subsequently used in the experiments.

**PCR Analysis.** PCR analysis was used for determination of integration of transected DNA into the genome of the host cell. PCR primers were chosen from different exons, thereby readily (by different sizes of the amplified products) distinguishing transfected p53 cDNA from endogenous genomic p53. The following primers were chosen for detection of p53: upstream primer, 5'-TGTGACGTGGGTTGATTC-3'; and downstream primer, 5'-GAGAGGAGCCTGTTTTCG-3'. DNA from cells was isolated as follows: 1 × 10⁷ cells were washed twice in PBS and then resuspended in 130 µl of PCR buffer with nonionic detergents and proteinase K (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% NP40, 45% Tween 20, and 0.06 µg proteinase K/ml). The mixture was incubated at 55°C for 1 h and then for 10 min at 95°C to inactivate the protease. Twenty-five µl of the mixture was used as template in a 40-cycles PCR reaction performed in a Perkin Elmer Cetus DNA thermal cycler using the primers described above. The amplified products were analyzed on a 2% agarose gel stained with ethidium bromide.

**Bioblastic Labeling and Immunoprecipitation.** Cells were harvested at exponential growth, washed once with Hanks’ balanced salt solution (GIBCO-BRL, Gaithersburg, MD) and then incubated for 30 min at 37°C in methionine- and cysteine-free RPMI 1640 supplemented with 10% dialyzed FBS (GIBCO-BRL) at a concentration of 2 × 10⁶ cells/ml in order to deplete the intracellular pools of methionine and cysteine. Subsequently, the cells (2 × 10⁶/ml) were incubated for 60 min at 37°C with identical medium supplemented with 7-10 µCi/ml of [³⁵S]methionine and [³⁵S]cysteine (DuPont-NEN, Wilmington, DE) to obtain labeling of newly synthesized proteins. Following labeling, all steps were performed at 4°C. The cells were resuspended in a lysis buffer consisting of 50 mM Tris HCl (pH 8.0), 0.15 mM NaCl, 5 mM EDTA (pH 8.0), 0.5% NP40 including the protease inhibitors aprotinin (1 µg/ml), phenylmethylsulfonyl fluoride (100 µg/ml), EDTA (0.5 mM), leupeptin (0.5 µg/ml), and pepstatin (1 µg/ml), followed by incubation on ice for 1 h prior to three sequential 30-s bursts of sonication using a sonicator (Kistner Lab, Stockholm, Sweden). After lysis, the DNA was removed by centrifugation at 37,500 × g for 1 h at 4°C. The supernatant was stored frozen at −20°C until immunoprecipitation. Immunoprecipitation was performed twice. The first immunoprecipitation (preadsorption) was nonspecific, aiming at removing from the supernatant proteins binding nonspecifically to the monoclonal antibodies. For this purpose, a polyclonal mouse IgG-agarose was used. The second immunoprecipitation was specific, aiming at extracting radioactively labeled p53 protein from the supernatant. Preadsorption was performed in the following way: 20 µl of mouse IgG-agarose (Sigma) was added to the supernatant, and immunocomplexes were allowed to form with mouse IgG at 4°C overnight. Next, the solution was centrifuged to remove the IgG-agarose and preadsorbed proteins. The supernatant was subjected to specific immunoprecipitation with the different monoclonal p53 and ras (negative control) antibodies in the same way, except that the immunocomplexes were adsorbed to a mixture of protein A- and protein G-Sepharose (Sigma). After centrifugation, the precipitate was washed four times with lysis buffer. The immunoprecipitated proteins were separated on a 7-20% SDS-PAGE. The gel was dried, and Hyperfilm MP (Amersham, Amersham, United Kingdom) was exposed for 6 days at −70°C after fluorographic amplification with Amplify (Amersham).

**In Vitro Translation of p53.** p53 cDNA from pc53SN3 was cloned into pBluescript (pBSK--; Stratagene). After linearization with Sml, 1 µg of p53BSK was subjected to in vitro transcription with T3 RNA polymerase using an in vitro
transcription kit (Promega, Madison, WI) according to the manufacturer's instructions. In vitro-transcribed RNA was in vitro-translated using rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. [35S]Cysteine (Amersham) was included to obtain labeling of the proteins.

**Determination of Growth Rate in Suspension Culture.** Cells at exponential growth were diluted at a concentration of 0.25 × 10^5/ml in RPMI×10% FBS and kept in a humidified 5% CO_2 atmosphere at 37°C. Aliquots were removed daily, and the number of cells and viability as judged by Trypan blue exclusion was determined.

**Assessment of Clonal Proliferation in Soft Agar.** Cells 5,000 or 10,000 at exponential growth were seeded in 1 ml of 0.3% agar on top of 1 ml of 0.5% agar in McCoy's medium (GIBCO-BRL) supplemented with 15% FBS in 35-mm tissue culture dishes. The cells were allowed to grow for 15 days in a humidified 5% CO_2 atmosphere at 37°C. The number of colonies containing more than 40 cells was then determined.

**Assessment of Differentiation and Apoptosis by Morphological Characterization.** Exponentially growing cells were incubated at 3 × 10^5/ml in RPMI 1640 with 10% FBS without addition (control cells) or with TNF (0.1 nm). After 24, 48, and 72 h, aliquots were withdrawn for cytospin preparation and staining with May-Grünewald-Giemsa for morphological characterization. For determination of the induction of apoptosis, 400 cells were counted on each cytospin preparation, and the percentage of cells displaying morphological criteria for apoptosis (such as chromatin condensation and appearance of membrane protuberances or apoptotic bodies; Ref. 37) was determined.

**Determination of Hemoglobin.** The amount of hemoglobin was determined as described (38). Briefly, cells at exponential growth were washed twice with PBS, then lysed at a concentration of 1 × 10^6 cells/ml in the same buffer with 1% NP40. After incubation on ice for 1 h, DNA was removed by centrifugation at 37,500 × g for 1 h at 4°C. Supernatants were stored frozen at −70°C until hemoglobin determination. Supernatants of 5 µl were mixed with 200 µl of 1% (w/v) tetrathylbenzotriazine (Sigma) solution in 90% acetic acid and with 200 µl of freshly prepared 1% (v/v) H_2O_2. After incubation at room temperature for 20 min, 2 ml of 10% acetic acid was added to stop the reaction, and the absorbance at 515 nm was determined within an hour. From a standard curve made from the lysate of the mock-transfected clone M1, the relative amount of hemoglobin was determined.

**Statistical Analysis.** Clonogenicity (percentage of colonies >40 cells relative to number of seeded cells), TNF-induced inhibition of clonogenic growth, relative amount of hemoglobin, and the increase of the amount of hemoglobin upon TNF incubation were compared between mock transfecteds and wild-type-like p53-transfectants using Student's t test.

**References**


