Wild-Type p53 Gene Expression Induces Granulocytic Differentiation of HL-60 Cells

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Overexpression of wild-type p53 gene in malignant cell lines has been shown to inhibit cell proliferation in a number of cases. However, endogenous p53 protein seems to play little role in normal cell-cycle control as suggested by the normal development of p53 null mice, and by the low p53 protein levels expressed in most cell types. Recently, increased expression of endogenous p53 protein has been observed during the cellular response to DNA damage, as well as during differentiation of human hematopoietic cells. To study the role of the p53 gene in hematopoietic differentiation, we introduced the wild-type p53 gene or the temperature-sensitive p53(Va1135) mutant into p53-deficient HL-60 promyelocytic leukemia cells. Morphological analysis, flow-cytometric determination of granulocytic or monocytic surface markers, and ability to reduce nitroblue tetrazolium (NBT) demonstrated that expression of exogenous wild-type p53 gene in HL-60 cells induces differentiation through the granulocytic pathway. Proliferation and cell-cycle analysis performed after early expression of wild-type p53 showed that induction of differentiation is not coupled with growth arrest, which suggests that p53 is involved in differentiation independently of its activity on the cell cycle.

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MATERIALS AND METHODS

Cells and plasmids. HL-60 promyelocytic leukemia cells were cultured in RPMI 1640 culture medium (GIBCO-BRL, Life Technology, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicine (Gibco-BRL, Life Technology). Plasmid php53, which carries the human wild-type p53 cDNA under the control of the constitutive SV40 promoter, pMMTVp53, which carries the human wild-type p53 cDNA under the control of the steroid-inducible mammary murine tumor virus promoter (kindly provided by R. Baserga), and pN53cG(va1135) [ie, the pLRp53cG(va1135) plasmid] with the selectable marker neo under the control of the RSV-promoter] were used. The selectable marker coded by pRSVneo was transfected alone or cotransfected with the first two p53-coding plasmids. Selection of stably transfected mass culture cells was performed with 1 mg/mL of G418 for 3 weeks. Cell clones were obtained by limiting dilutions. The transfecants were maintained in culture medium supplemented with 200 μg/mL of G418.

Transferin receptor-mediated transfection. Approximately 2 × 10^6 exponentially growing cells, maintained in 5 mL of RPMI 1640 with 10% dialyzed, heat-inactivated FBS and gentamicin (transfection medium), were transfected by transferrin receptor-mediated uptake of plasmid DNA. Briefly, 6 μg of plasmid pRSVneo or a mix of php53 or pMMTVp53 and pRSVneo, at a molar ratio of 10:1, or pN53cG(va1135) were conjugated with 3 μg of dextrin transferrin.
purified fluorescien isothiocyanate (FITC)-IgG F(ab'), (Cappel, bometrics %p.a., Milan, Italy), washed twice with phosphate-buffered acetate (Sigma), and incubated at 37°C for the establishment of HL-60 cells expressing wild-type p53.

To explore the physiological role of p53 in hematopoietic differentiation, we introduced the wild-type p53 gene in HL-60 promyelocytic leukemia cells by cotransfection of either the constitutive expression vector pHSV53 or the inducible expression vector pMMTVp53 and the selectable marker pRSVneo. pRSVneo alone was transfected as control. After 3 weeks of G418 selection, stable transfected cells were grown as mass cultures. Integration of exogenous p53 cDNA was assessed on cell lysates by PCR amplification of DNA sequences corresponding to p53 exons 7 and 8. The two primers used for PCR (see Methods) encompass a 288-bp fragment of exogenous p53 cDNA, and a 608-bp fragment, comprising the intron between exons 7 and 8, of genomic DNA. The 288-bp fragment was amplified from both HL-SVP53 and HL-MMTVp53 transfecteds, and was absent in the parental and pRSVneo-transfected HL-60 cells. The 608-bp fragment could not be amplified from any of the cell cultures analyzed, confirming the homozygous deletion of the p53 gene in the HL-60 genome (data not shown). The

RESULTS

Stable gene transfer by receptor-mediated DNA uptake. We have previously reported that a DNA delivery system based on transferrin receptor-mediated endocytosis was an efficient method to introduce oligomers into HL-60 cells, which express high levels of transferrin receptors. Because of the difficulty of transfecting hematopoietic cells with standard procedures, and the recent demonstration that receptor-mediated uptake of plasmid DNA gives satisfactory results for transient gene expression in K562 cells, we used the transferrin receptor-mediated system in an attempt to stably transfect the HL-60 cells. Transferrin/pRSVneo complexes were added daily to growing HL-60 cells maintained in transfection medium (see Methods). Different groups of cells were treated with transferrin/pRSVneo complexes for different periods of time ranging from 1 to 10 days. Cells incubated with transferrin alone were used as controls. One day after completing treatment, the cells underwent G418 selection for isolation of stable transfecteds.

Establishment of HL-60 cells expressing wild-type p53. To explore the physiological role of p53 in hematopoietic differentiation, we introduced the wild-type p53 gene in HL-60 promyelocytic leukemia cells by cotransfection of either the constitutive expression vector pHSV53 or the inducible expression vector pMMTVp53 and the selectable marker pRSVneo. pRSVneo alone was transfected as control. After 3 weeks of G418 selection, stable transfecteds were grown as mass cultures. Integration of exogenous p53 cDNA was assessed on cell lysates by PCR amplification of DNA sequences corresponding to p53 exons 7 and 8. The two primers used for PCR (see Methods) encompass a 288-bp fragment of exogenous p53 cDNA, and a 608-bp fragment, comprising the intron between exons 7 and 8, of genomic DNA. The 288-bp fragment was amplified from both HL-SVP53 and HL-MMTVp53 transfecteds, and was absent in the parental and pRSVneo-transfected HL-60 cells. The 608-bp fragment could not be amplified from any of the cell cultures analyzed, confirming the homozygous deletion of the p53 gene in the HL-60 genome (data not shown).
tracts containing equal amounts of proteins were incubated with template-DNA served as a negative control, one with 1 ng p53 plasmid as a positive control. (B) Wild-type p53 protein expression. The cells were metabolically labeled with [35S]-methionine. Cell lysates was subjected to PCR. Two primers that amplify a 288-bp plasmid as a positive control. (B) Wild-type p53 protein expression. The cells were metabolically labeled with [35S]-methionine. Cell extracts containing equal amounts of proteins were incubated with PAb421 to immunoprecipitate p53 proteins. Immunocomplexes were separated on a denaturing 10% acrylamide gel, and subjected to fluorography.

Fig 1. (A) Integration of p53 cDNA. Genomic DNA from total cell lysates was subjected to PCR. Two primers that amplify a 288-bp fragment from p53 cDNA were used. Ten microliters of PCR products were loaded on a 4% agarose gel and blotted onto nylon filters. Hybridization was performed with a synthetic oligonucleotide complementary to part of the p53 exon 7. A PCR performed without template-DNA served as a negative control, one with 1 ng php53 plasmid as a positive control. (B) Wild-type p53 protein expression. The cells were metabolically labeled with [35S]-methionine. Cell extracts containing equal amounts of proteins were incubated with PAb421 to immunoprecipitate p53 proteins. Immunocomplexes were separated on a denaturing 10% acrylamide gel, and subjected to fluorography.

Specificity of the amplified sequences was confirmed by hybridizing the PCR products with an oligomer complementary to a p53 sequence internal to the primers used for PCR amplification (Fig 1A).

The expression of exogenous p53 proteins in HL-SVp53 and HL-MMTVp53 cells was evaluated by immunoprecipitation from metabolically labeled cells. The monoclonal antibody PAb421, which recognizes a p53 epitope shared by different mammalian species, was used. HL-SVp53 cells expressed low but clearly detectable levels of p53 protein. As expected, parental and HL-neo cells did not express detectable p53 protein (Fig 1B). In our hands, the HL-60 cells transfected with pMMTVp53, which should transcribe the p53 gene after induction of the MMTV promoter with steroid hormones, constitutively expressed p53 (Fig 1B). We were not able to detect any increase in p53 protein expression after dexamethasone treatment of pMMTVp53 transfectants (data not shown). Thus, for the following experiments, both HL-SVp53 and HL-MMTVp53 cells were considered to express exogenous p53 protein in a constitutive way.

p53 expression is associated with induction of granulocytic differentiation. The pRSVneo transfections of HL-60 cells produced G418-resistant populations that could be easily maintained in culture. Although it was possible to obtain HL-60 cells expressing low levels of p53 protein from each of the six different transfections performed, these cells could not be maintained in culture longer than 10 or 12 weeks. After this period, the cells either completely stopped growing or ceased expressing p53, becoming morphologically indistinguishable from the parental HL-60 cells. However, the phenotypical changes developed by these cells during this period could be studied.

During the transient expression of p53, starting form the third day of incubation with transferrin/DNA complexes, we compared the growth rate of the p53-expressing HL-60 cells to those of parental and pRSVneo-transfected cells. A moderately reduced growth rate was observed in both HL-SVp53 and HL-MMTVp53 transfectants compared with control cells. The doubling time was 63 hours for parental and pRSVneo-transfected HL-60 cells, and 86.4 hours and 80 hours for HL-SVp53 and HL-MMTVp53 transfectants, respectively. This result is similar to that reported for L12 and K562 leukemia cell lines in which wild-type p53 transfection induced differentiation.

In the 4 weeks following G418 selection, we analyzed the morphological characteristics of the p53-expressing HL-60 cells on cytopsin preparations stained with Wright or May-Grunwald Giemsa. The HL-SVp53 and HL-MMTVp53 transfectants showed a subset of cells, ranging from 18% to 39% of the living cells in different preparations, with morphological characteristics of granulocytic differentiation. These included, compared with control cells, a decreased nuclear to cytoplasmic ratio, less basophilic cytoplasm, less prominent nucleoli, and segmented and plurisegmented nuclei (Fig 2A through C). Although a small percentage of cultured HL-60 cells can spontaneously differentiate to more mature myeloid cells, in control cultures the fraction of differentiated cells was no greater than 5%, and no plurisegmented nuclei were observed.

HL-60 cell differentiation was also evaluated by the capacity to phagocytize and reduce NBT. Results from different preparations demonstrated that 13% to 33% of the cells from the p53-expressing mass cultures reduced NBT. No more than 3% of the parental HL-60 cells were positive for NBT reduction (data not shown).

CD15 and CD14 cell surface antigens are markers of granulocytic and monocytic differentiation, respectively. To confirm specific differentiation of the p53-expressing HL-60 cells through the granulocytic pathway, with the exclusion of the monocytic one, the expression of CD15 and CD14 was measured by flow cytometry. A comparison between the parental HL-60 and the HL-SVp53 cells showed an increase, in the p53 transfectants, of the number of cells expressing CD15 antigen, while expression of CD14 was un-
Wt-p53-MEDIATED DIFFERENTIATION OF HL60 CELLS

Fig 2. Morphological features of parental and p53-transfected HL-60 cells. Cytocentrifuge preparations of (A) HL-60, (B) HSVP53, and (C) HL-MMTVp53 cells were stained with May-Grünwald Giemsa. (Original magnification × 787.5.)

changed. Comparable results were obtained with HL-60 cells induced to differentiate into granulocytes by retinoic acid (Fig 3). The HL-60 parental cell line expresses CD15 antigen on a high percentage of cells (50% to 80% in different experiments). Thus, it is possible, although we used transfected maintained in mass culture, that elimination of CD15 cells occurred through posttransfection selection.

Taken together, morphological, functional, and fluorimetric analyses show that p53 gene expression in HL-60 cells induces differentiation through the granulocytic pathway.

Kinetics of p53-mediated differentiation. The constitutive nature of p53 expression in the experiments described here did not allow us to study the kinetics of the early modifications induced by p53. To obtain an inducible wild-type p53 activity, we transfected HL-60 cells with the Val135 mutant of the murine p53 gene. This is a ts mutant (ts-p53) that behaves like wt-p53 at 32°C, but not at 37°C. We stably introduced ts-p53 in HL-60 cells by transferrin receptor-mediated DNA uptake, and G418-resistant cells were cloned by limiting dilutions at 37°C. A ts-p53 clone (HL-tsp53/2), stably growing at 37°C, was isolated. These cells expressed low levels of p53 protein comparable to those observed in the HL-SVP53 and HL-MMTVp53 mass cultures (data not shown).

To study the kinetics of p53-mediated differentiation, the HL-tsp53/2 cells and the parental HL-60 cells were incubated at either 37°C or 32°C for 2 weeks. Because of increased mortality of HL-60 cells incubated at 32°C, particularly at high cell density (see next paragraph), cell confluence was avoided, the medium was replaced twice a week, and the presence of differentiated cells was morphologically evaluated on cytospin preparations stained with May-Grünwald Giemsa. The percentage of differentiated cells was calculated after exclusion of the dead cell bodies. The number of myelocytes and metamyelocytes began to increase after the first 2 days at 32°C only in the HL-tsp53/2 cells and remained constant after the fifth day (Fig 4A). An increased number of segmented neutrophils was also present (Fig 4B). As observed in the HL-SVP53 and HL-MMTVp53 transfec-
tants, cell differentiation was not synchronous and involved approximately 50% of living cells.

Expression of the granulocytic differentiation marker CD15 was also assessed by cytofluorimetric analysis. The HL-tsp53/2 clone expressed this antigen on 15% to 20% of the cells when grown at 37°C. An augmented and persistent expression of CD15 antigen was observed in the HL-tsp53/
Fig 4. Kinetics of p53-mediated cell differentiation. The cells were prepared as in Fig 2. Two hundred cells were counted from each cytocentrifuge preparation. The indicated fractions of (A) myelocytes plus metamyelocytes, or (B) segmented neutrophils are calculated on living cells. (H) HL-tsp53/2 incubated at 32°C; (m) HL-tsp53/2 incubated at 37°C; (H) HL-60 incubated at 32°C; (m) HL-60 incubated at 37°C. (C) Kinetics of CD15 antigen expression following induction of wt-p53 activity. The cells were prepared and analyzed as in Fig 3. The percentages of positive cells grown at 32°C (H), or 37°C (m) are plotted.

2 cells only when incubated at 32°C. In contrast, the expression of CD15 antigen in the parental HL-60 cell line (50% to 80% of the cells) was not meaningfully affected by temperature (Fig 4C). The expression of CD15 antigen on less than 20% of the cells in the HL-tsp53/2 clone can be due to the cloning procedure after ts-p53 transfection. The selection from HL-60 population of a low CD15 expressor argues against the possibility that exogenous p53 might be preferentially expressed in CD15+ cells, as we discussed for HL-SVp53 mass cultured cells.

To verify the continuous expression of exogenous p53 gene, the HL-tsp53/2 cells were incubated at the permissive temperature for a period of 2 weeks. Cell lysates analyzed by Western immunoblotting with the p53-reacting antibody PAb240 showed that HL-tsp53/2 cells continued to express p53 proteins after 2 weeks at 32°C (data not shown).

differentiation is not coupled with growth arrest. p53 can inhibit cell proliferation, apparently inducing a cell accumulation in G1 phase. Thus, it is conceivable that p53-transfected HL-60 cells underwent spontaneous differentiation, because p53 restored a cell-cycle control whose lack can inhibit cell differentiation. We evaluated the growth rate of either HL-tsp53/2 or HL-60 cells maintained at 37°C or 32°C. A reduced growth rate was observed in the HL-tsp53/2 cells during the first 2 days of incubation at 32°C. After this period, the HL-tsp53/2 cells proliferated at a similar rate as the parental cells grown at the same temperature (Fig 5A). The DNA content analysis showed no modifications in the cell-cycle profile either during the first 24 hours or after 6 days of wt-p53 expression (data not shown). The reduced cell number observed in the HL-tsp53/2 cells during the first 2 days of incubation at 32°C was associated with, and presumably explained by, a reduction in cell viability (Fig 5B). Taken together, these results show that in HL-
60 cells, p53-mediated maturation is not preceded by a G1 arrest, indicating that differentiation is not merely the result of restoration of critical cell-cycle controls.

The main effects that have so far been observed after exogenous expression of wt-p53 in different hematopoietic cell lines are apoptotic cell death,\textsuperscript{8,15} or cell differentiation.\textsuperscript{8,12} Cell death is usually observed suddenly after expression of wt-p53 activity.\textsuperscript{13} Thus, we asked whether the 15% reduction in cell viability observed during the first 2 days of culture at 32°C was due to apoptosis. DNA fragmentation was indeed observed in HL-tsp53/2 cells maintained at 32°C for 24 hours (Fig 6). Morphological signs of apoptosis were observed in all the cytospin preparations used to evaluate the kinetics of myeloid differentiation. However, HL-60 cells normally undergo apoptosis during the process of differentiation.\textsuperscript{22} Thus, after the first day of wt-p53 expression, it is impossible to assign the apoptotic effects to p53 or the differentiation process per se.

**DISCUSSION**

To explore the physiological function of the wt-p53 gene, we induced its expression in HL-60 promyelocytic leukemia cells. The advantages offered by these cells are their capacity to differentiate into granulocytes or monocytes, and the absence of endogenous p53 due to homozygous deletion of the p53 gene. We selected HL-60 cells that stably expressed p53 for approximately 3 months, and a ts-p53\textsuperscript{+} cell clone that could be induced to express wt-p53 activity by incubation at 32°C. We found that both types of HL-60 transfectants differentiated along the granulocytic pathway. This conclusion was based on morphological changes typical of granulocytic cells, on the matured ability to phagocytize and reduce NBT, and on the increased expression of CD15 antigen. These changes constitute evidence that p53 participates in the pathways leading to functional cell differentiation.

Sustained proliferation is incompatible with terminal differentiation in a number of cell systems.\textsuperscript{26} The wt-p53 can induce growth arrest and cell accumulation in the G1 phase of the cell cycle.\textsuperscript{14,18} Thus, it is conceivable that p53-
transfected HL-60 cells underwent spontaneous differentiation because p53 reestablished those cell-cycle controls whose lack blocked cell differentiation. A similar mechanism was proposed for HL-60 cells treated with anti-myc oligonucleotides. The resulting downregulation of myc induced growth suppression followed by cell differentiation. To evaluate whether p53 is a differentiation gene per se or whether it induces differentiation by allowing the cells to cease proliferation, we studied the kinetics of cell differentiation and proliferation in the early period of wt-p53 activation. No major differences in the growth rate and the cell-cycle profile were found between cells grown at the permissive and restrictive temperatures. These results show that in HL-60 cells, p53-mediated maturation is not preceded by a G1 arrest, which indicates that differentiation is not merely the result of restoration of critical cell-cycle controls. The kinetics of cell differentiation showed an increase in the number of myelocytes and metamyelocytes beginning 48 hours after the induction of wt-p53 activity. These maturation stages do not require cell-cycle arrest during normal hematopoiesis. The fact that p53 can induce this type of maturation without affecting cell proliferation suggests that p53 is involved in differentiation independently of its activity on the cell cycle.

Two main effects have so far been observed after artificial expression of wt-p53 in different hematopoietic cell lines: apoptotic cell death and cell differentiation. The wt-p53 activity induced DNA fragmentation coupled with loss of viability in approximately 15% of the HL-tsp53/2 cells during the first 24 hours of incubation at 32°C. However, wt-p53 expression led mainly to differentiation. The hematopoietic cell lines that have been used to study the wt-p53 functions have in common the characteristic of not expressing endogenous p53. However, the mechanisms involved in the transformation of these cells are different, and the cells are at different stages of differentiation. Each particular phenotype offers a cellular environment in which p53 might act with different effects. Alternatively, the observation of divergent phenotypes might be explained by expression of different levels of exogenous p53 in the transfecants. Indeed, we did not find DNA fragmentation in HL-tsp53/2 cells grown at the intermediate temperature of 34°C, whereas we continued to observe cell differentiation (S.S. and G.B., unpublished data). It has been reported that temperature-sensitive genes can induce intermediate phenotypes when the cells bearing them are cultured at temperatures between the permissive and restrictive ones. Intermediate temperatures are thus used to make the ts mutants "leak" to varying degrees.

The increased expression of wt-p53 observed after x-ray-- or drug-induced DNA damage has suggested that p53 has growth-controlling functions in stressed cells. The increased expression of p53 observed during hematopoiesis, as well as the demonstration that exogenous p53 can induce hematopoietic maturation, suggests that p53 plays a role in cell differentiation. In an attempt to unify these apparently separated functions of p53, we propose that p53 increases during differentiation, similarly to induced DNA damage, in response to the presence of single-stranded nicks in the DNA, which occur physiologically during hematopoietic maturation.

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