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Cytokines Inhibit p53-Mediated Apoptosis but Not p53-Mediated G_1 Arrest

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Murine erythroleukemia cells that lack endogenous p53 expression were transfected with a temperature-sensitive p53 allele. The temperature-sensitive p53 protein behaves as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C. Three independent clones expressing the temperature-sensitive p53 protein were characterized with respect to p53-mediated G_1 cell cycle arrest, apoptosis, and differentiation. Clone ts5.203 responded to p53 activation at 32°C by undergoing G_1 arrest, apoptosis, and differentiation. Apoptosis was seen in cells representative of all phases of the cell cycle and was not restricted to cells arrested in G_1. The addition of a cytokine (erythropoietin, c-kit ligand, or interleukin-3) to the culture medium of ts5.203 cells blocked p53-mediated apoptosis and differentiation but not p53-mediated G_1 arrest. These observations indicate that apoptosis and G_1 arrest can be effectively uncoupled through the action of cytokines acting as survival factors and are consistent with the idea that apoptosis and G_1 arrest represent separate functions of p53. Clones ts15.15 and tsCB3.4 responded to p53 activation at 32°C by undergoing G_1 arrest but not apoptosis. We demonstrate that tsCB3.4 secretes a factor with erythropoietin-like activity and that ts15.15 secretes a factor with interleukin-3 activity and suggest that autocrine secretion of these cytokines blocks p53-mediated apoptosis. These data provide a framework in which to understand the variable responses of cells to p53 overexpression.

The product of the p53 tumor suppressor gene plays a critical role in mediating cell cycle arrest in G_1 or apoptosis in response to DNA damage. In certain cells, induction of p53 protein in response to irradiation can lead to transient arrest in the G_1 phase of the cell cycle (23). Transient arrest in G_1 is believed to provide time for DNA repair prior to the onset of DNA synthesis and mitosis. In human diploid fibroblasts, however, a prolonged arrest in G_1 is observed following irradiation, suggestive of a permanent rather than a transient cell cycle arrest (9). Other cells (thymocytes, quiescent lymphocytes) rapidly undergo p53-dependent apoptosis in response to irradiation (7, 31, 48). These data lead to two distinct models of p53 protein function. In one model, p53 mediates a transient arrest in G_1 in order to facilitate DNA repair following DNA damage. Cells that lack p53 expression and enter S phase inappropriately after treatment with metabolic inhibitors that normally arrest cells in G_1 have been shown to have a higher incidence of genetic instability (29, 54). In the second model, p53 prevents the proliferation of cells that have sustained DNA damage either by promoting a permanent block in G_1 or by promoting apoptosis. The observation made in clonogenic survival assays that cells expressing wild-type p53 are more sensitive to ionizing radiation than cells expressing mutant p53 or cells lacking p53 supports the second model (3, 25, 33, 37).

The overexpression of p53 protein that occurs in irradiated cells can be mimicked by ectopic p53 expression following p53 gene transfer into nonirradiated recipient cells. Nonirradiated cells forced to express high levels of wild-type p53 protein have been shown to undergo either cell cycle arrest in G_1 (10, 28, 32) or apoptosis (44, 56). Overexpression of p53 has also been shown to promote differentiation in certain cell types (1, 5, 11, 21, 43, 46).

A key and as yet unresolved question arising from these studies is why certain cells such as thymocytes undergo apoptosis in response to p53 protein overexpression whereas other cells such as fibroblasts undergo p53-dependent G_1 arrest. Several studies have suggested that p53-mediated cell death is not dependent on the induction of prior growth arrest and that growth arrest and apoptosis represent separate functions of p53 (4, 6, 45, 50, 55).

Friend virus-transformed murine erythroleukemia cells that lack endogenous p53 protein expression and express a temperature-sensitive p53 (p53ts) mutant allele provide a good model with which to investigate the role of p53 in regulating G_1 arrest and apoptosis. The p53ts protein contains valine instead of alanine at amino acid position 135 and behaves as a mutant polypeptide at 37°C and as a wild-type polypeptide at 32°C (34). We previously showed that the DP16-1 erythroleukemia cell line expressing p53ts protein grows well at 37°C. At 32°C, however, these cells arrest in G_1, undergo apoptosis, and express globin, a marker for erythroid differentiation (21). In this report, we characterize the response of three p53ts-expressing clones derived from two different erythroleukemia cell lines and demonstrate that G_1 arrest and apoptosis can be effectively uncoupled by various cytokines acting through autocrine or nonautocrine pathways. p53-mediated G_1 arrest is insensitive to cytokines, whereas p53-mediated apoptosis is blocked in the presence of cytokines. In addition, we demonstrate that cells in all phases of the cell cycle undergo apoptosis when p53 protein is activated at 32°C. These findings are consistent with the idea that cell cycle arrest and apoptosis represent distinct and independent responses to p53 activation. Moreover, they highlight the importance of the cellular environment in determining the cellular response to p53 protein expression.

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MATERIALS AND METHODS

Cell lines, culture methods, and cytokines. The DP16-1 cell line was derived from the spleen cells of a mouse infected by the Friend virus complex consisting of spleen focus-forming virus ($SFFV_p$) and Friend murine leukemia virus (F-MuLV). The CB3 cell line was derived from the spleen cells of a mouse infected at birth by F-MuLV alone. The endogenous p53 genes are structurally rearranged in both DP16-1 and CB3 (36), and as a result, these cell lines do not express endogenous p53 protein. DP16-1.p53ts cells were derived from the DP16-1 cell line by coelectroporation of a plasmid containing the temperature-sensitive $p53^{Val\text{-}135}$ allele and pSV2neo, which encodes drug resistance to G418 (21). ts5.203 and ts15.15 are two independent clones obtained from DP16-1.p53ts cells by subcloning in methylcellulose. The tsCB3.4 clone was derived from CB3 by coelectroporation with $p53^{Val\text{-}135}$ and pSV2neo as described for the DP16-1.p53ts clones. All cell lines were cultured at 37°C in alpha minimal essential medium (α-MEM) containing 10% fetal calf serum (FCS). Cell viability was determined by eosin stain exclusion. Cells that expressed hemoglobin were identified by staining with diaminofluorene (DAF) (22). Recombinant human erythropoietin (EPO), murine interleukin-3 (IL-3), and c-kit ligand (KL) were obtained from Mark Minden and Norman Iscove (Ontario Cancer Institute/Princess Margaret Hospital, Toronto, Ontario, Canada). These cytokines were obtained as media conditioned by cells transfected with expression vectors containing full-length cDNA clones for each of the cytokine genes. Each factor was titrated by using a mouse bone marrow colony assay (49). Purified murine EPO was purchased from Boehringer Mannheim, Québec, Québec, Canada. Where indicated, these cytokines were added to the culture medium at a concentration of 1 U/ml. Monoclonal antibody specific for mouse IL-3 was purchased from Genzyme, Cambridge, Mass.

Clonogenic cells were detected by their capacity to give rise to colonies in 0.8% methylcellulose with α-MEM and 10% FCS. Clonogenic cell survival in the suspension cultures was determined by plating fixed volumes in methylcellulose and incubating the dishes at 37°C. Colonies containing more than 40 cells were counted 6 days after plating.

Detection of EPO activity. EPO activity in cell culture supernatants was assessed by using a CFU-E (colony-forming unit–erythroid) assay (19, 49) with recombinant EPO as a standard. Briefly, EPO (1 U/ml) or culture supernatant (16% of final volume) was mixed with 2×10^5 mouse bone marrow cells in Iscove's modified Dulbecco's medium containing lipid, insulin, transferrin, and 0.8% methylcellulose to a final volume of 4 ml. The mixture was distributed in four dishes and incubated at 37°C with 5% CO_2. Two days after plating, erythroid colonies (CFU-E) with more than eight cells were counted under the microscope. In the same assay, non-CFU-E colonies consisting of more than 50 cells were enumerated 7 days after plating.

Single-cell culture. Cells were diluted in α-MEM supplemented with 10% FCS and plated in 96-well dishes with one cell per well. The dishes were then incubated at 37 or 32°C and inspected daily for 3 days for growth or differentiation. The wells were inspected immediately after plating, and wells that did not contain a single cell were excluded from further analysis. When nuclear condensation appeared in a cell, DAF staining was performed to determine if the cell contained hemoglobin (22). At the end of the third day, all cells were stained with DAF for hemoglobin. The wells were then classified into three groups: growth, in which the cell number increased to four or more with no positive DAF staining; arrest, in which the cell number was less than four with no positive DAF staining; and differentiation, in which at least one cell per well stained positive with DAF.

Indirect immunofluorescent antibody staining. Cells were grown on glass coverslips for 16 h at 32 or 37°C in α-MEM in the presence or absence of EPO (1 U/ml). The cells were then fixed in acetone at −20°C for 15 min. The cells were incubated with the anti-p53 monoclonal antibody PAb122 (15) at 37°C for 60 min and then with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G at room temperature for 60 min. The cells were counterstained in diluted Evans blue (1:1,000), and the coverslip was mounted in a solution containing 90% glycerol, 10% phosphate-buffered saline (PBS), and 0.1% *p*-phenylenediamine. p53 protein staining was examined by UV microscopy.

Metabolic labeling and immunoprecipitation. A total of 10^7 cells were labeled for 60 min at 37 or 32°C with 250 μCi of [^{35}S]methionine in 200 μl of methionine-free α-MEM containing 10% dialyzed FCS. Cells were then washed twice in PBS and lysed on ice for 30 min in 400 μl of lysis buffer (1% Nonidet P-40 [NP-40], 150 mM NaCl, 20 mM Tris [pH 8], 0.5 mM phenylmethylsulfonyl fluoride, 1 μg each of pepstatin, aprotinin, and leupeptin per ml). The lysate was centrifuged at 12,000 rpm at 4°C for 15 min to remove the cell debris, and the supernatant was precleared by incubation with normal mouse immunoglobulin G followed by 20 μl (pellet volume) of formalin-fixed *Staphylococcus aureus* cells. Proteins were immunoprecipitated with the following monoclonal antibodies: PAb419, a control antibody against simian virus 40 large T antigen (17); PAb421, a panspecific antibody against p53 (17); PAb246, a murine p53-specific antibody that recognizes wild-type p53 protein (35, 53); and PAb240, an antibody that recognizes denatured and mutant p53 protein (13). The immune complexes were precipitated with 50 μl of protein A-Sepharose beads (Pharmacia, Biotech, Baie d'Urfé, Québec, Canada). p53 protein was eluted by boiling for 5 min. The eluate was resolved by electrophoresis in a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS), and proteins were visualized by autoradiography.

Electrophoretic gel mobility shift assay. To prepare nuclear extracts, cells were rinsed in PBS and lysed for 5 min on ice in buffer A (20 mM Tris [pH 7.4], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml). The supernatant was discarded, and the nuclear pellet was resuspended in 3 volumes of buffer A modified to contain 100 mM NaCl, 1% NP-40, and 5 mM adenylylimidodiphosphate. The nuclear extract was maintained on ice for 30 min and centrifuged at 12,000 rpm for 15 min. The supernatant was retained and used immediately. A 10-μl DNA binding reaction mixture contained 1 μl of PAb421, 2 μl of nuclear lysate (about 10 μg of protein), 1 μg of poly(dI-dC), and 5×10^4 cpm ^{32}P-labeled double-stranded p53CON oligonucleotide containing the p53 consensus binding sequence defined by Funk et al. (12) shown underlined (GGATCCAAGCTTGGACATGCCCGGGCATGTCCCTCGAGGGATCC) in a final concentration of 20 mM Tris (pH 7.4), 100 mM NaCl, 5 mM EDTA, 5 mM adenylylimidodiphosphate, 0.2% NP-40, and 10% glycerol. Adenylylimidodiphosphate, a nonhydrolyzable analog of ATP, was included in the reaction mixture since it was shown to inhibit the conversion of temperature-sensitive $p53^{Val\text{-}135}$ protein from the mutant conformation to the wild-type conformation (16). The oligonucleotide was labeled with Klenow DNA polymerase and [α-^{32}P]dCTP in a fill-in reaction after annealing with a short antisense oligonucleotide (GGATCCCTCGAG). In control binding reactions, an unrelated oligonucleotide (oligonucleotide C) having the same nucleotide composition as p53CON was used as the substrate or competitor. Binding reaction mixtures were incubated at 32°C for 30 min, and samples were analyzed on a 4% nondenaturing polyacrylamide gel run at 200 V for 2.5 h. Gels were dried and exposed to X-ray film overnight at room temperature.

In situ terminal deoxynucleotidyltransferase assay for apoptosis. The method of Gorczyca et al. (14) was used to identify apoptotic cells and simultaneously relate their position in the cell cycle.

RESULTS

p53 expression results in G_1 arrest, differentiation, and apoptosis in DP16-1 Friend erythroleukemia cells. The p53-nonproducing murine erythroleukemia cell line DP16-1 transfected with a p53ts allele provides a good model system with which to assess the consequence of p53 protein expression in a tumor cell. At 37°C, transfected cells (DP16-1.p53ts) express p53 protein in a mutant conformation; at 32°C, the mutant p53 protein undergoes a conformational change and behaves like wild-type p53. Previous studies from our laboratory (21) and others (40) have shown that DP16-1 cells expressing the p53ts protein proliferate at 37°C and appear phenotypically indistinguishable from parental DP16-1 cells. At 32°C, however, these cells arrest in the G_1 phase of the cell cycle, rapidly lose viability, and undergo differentiation and apoptosis. We have noticed that this phenotype of DP16-1.p53ts is not stable. After several months in culture at 37°C, these cells failed to respond in the typical manner when the incubation temperature was reduced to 32°C. As a result, it became necessary to isolate and characterize subclones which retained the phenotype of the original transfected cells. The behavior of one representative subclone (ts5.203) is shown in Fig. 1. These cells, upon p53 protein activation at 32°C, rapidly lost viability (Fig. 1A) and underwent morphological changes associated with apoptosis, including chromatin condensation and nuclear fragmentation, as revealed by acridine orange staining (Fig. 1B), as well as internucleosomal DNA fragmentation as shown by agarose gel electrophoresis (Fig. 1C).

Cell death was accompanied by an increase in the number of cells that produced hemoglobin, a marker of erythroid differentiation (see below). The increase occurred within 24 h of the temperature shift from 37 to 32°C and reached a peak after 48 h of incubation at the lower temperature.

Relationship between p53-mediated G_1 arrest and p53-mediated apoptosis. In response to a variety of DNA-damaging agents, endogenous wild-type p53 protein expression has been shown to increase, leading to apoptosis in some cells or to G_1 cell cycle arrest in other cells. The relationship, if any, between these two p53-dependent processes is not understood. Previous

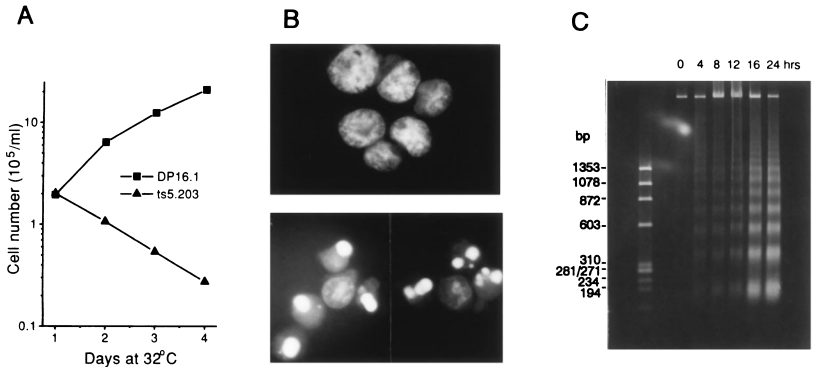

FIG. 1. Expression of p53ts protein at 32°C promotes apoptosis in ts5.203 cells. (A) ts5.203 and parental DP16-1 cells maintained in culture at 37°C were transferred to 32°C, and the viable cell number was determined each day for 4 days on the basis of eosin stain exclusion. (B) ts5.203 cells were cultured at 37°C (top) or 32°C for 36 h (bottom), fixed with 4% (vol/vol) formaldehyde in ethanol for 30 min, stained with acridine orange (10 μg/ml) for 10 min, and visualized by microscopy. (C) ts5.203 cells were cultured at 32°C, and at the time points indicated, 3×10^6 cells were collected and chromosomal DNA was isolated (41). Three micrograms of DNA from each sample was separated on a 1.5% agarose gel at 1 V/cm for 10 h. *Hae*III-digested ϕX174RF DNA fragments were applied to the first lane and served as molecular size markers. The size of each fragment is shown on the left.

studies using p53ts protein have suggested that p53-mediated cell death is not dependent on the induction of prior growth arrest (55) and that p53-mediated growth arrest and apoptosis represent separate functions of p53 (40). Both of these studies, however, also report that p53 induces cell death predominantly in G_1.

To examine the relationship between p53-mediated G_1 arrest and p53-mediated apoptosis in ts5.203 cells, we used a recently described flow cytometric assay that can identify apoptotic cells and simultaneously relate their positions in the cell cycle (14). The method relies on the use of formaldehyde to fix the cells. This fixative minimizes the loss of fragmented DNA from cells undergoing apoptosis. Terminal deoxynucleotidyltransferase was added to the fixed cells to label the DNA strand breaks in situ with biotinylated dUTP. DNA content was determined by staining with propidium iodide, and the incorporated biotinylated dUTP was measured with fluoresceinated streptavidin. ts5.203 cells were shifted to 32°C and sampled at different time points. In Fig. 2A, the DNA content profiles resulting from propidium iodide fluorescence alone are shown. An increase in the proportion of cells in G_1 and a decrease in the proportion of S-phase cells is evident, indicative of a block in G_1. A population of cells in G_2/M was also observed 12 h after the temperature shift. These cells may be cycling but have not had sufficient time to traverse the cell cycle and enter G_1 following the temperature shift. Alternatively, they may be blocked in G_2/M. Evidence for a second cell cycle block at G_2/M mediated by p53 was previously presented by Stewart et al. (47). In Fig. 2B, the same cell population was assessed by dual-parameter flow cytometry on the basis of DNA content (propidium iodide staining) and incorporation of biotinylated dUTP. These data demonstrate the presence of apoptotic cells as early as 6 h following the temperature shift. Cells in all phases of the cell cycle contain DNA strand breaks and can be considered to be undergoing apoptosis. Apoptosis does not occur preferentially in cells arrested in the G_1 phase of the cell cycle.

Abrogation of p53-mediated differentiation and apoptosis by EPO, IL-3, and KL. Although parental DP16-1 cells and ts5.203 cells do not require EPO for growth at 37°C, ts5.203 cells could be shown to respond to EPO at 32°C. The addition of recombinant human EPO or purified murine EPO to the culture medium of these cells prolonged cell survival (Fig. 3A), blocked p53-mediated apoptosis, and suppressed p53-mediated differentiation (Fig. 3B). This effect was not restricted to EPO and was also seen upon addition of two other cytokines, IL-3 and KL. IL-3 alone had a weaker but reproducible effect compared with EPO or KL (Fig. 3). These results demonstrate that IL-3, KL, and EPO, three cytokines that can promote the

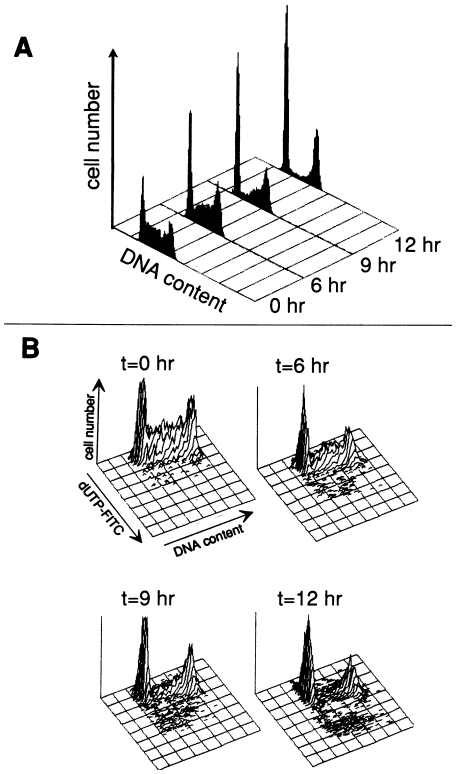

FIG. 2. Relationship between p53-mediated G_1 arrest and apoptosis. ts5.203 cells were transferred from 37 to 32°C and, at the indicated time points, were fixed, labeled in situ with biotin-dUTP by using terminal deoxynucleotidyltransferase, and stained with fluoresceinated streptavidin to measure DNA strand breaks and with propidium iodide to measure DNA content. Single-parameter DNA cell cycle analysis (fluorescence due to propidium iodide) is shown in panel A. Dual-parameter cell cycle analysis is shown in panel B. Fluorescence due to dUTP-fluorescein isothiocyanate is shown on a log scale, and the fluorescence due to propidium iodide is shown on a linear scale.

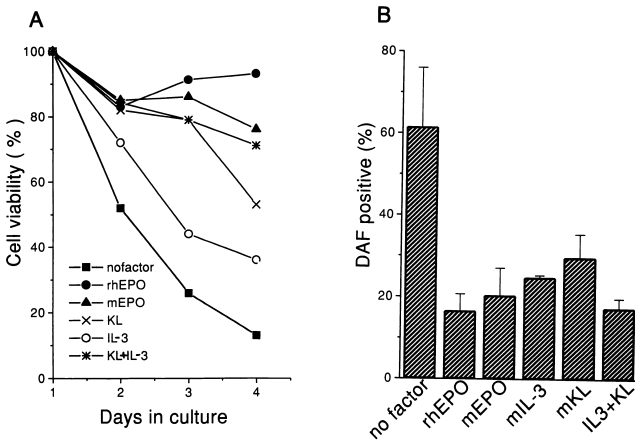

FIG. 3. EPO, IL-3, and KL suppress p53-mediated apoptosis and differentiation. (A) ts5.203 cells were cultured at 32°C in the presence or absence of cytokines, and on the days indicated, cell viability was determined on the basis of eosin stain exclusion. (B) DAF staining was used to determine the proportion of hemoglobin-positive ts5.203 cells present in the population 72 h after the cells were transferred to 32°C. The means and standard deviations of three experiments are shown. rhEPO, recombinant human EPO; mEPO, purified murine EPO; mKL and mIL-3, recombinant murine KL and IL-3, respectively.

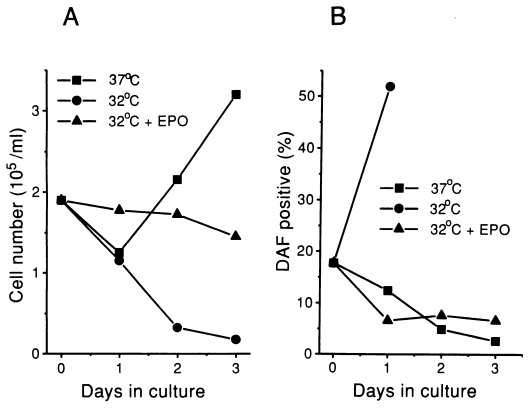

FIG. 4. Reversibility of p53-mediated growth arrest. ts5.203 cells were maintained at 32°C for 5 days in the presence of EPO (1 U/ml). The cells were then washed to remove the EPO, and equal numbers of cells were cultured at 37 or 32°C in α-MEM containing 10% FCS or at 32°C in α-MEM supplemented with 10% FCS and EPO (1 U/ml). On the days indicated after the cells were returned to culture, viable cell number was determined by eosin stain exclusion (A), and the proportion of hemoglobin-positive cells was determined by DAF staining (B).

growth and maturation of normal erythroid precursor cells, can also promote the survival of cells induced to undergo p53-mediated apoptosis and differentiation. p53-mediated growth arrest, however, was not abrogated by the cytokines. While EPO and KL prolonged the survival of ts5.203 cells at 32°C, an overall increase in cell number was not observed and these cells remained arrested primarily in G_1 (data not shown).

Single-cell assay. To investigate the fate of individual ts5.203 cells exposed to 32°C in the presence or absence of EPO, DP16-1 and ts5.203 cells were diluted appropriately, and single cells were deposited into individual wells of 96-well plates. Immediately after plating, all wells were inspected microscopically, and any well not containing a single cell was excluded from further analysis. The fate of individual cells incubated at 32 or 37°C was then monitored over a 3-day period, at the end of which time each well was scored for cell growth, lack of cell growth (arrest), or differentiation on the basis of staining with DAF as described in Materials and Methods. The results of this analysis are presented in Table 1 and can be summarized as follows: (i) there was no growth of ts5.203 cells at 32°C in the presence or absence of EPO; (ii) in the absence of EPO, a majority of ts5.203 cells (97%) became DAF positive at 32°C and resembled apoptotic cells; and (iii) EPO reduced the number of cells undergoing differentiation at 32°C. These observations, made on single cells, are consistent with the results from bulk cultures presented in Fig. 1 and 3. We conclude that the increased number of hemoglobin-positive ts5.203 cells present at 32°C cannot be attributed to the small proportion (7%) of preexisting differentiated cells present in the population and that p53 is directly involved in promoting growth arrest and differentiation. We also note that differentiation and apoptosis appear to be coupled processes in Friend cells forced to express p53 protein, since all DAF-positive cells contained condensed nuclei characteristic of apoptotic cells.

TABLE 1. Single-cell analysis of ts5.203 and parental DP16-1 cells at 37 and 32°C in the presence or absence of EPO

Determination	% of wells[a]					
	DP16-1			ts5.203		
	37°C	32°C	32°C + EPO	37°C	32°C	32°C + EPO
Growth[b]	87	71	71	81	0	0
Arrest[c]	6	21	24	12	3	62
Differentiation[d]	7	8	5	7	97	38

[a] Proportion of wells initially seeded with a single cell that showed evidence of cell growth, arrest, or differentiation.
[b] Cell number increased to four or more.
[c] Cell number remained less than four.
[d] Differentiation was determined by positive DAF staining.

Reversibility of p53-mediated growth arrest. The ability of EPO to maintain p53ts-expressing cells in a growth-arrested but viable and undifferentiated state at 32°C prompted us to examine whether these cells retained proliferative capacity when returned to 37°C. Specifically, we wished to know whether these cells were arrested at a stage preceding or following their commitment to differentiate and undergo apoptosis. ts5.203 cells were incubated with EPO at 32°C for 5 days. Growth-arrested cells were then pelleted, resuspended in fresh medium in the presence or absence of EPO, and incubated at 37 or 32°C for a further 3 days. The response of cells to incubation at 32°C with or without EPO was similar to that described in Fig. 3. Cells quickly lost viability in the absence of EPO, and a sharp increase in the proportion of hemoglobin-positive cells was observed. Determination of the cell cycle/apoptosis profiles of these cells by dual-parameter flow cytometric analysis indicated that cells in G_1 at the time of EPO withdrawal underwent apoptosis in G_1 (data not shown). Hence, exit from G_1 into S phase does not appear to be required for the onset of apoptosis. Addition of EPO maintained cell viability and reduced the number of hemoglobin-positive cells (Fig. 4). When the cells were transferred to 37°C, proliferation was observed after a short lag (Fig. 4). We conclude that the growth arrest mediated by p53 expression in the presence of EPO was reversible and that these cells were not committed to undergo cell death or differentiation.

p53 protein expression, conformation, cellular localization, and sequence-specific DNA binding activity are unchanged in the presence of EPO. The inability of p53 protein to promote apoptosis in cells grown in the presence of EPO suggested that

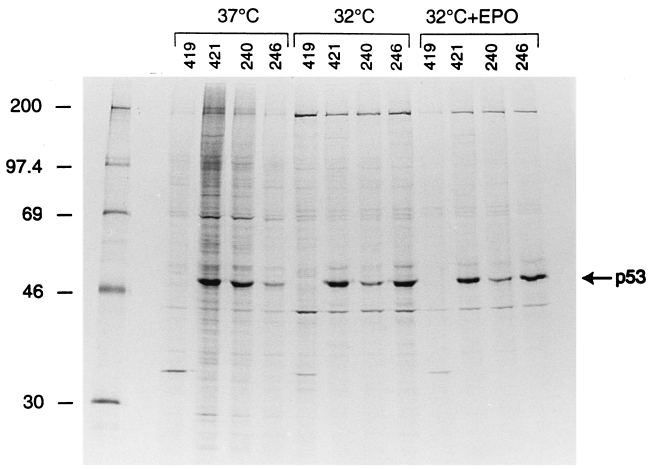

FIG. 5. Metabolic labeling and immunoprecipitation of p53ts protein in ts5.203 cells. ts5.203 cells were cultured at 37 or 32°C in the presence or absence of EPO for 12 h. Cells were labeled with [^{35}S]methionine, lysates were prepared, and p53 protein was immunoprecipitated as described in Materials and Methods with the following monoclonal antibodies: PAb419, a control antibody recognizing the simian virus 40 large T antigen; PAb421, a p53-specific antibody recognizing both wild-type and mutant p53; PAb240, which recognizes mutant p53; and PAb246, which recognizes murine wild-type p53. The immune complexes were collected with protein A-Sepharose, and the proteins were resolved by electrophoresis on a 10% polyacrylamide gel containing SDS. Molecular size markers in kilodaltons are shown on the left.

EPO and other cytokines might be modulating this function of p53. p53 protein conformation and expression were compared in ts5.203 cells grown in the presence or absence of EPO. Metabolic labeling and immunoprecipitation using PAb421, a monoclonal antibody which recognizes both the wild-type and mutant forms of p53 protein (17), showed similar levels of p53 protein synthesis at 37, 32, and 32°C with EPO, indicating that p53 protein synthesis was not downregulated at 32°C in the presence of EPO (Fig. 5). At 37°C, p53 protein was predominantly in the mutant conformation, as revealed by immunoprecipitation with PAb240, a monoclonal antibody that recognizes preferentially the mutant conformation of p53 protein (13). At 32°C, p53 protein was predominantly in a wild-type conformation, as shown by immunoprecipitation with PAb246, a monoclonal antibody that recognizes preferentially the wild-type conformation of p53 (53). In the presence of EPO, p53 protein remained predominantly in the wild-type, PAb246-positive conformation at 32°C (Fig. 5).

Translocation of p53 protein into the cell nucleus is believed to be critical for its tumor suppressor activity. Hence, we examined the possibility that during EPO-mediated signal transduction, p53 protein is modified and retained in the cytoplasm of ts5.203 cells, precluding its ability to promote apoptosis. We compared the localization of p53 protein at 37, 32, and 32°C with EPO by indirect immunofluorescent antibody staining using PAb122, a monoclonal antibody that recognizes an epitope at the carboxyl terminus of p53 protein (15). At 37°C, p53ts protein was detected in both the cytoplasm and the nucleus, whereas at 32°C, p53ts protein was localized exclusively in the nucleus. In cells incubated at 32°C with EPO, the localization of p53ts protein in the nucleus was unchanged (Fig. 6).

The site-specific DNA binding activity of p53ts protein expressed in ts5.203 cells was examined in a gel mobility shift assay modified to contain adenylylimidodiphosphate as described by Hainaut and Milner (16). Nuclear extracts were prepared from ts5.203 cells grown at 32°C in the presence or absence of EPO and mixed with a ^{32}P-labeled double-stranded oligonucleotide containing a p53 binding consensus sequence, p53CON (12). DNA binding activity was not perturbed in cells treated with EPO (Fig. 7). Competition with an excess of unlabeled p53CON oligonucleotide but not with an equivalent amount of unrelated oligonucleotide having the same nucleotide composition as p53CON confirmed that binding was specific (Fig. 7).

Together, these results indicate that the inclusion of EPO in the culture medium of ts5.203 cells incubated at 32°C did not

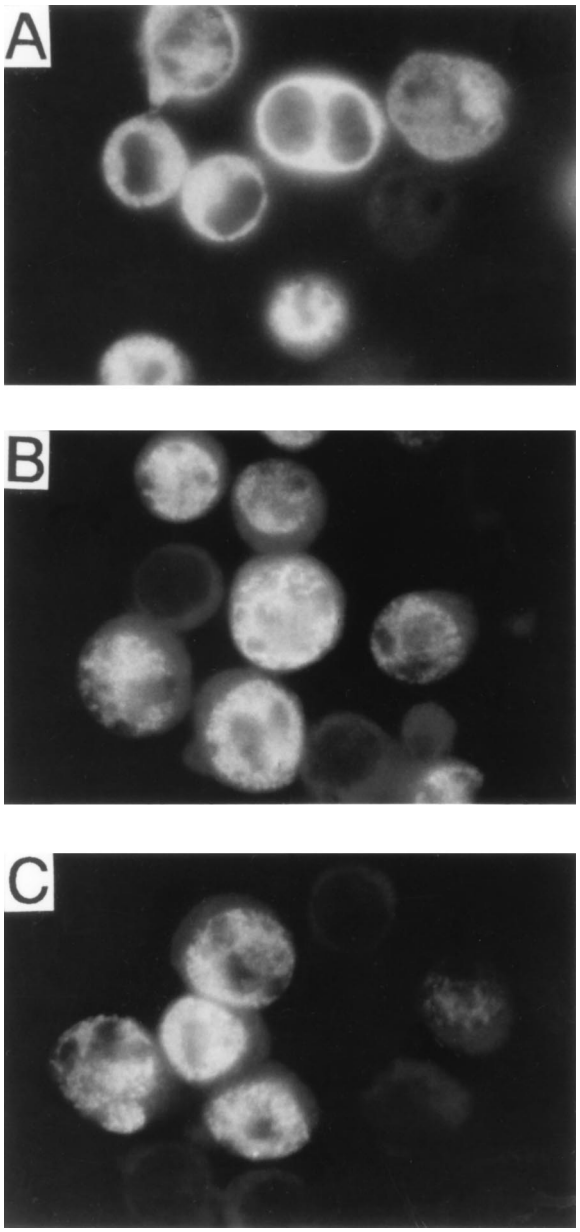

FIG. 6. Subcellular localization of p53 protein. ts5.203 cells were cultured at 37 or 32°C for 16 h in the presence or absence of EPO. The cells were fixed in acetone and immunostained with the p53-specific antibody PAb122, using a fluorescein-labeled second antibody. Cells were counterstained with Evans blue. p53 was detected primarily in the cytoplasm of cells cultured at 37°C (A) or in the nuclei of cells incubated at 32°C without (B) or with (C) EPO. The black-and-white photographs were derived from color slide film. The faint cytoplasmic staining in panels B and C is due to the Evans blue counterstain.

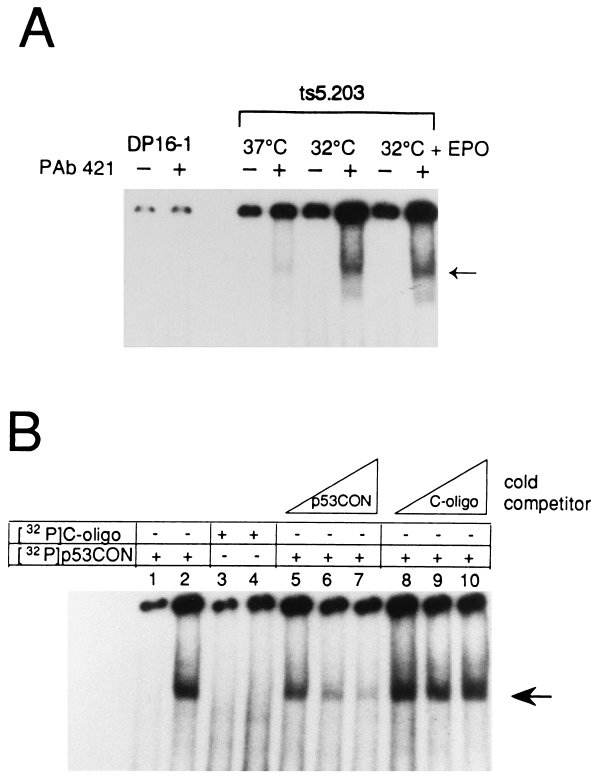

FIG. 7. DNA binding of p53ts protein expressed in ts5.203 cells. (A) Electrophoretic mobility shift assays were performed with nuclear extracts prepared from ts5.203 cells that were cultured at 37 or 32°C in the presence or absence of EPO for 12 h. Parental DP16-1 cells, which lack p53 protein, were used as a negative control. Nuclear extracts contained approximately 10 μg of total protein, and the extracts prepared from ts5.203 cells were adjusted to contain equivalent amounts of p53 protein on the basis of immunoblot analysis. Binding reactions were performed with a ^{32}P-labeled double-stranded oligonucleotide containing the p53 consensus binding sequence (p53CON) with (+) or without (−) the p53-specific monoclonal antibody PAb421. The binding reactions were run on a 4% nondenaturing polyacrylamide gel. The arrow on the right indicates the position of the supershifted PAb421-p53 protein-DNA complex. (B) Control binding reactions in which increasing amounts of nonlabeled p53CON or an unrelated double-stranded oligonucleotide, oligonucleotide C (C-oligo), were used to compete with labeled p53CON for binding to p53 protein present in nuclear extracts prepared from ts5.203 cells cultured at 32°C for 12 h. Unlabeled (cold) competitor was added at 10-fold (lanes 5 and 8), 50-fold (lanes 6 and 9), and 100-fold (lanes 7 and 10) molar excess over the labeled p53CON substrate. All reactions except those applied to lanes 1 and 3 contained antibody PAb421.

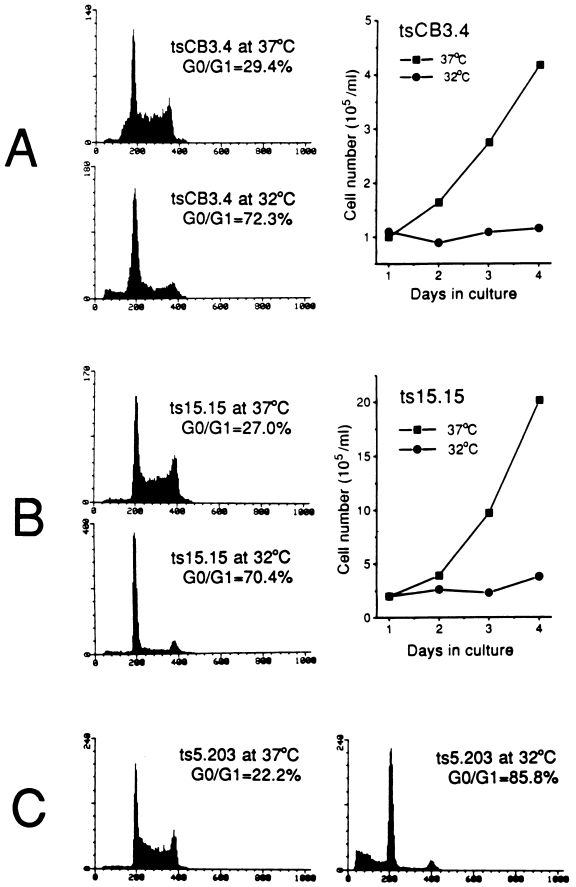

FIG. 8. p53-mediated growth arrest in mouse erythroleukemia cell lines tsCB3.4 (A), ts15.15 (B), and ts5.203 (C). (A and B) In panels on the right, cultures were initiated at a concentration of 10^5 cells per ml at either 37 or 32°C, and on the days indicated, viable cell number was determined by eosin stain exclusion. Panels on the left show flow cytometric analysis of cells grown at 37 or 32°C for 24 h. The flow cytometric analysis of ts5.203 cells cultured at 37 or 32°C for 24 h is shown in panel C for comparison. Cells were fixed in ethanol and stained with propidium iodide as described in the text. The proportion of cells in G_0/G_1 was determined by using the SOBR model of the CellFIT program.

affect p53 protein translocation into the nucleus, the level of p53 protein synthesis, p53 protein conformation, or its sequence-specific DNA binding activity. ts5.203 cells treated with EPO, however, failed to proliferate at 32°C and had arrested predominantly in G_1. Thus, EPO does not interfere with the ability of p53 to mediate G_1 arrest and block entry into S phase.

p53-mediated growth arrest in CB3 cells. DP16-1 cells were derived from mouse spleen cells infected with $SFFV_p$, and F-MuLV, whereas CB3 cells were derived from mouse spleen cells infected with F-MuLV alone. Both cell lines were derived from infected erythroid precursors and are EPO independent. In addition, both cell lines fail to express endogenous p53 protein as the result of rearrangement of the p53 alleles (36). In DP16-1 cells, the *env* gene product, gp55, encoded by $SFFV_p$ is synthesized (21), and in previous studies this viral protein was shown to bind and activate the EPO receptor, resulting in EPO-independent cell growth (27, 39, 57). Having characterized the response of DP16-1 cells to ectopic p53 expression, and having shown that these cells are responsive to added EPO even though they express gp55, we wished to determine if the CB3 cell line, which does not express $SFFV_p$-encoded gp55, would display a similar response to p53ts expression.

After coelectroporation of the p53ts allele and pSV2neo into CB3 cells, a p53-positive and G418-resistant clone (tsCB3.4) was isolated by successive subcloning at 37°C. At 37°C, tsCB3.4 cells grew in the absence of EPO as did the parental CB3 cells. At 32°C, these cells ceased to proliferate and accumulated in G_1 (Fig. 8A). However, a decrease in cell number was not observed over a 4-day period of incubation at 32°C, and few cells with a hypodiploid DNA content characteristic of apoptotic cells were visible after propidium iodide staining and flow cytometry (Fig. 8A) compared with ts5.203 cells (Fig. 8C).

EPO-like activity in CB3 culture supernatants. The response of CB3 cells to p53 expression is similar to that of DP16-1 cells expressing p53 in the presence of EPO. This observation raised the possibility that tsCB3.4 cell survival was the result of autocrine cytokine production. We examined this possibility by testing the culture supernatants of tsCB3.4 and

TABLE 2. Methylcellulose colony assay of mouse bone marrow cells in the presence of cytokines or conditioned media from Friend cell cultures

Condition	Total colony count/2 × 10^5 bone marrow cells	
	CFU-E[a] (day 2)	Non-CFU-E[b] (day 7)
Cytokines		
None	0	0
EPO (1 U/ml)	276	0
IL-3 (1 U/ml)	0	243
KL (1 U/ml)	0	118
Conditioned media (16%)		
CB3	44	0
tsCB3.4	100	0
DP16-1	0	10
ts5.203	0	24
ts15.15	0	136

[a] Each colony contained more than eight cells.
[b] Each colony contained more than 50 cells.

parental CB3 cells for EPO-like activity in a mouse bone marrow colony assay. The culture supernatants were mixed with fresh mouse bone marrow cells and examined for the ability to promote the development of early erythroid colonies in methylcellulose. Since early erythroid progenitors (CFU-E) are highly dependent on EPO, this assay serves as a sensitive test for EPO or EPO-like activity. The results are summarized in Table 2 and indicate that the culture supernatants from CB3 and tsCB3.4 cells supported the development of erythroid colonies (CFU-E) from bone marrow cells. EPO activity was not detected in the culture supernatants obtained from parental DP16-1 cells or from its p53ts-expressing derivatives (ts5.203 and ts15.15). Northern (RNA) blotting indicated that CB3 cells but not DP16-1 cells expressed EPO mRNA (data not shown), suggesting that the secreted EPO-like activity might indeed be due to EPO.

p53-mediated growth arrest in ts15.15 cells. The ts15.15 cell line was derived independently from DP16-1 cells by electroporation of the p53ts allele in the same way as ts5.203 cells. Incubation at 32°C for 24 h resulted in an accumulation of cells in G_1, and few cells with a hypodiploid DNA content characteristic of apoptotic cells were visible after propidium iodide staining and flow cytometry (Fig. 8B). There was little change in cell number over a 4-day period of incubation at 32°C (Fig. 8B). This response was similar to that of tsCB3.4 cells and different from that of ts5.203 cells.

Involvement of an autocrine loop in blocking p53-mediated apoptosis in ts15.15 cells. Using a methylcellulose-based mouse bone marrow colony assay, we determined that these cells do not secrete an EPO-like activity (Table 2). They do, however, secrete an activity that supports the growth of non-CFU-E colonies (Table 2). A similar activity was detected in the culture supernatants obtained from DP16.1 and ts5.203 cells but at levels 6- to 14-fold lower than in ts15.15 cells. This activity was not detected in the culture supernatants obtained from CB3 or tsCB3.4 cells. In addition, ts15.15-conditioned medium promoted the proliferation of the murine IL-3-dependent myeloid progenitor cell line, 32D (data not shown). Importantly, we have tested conditioned medium collected from ts15.15 cells for its ability to suppress p53-mediated apoptosis in ts5.203 cells cultured at 32°C. ts15.15-conditioned medium was effective and could replace EPO, KL, or IL-3 in this assay (data not shown).

Cells whose growth or survival is contingent upon autocrine secretion of a factor often display cell density-dependent effects. To assess the dependency of ts15.15 cells on the secreted cytokine, cells were seeded at various cell densities and cultured in suspension medium (α-MEM and 10% FCS) for several days at either 37 or 32°C. Clonogenic cell survival in the suspension cultures as a function of time was determined by plating fixed volumes in methylcellulose supplemented with α-MEM and 10% FCS and incubating the dishes at 37°C. ts15.15 cells proliferated in suspension culture at 37°C even at initial densities as low as 100 cells per ml, and clonogenic cell recovery increased in the methylcellulose colony assay (data not shown). Clonogenic cell recoveries were proportionally similar in suspension cultures initially seeded at 10^5 cells per ml and cultures initially seeded at 100 cells per ml. Hence, clonogenic cell recovery from suspension cultures kept at 37°C was not dependent on cell density, and as a result, it is unlikely that secreted cytokines are required for the proliferation or viability of ts15.15 cells at 37°C. At 32°C, however, clonogenic cell recovery was dependent on the initial cell density of the suspension culture and decreased with the duration of incubation in suspension at 32°C (Fig. 9). It is apparent that high cell density in the suspension culture protected clonogenic cells from p53-mediated cell killing. We conclude that the failure of ts15.15 cells to undergo p53-mediated apoptosis is cell density dependent and is therefore likely to be a direct consequence of autocrine cytokine release.

To determine if secreted IL-3 was responsible for blocking p53-mediated apoptosis and promoting survival of ts15.15 cells at 32°C, an anti-IL-3 antibody was added directly to cultures of ts15.15 cells incubated at either 32 or 37°C. Cell viability was then assessed by using eosin staining and compared with that of control cultures grown without the anti-IL-3 antibody (Fig. 10). The anti-IL-3 antibody effectively inhibited the survival of the ts15.15 cells cultured at 32°C. In contrast, no effect of the anti-IL-3 antibody, even when added at high levels, was observed on the growth of ts15.15 cells maintained at 37°C, excluding the possibility that the anti-IL-3 antibody was directly toxic to ts15.15 cells (Fig. 10, inset).

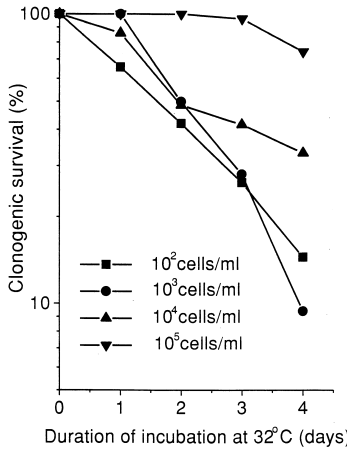

FIG. 9. Clonogenic cell recovery of ts15.15 cells cultured in suspension at 32°C at various cell densities. ts15.15 cells were incubated at 32°C at various initial cell densities in suspension culture consisting of α-MEM and 10% FCS. On 4 consecutive days, clonogenic cell survival in the suspension cultures was determined by plating fixed volumes in a methylcellulose colony assay at 37°C as described in Materials and Methods.

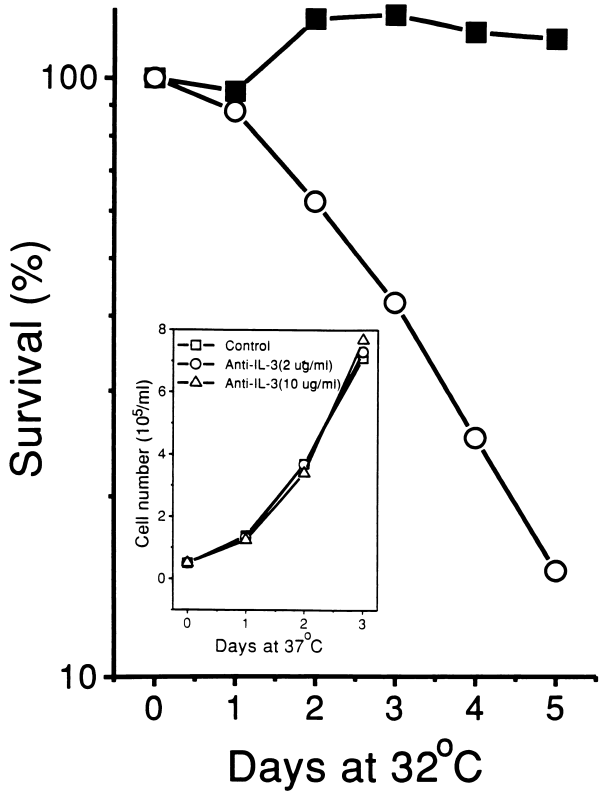

FIG. 10. Effect of anti-IL-3 antibody on the survival of ts15.15 cells. ts15.15 cell cultures, at a concentration of 1.5×10^5 cells per ml, were transferred to 32°C. A monoclonal antibody against mouse IL-3 was not added (■) or was added to the cultures at the start of the experiment (day 0) at a concentration of 2 μg/ml (○). Survival was measured by eosin stain exclusion. Each point represents the mean of triplicate measurements. The inset shows the growth of ts15.15 cells at 37°C in the presence or absence of the IL-3 antibody.

DISCUSSION

We have compared the responses of three different Friend cell clones expressing a p53ts protein at 32°C, the temperature at which the mutant p53 protein becomes active probably owing to a change in conformation. Clone ts5.203 underwent an arrest in the G_1 phase of the cell cycle that was accompanied by loss of cell viability, apoptosis, and differentiation. Consistent with previous reports, p53ts-expressing cells already in S or G_2/M at the time of the temperature shift continued to traverse the cell cycle until they entered G_1 and arrested (21, 32, 40). Cells containing DNA strand breaks characteristic of apoptotic cells were detected in all phases of the cell cycle within 6 h after the temperature was shifted to 32°C. Clones ts15.15 and tsCB3.4 accumulated in G_1; however, viability was preserved and little apoptosis was observed.

In ts5.203 cells, apoptosis and differentiation were effectively suppressed by the inclusion of various cytokines in the culture medium of ts5.203 cells, including EPO, KL, and IL-3. The G_1 arrest, however, was unperturbed in the presence of EPO. We confirmed that the p53ts protein was expressed and that it retained double-stranded DNA binding activity in these cells at 32°C in the presence of EPO. Hence, EPO did not abrogate p53-mediated apoptosis through downregulation of p53 expression or change in p53 protein conformation. These data demonstrate that p53-mediated apoptosis and p53-mediated G_1 arrest can be uncoupled in erythroleukemia cells by the action of certain cytokines.

This idea was corroborated by the analysis of ts15.15 and tsCB3.4 cells. tsCB3.4 was derived from the CB3 murine erythroleukemia cell line. We determined that these cells express EPO mRNA and secrete EPO or a factor with EPO-like activity into the culture medium. It is probable that this secreted factor suppresses p53-mediated apoptosis but not p53-mediated G_1 arrest in these cells in the same way that EPO suppresses apoptosis when it is added to ts5.203 cells. ts15.15, like ts5.203, was derived from the Friend virus-transformed murine erythroleukemia cell line DP16-1. The ability of ts15.15 cells to survive at 32°C without undergoing apoptosis was shown to be cell density dependent. High cell density afforded protection from apoptosis, whereas at low cell density, ts15.15 cells were sensitive to p53-mediated cell death. Low cell density had no effect on the growth of ts15.15 cells at 37°C. The ts15.15 cell line does not secrete EPO; it does, however, secrete a factor that promotes the formation of non-CFU-E colonies from progenitors present in mouse bone marrow and sustains the proliferation of the IL-3-dependent 32D cell line. Moreover, conditioned medium collected from ts15.15 cells suppressed p53-mediated apoptosis in ts5.203 cells. The demonstration that the survival of ts15.15 cells at 32°C could be blocked by an anti-IL-3 antibody provides strong evidence that externally secreted IL-3 is required for the survival of ts15.15 cells.

The ability of at least three different cytokines acting through different receptors to suppress p53-mediated apoptosis in erythroleukemia cells suggests that different cell types may also respond differently to p53 protein expression, depending on culture conditions and the inherent capacity of each line to secrete and respond to growth factors. Enhanced cytokine secretion or responsiveness in p53ts-expressing DP16-1 cells may also explain why these cells invariably lose the ability to undergo p53-mediated apoptosis after prolonged culture at 37°C.

In their ability to suppress p53-mediated apoptosis, EPO, KL, and IL-3 are acting as survival factors rather than as proliferative factors. Koury and Bondurant (24) previously demonstrated that EPO inhibits apoptosis in murine proerythroblasts infected with the anemia-inducing strain of Friend virus. EPO has also been shown to promote the survival but not the growth of 32D cells deprived of IL-3 (2). IL-3-deprived 32D cells that had extended survival in EPO were reversibly arrested in the G_1 phase of the cell cycle. Hence, EPO is providing a survival function in 32D cells but is unable to promote cell cycle progression. While the role of p53 in mediating apoptosis in erythroid progenitors and in 32D cells is not certain, these studies do reveal a role for EPO in blocking apoptosis without promoting proliferation and are consistent with the findings reported here. The ability of EPO to maintain cells in a viable G_1-arrested state as shown here and by Askew et al. (2) may be critical for its function as a survival factor. The growth arrest phenotype of ts5.203 cells at 32°C in the presence of EPO was reversible even after 5 days, with viable cell numbers increasing upon the return of the cultures to 37°C. This finding indicates that the cells were arrested early in the cell cycle at a stage prior to their commitment to undergo apoptosis or differentiation.

Yonish-Rouach et al. have shown that IL-6 can inhibit p53-mediated apoptotic cell death in mouse M1 myeloid cells (56). IL-6 normally induces myeloid differentiation of M1 cells in culture. However, when these cells were transfected with a p53ts allele and forced to express p53 protein in its wild-type conformation at 32.5°C, the inclusion of IL-6 in the culture medium prevented cell death. IL-3 also served as a survival factor provided IL-3 receptors were present. Survival of these cells may be explained by the observation that M1 cells exited the cell cycle and were arrested in a G_0-like quiescent state

upon activation of p53 and addition of IL-6 (26). Thus, retention of p53-expressing cells in a non-cycling G_0/G_1-arrested state by cytokines prevents apoptosis. In contrast with our findings with ts5.203 cells and EPO, M1 cells exposed to activated p53 and IL-6 became irreversibly factor dependent, as reflected by loss of cell viability when the cells were placed in cytokine-free medium at 37.5°C (55).

Oncogenes such as c-*myc* and E1A can induce apoptosis particularly after serum depletion, and this form of apoptosis has been shown to be dependent on p53 (8, 30, 50). Myc-induced apoptosis in low serum can be inhibited by insulin-like growth factors and platelet-derived growth factor (18). In addition, expression of a survival gene such as *bcl-2* or E1B will block p53-dependent apoptosis (6, 8, 48, 51). Bcl-2 expression in baby rat kidney cells expressing p53ts protein at 32°C blocked p53-dependent apoptosis and was associated with reversible and complete growth arrest. These cells were not arrested at any single point in the cell cycle but rather were arrested in G_1, S, and in G_2/M (6). It will be interesting to determine if certain growth factor-receptor interactions activate signalling pathways that ultimately lead to the expression of *bcl-2* or *bcl-2*-like survival genes that override the induction of the cell death program.

One commonality that emerges from this discussion is the requirement for cells destined to undergo p53-dependent apoptosis to be actively cycling. In this regard, Wu and Levine (52) have demonstrated that p53ts-expressing mouse embryo fibroblasts undergo a reversible G_1 arrest at 32°C. Coexpression of the transcription factor E2F-1 in these cells resulted in a rapid loss of cell viability through a process of apoptosis. Deregulated overexpression of E2F-1 has been shown to promote S-phase entry (20), leading to apoptosis (38, 42).

DP16-1 cells express the SFFVp *env* gene product, gp55, which binds to the EPO receptor. This interaction leads to receptor activation and mitogenesis in the absence of EPO. Deregulated and constitutive expression of gp55 in DP16-1 cells may give rise to persistent signalling through the EPO receptor. Sustained activation of the EPO receptor signalling pathway in response to gp55 may be incompatible with wild-type p53 expression leading to apoptosis in a manner analogous to deregulated E2F-1 expression, which leads to inappropriate cell cycle progression and apoptosis dependent on p53. The ability of EPO to block apoptosis could mean that the signal transmitted from EPO receptors activated by binding to gp55 is different from that of EPO receptors bound to EPO. EPO binding to its receptor may result in transient and regulated activation of a signalling pathway that promotes survival.

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