Characterization of the Molecular Mechanisms for p53-mediated Differentiation[1]

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Abstract

The p53 tumor suppressor protein can induce both apoptosis and cell cycle arrest. Moreover, we and others have shown previously that p53 is a potent mediator of differentiation. For example, expression of ptsp53, a temperature-inducible form of p53, induces differentiation of leukemic monoblastic U-937 cells. The functions of p53 have for long been believed to be dependent on the transactivating capacity of p53. However, recent data show that both p53-induced cell cycle arrest and apoptosis can be induced independently of p53-mediated transcriptional activation, indicating alternative pathways for p53-induced apoptosis and cell cycle arrest. The *bcl-2* proto-oncogene contributes to the development of certain malignancies, probably by inhibition of apoptosis. Interestingly, Bcl-2 has been shown to inhibit p53-mediated apoptosis as well as p53-mediated transcriptional activation. Asking whether Bcl-2 would interfere with the p53-mediated differentiation of U-937 cells, we stably transfected *bcl-2* to U-937 cells inducibly expressing p53. Although the established Bcl-2-expressing clones were resistant to p53-mediated apoptosis, we did not observe any interference of Bcl-2 with the p53-mediated differentiation, suggesting separable pathways for p53 in mediating apoptosis and differentiation of U-937 cells. Neither did expression of Bcl-2 interfere with p53-induced expression of endogenous p21, suggesting that p53-induced differentiation might be dependent on the transcriptional activity of p53. To further investigate whether the p53-mediated differentiation of U-937 cells depends on the transcriptional activity of p53, we overexpressed *trans*-activation-deficient p53, a transcriptionally inactive p53 mutant in these cells. However, in contrast to the effects of wild-type p53, expression of *trans*-activation-deficient p53 did neither induce signs of apoptosis nor of differentiation in U-937 cells. Our results indicate that the transcriptional activity of p53 is essential both for p53-mediated apoptosis and differentiation of U-937 cells.

Introduction

One of the key proteins protecting tissues from malignant transformation is the tumor suppressor p53 (1). The tumor-suppressing activity of p53 is usually explained by its ability to prevent expansion of potentially malignant cells by either induction of apoptosis or an arrest in the G_1 phase of the cell cycle (1–3). p53 is a transcription factor and can transactivate genes of importance both for apoptosis (*i.e., bax*; Ref. 4) and G_1 arrest (*i.e., p21*; Refs. 2 and 5).

Apart from its role in apoptosis and cell cycle regulation, p53 has also been shown to participate in the differentiation process of a number of tissues such as pancreatic carcinoma cells, muscle cells, keratinocytes, neurons, thyroid cells (6–8), and various hematopoietic cell lines. For example, expression of p53 induces differentiation of leukemic L12 pre-B-cells, erythroleukemic K562 cells, Friendvirus transformed erythroleukemic cells, and promyelocytic HL-60 cells (9–12). Our own results show that inducible overexpression of p53 induces differentiation *per se* as well as promotes induction of differentiation with Vit D3[3] in monoblastic U-937 cells (13). The molecular mechanisms for p53-mediated differentiation are not clear but do not seem to rely on induction of p21 (14) or on a cell cycle arrest mediated by the hypophosphorylated form of the retinoblastoma protein (15).

The proto-oncogene *bcl-2* was first identified in the t(14;18) chromosomal translocation involved in 70% of human follicular B-cell lymphomas (16–18). Overexpression of Bcl-2 in lymphoid cells in culture and in transgenic mice prevents apoptosis induced with a wide variety of agents, such as steroids, gamma irradiation, or growth factor deprivation (19, 20). Importantly, Bcl-2 has also been shown to inhibit p53-induced apoptosis (21–23). Besides its antiapoptotic properties, Bcl-2 has a cell cycle-inhibitory function separable from its promotion of cell survival (24–26). Although levels of Bcl-2 fall during myeloid differentiation of normal bone marrow cells, consistent with an inhibitory role for Bcl-2 in hematopoietic differentiation (27), Bcl-2 does not seem to be a potent inhibitor of induced myeloid differentiation. Hence, overexpression of

Received 5/25/00; revised 9/25/00; accepted 9/27/00.
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[1] This work was supported by the Swedish Cancer Foundation, the Swedish Childhood Cancer Foundation, The Tobias Foundation, The Swedish Medical Research Council (Project 11546), Funds of Lunds sjukvårdsdistrikt, and the Gunnar, Arvid and Elisabeth Nilsson Foundation.
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[3] The abbreviations used are: Vit D3, vitamin D_3, 1α,25-dihydroxycholecalciferol; FACS, fluorescence activated cell sorter; NBT, nitroblue tetrazolium; TADp53, *trans*-activation-deficient p53; moAb, monoclonal antibody; IP, immunoprecipitation.

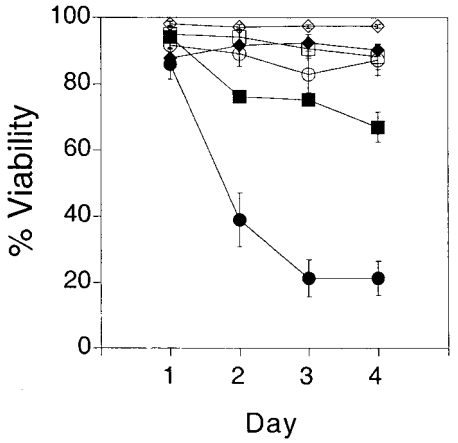

Fig. 1. Temperature dependence of the cell death-related properties of ptsp53(Val135). The U-937/ptsp53 clone A2 and the mock-transfected U-937 clone M2 at an initial concentration of 0.2×10^6 cells/ml were grown in suspension culture at 31.5°C, 32.5°C (*i.e.,* temperatures permissive for wild-type p53 activity) and 37°C (*i.e.,* the nonpermissive temperature for wild-type p53-activity) for 4 days. Viability, as judged by trypan blue exclusion, was determined daily. ●, ptsp53/A2 31.5°C; ■, ptsp53/A2 32.5°C; ♦, ptsp53/A2 37°C; ○, M2 31.5°C; □, M2 32.5°C; ◇, M2 37°C. Mean values are from three separate experiments; *bars,* SE.

Bcl-2 in HL-60 cells does not inhibit differentiation induced with all-*trans* retinoic acid (19, 28), and neutrophils from transgenic mice overexpressing Bcl-2 do not have a defect differentiation (29).

Several mechanisms for Bcl-2-mediated inhibition of p53 activity have been reported. For example, Bcl-2 binds to the protein product from the p53-target gene *bax* and inhibits its death-inducing activity (4, 23, 30). Moreover, Bcl-2 inhibits nuclear import of p53 in some cells (31). High levels of Bcl-2 can also inhibit the induction of the p53-regulated genes *p21*, *bax,* and *gadd45* after genotoxic stress (32). However, Bcl-2 has been shown not to interfere with p53-mediated G_1 arrest (22). Interestingly, the relationship between p53 and Bcl-2 seems reciprocal in that p53 can transcriptionally repress the expression of Bcl-2 (4, 23, 33, 34).

Both p53-mediated cell cycle arrest (35, 36) and apoptosis (37–39) can be induced independently of the transcriptional activity of p53. For example, by means of a potential SH3-domain binding site, p53 has been suggested to participate in a growth-arresting signal transduction pathway (35, 36).

Given the ability of Bcl-2 to inhibit p53-mediated apoptosis, potentially by means of its reported inhibitory effect on p53-mediated transactivation (32, 40), we set out to determine whether expression of Bcl-2 would interfere with p53-mediated differentiation. We show that Bcl-2 does not interfere with p53-mediated differentiation or with the transcriptional activity of p53. To further investigate whether the transactivating properties of p53 are necessary for p53-mediated differentiation, we expressed a temperature-sensitive transcriptionally inactive mutant of p53 [*i.e.*, p53(25, 26, Val135)] (Ref. 41) in U-937 cells. Our results show that the transcriptional activity of p53 is essential for induction of differentiation of U-937 cells.

Table 1 Percentage of ptsp53-expressing U-937 cells present in the sub-G_1 phase of the cell cycle after incubation at different temperatures

Control and U-937/ptsp53 A2 cells were seeded at 200,000 cells/ml in culture medium and incubated at 37°C, 32.5°C, and 31.5°C. After 48 h, viability was assessed by trypan blue exclusion. Concomitantly, cells were subjected to cell cycle analysis by flow cytometry as described in "Materials and Methods." Mean values from three experiments are shown. Viability of control cells was always >95%, and the fraction of control cells with a sub-G_1 DNA was always <10%.

Incubation temperature	Percentage of cells in sub-G_1 phase ± SE	Percentage of cells excluding trypan blue ± SE
37°C	8.58 ± 3.2	99 ± 0.6
32.5°C	17.7 ± 3.1	86 ± 2
31.5°C	38.3 ± 4.7	47 ± 4

Results

The Cell Death and Proliferation-related Properties of ptsp53(Val135) Depend on Small Shifts of the Temperature. Neither p53 protein (42–44) nor mRNA (44) can be detected in monoblastic U-937 cells, whose gene for p53 is altered by a point mutation (45). Therefore, to obtain inducible expression of p53 on a p53-null background, we overexpressed a temperature-inducible form of p53 [(*i.e.,* ptsp53(Val135)] in U-937 cells as described previously (13). When incubated at 32°C, U-937/ptsp53/A2 cells show signs of differentiation, cell cycle arrest, and apoptosis (13), reflecting the wild-type p53 activity of ptsp53. However, a difference in ptsp53 function was observed during induction of wild-type p53 activity of ptsp53 by incubation of U-937/ptsp53/A2 cells at temperatures around 32°C. When incubated at 32.5°C, mainly p53-mediated differentiation (data not shown) with almost no signs of cell death was observed, whereas incubation at 31.5°C induced pronounced p53-mediated cell death measured both by trypan blue exclusion (Fig. 1) and as fraction of cells with a sub-G_1 DNA content (Table 1). Because this difference in p53 function might suggest that ptsp53 achieves a higher wild-type p53 activity when incubated at 31.5°C than at 32.5°C, we wanted to determine whether this functional difference extended to the cell cycle regulatory properties of p53. For this purpose, mock-transfected and ptsp53-expressing U-937 cells were incubated at 37°C, 32.5°C, and 31.5°C. Each day, cells were harvested and subjected to a cell cycle analysis by flow cytometry (Table 2). Interestingly, after 24 h, the fraction of ptsp53-expressing cells present in S-phase after incubation at 31.5°C was lower than the S-phase fraction of ptsp53-expressing cells incubated at 32.5°C in repeated experiments. However, this temperature-related difference disappeared with prolonged culture, because the cell cycle distributions of U-937/ptsp53/A2 cells incubated at 31.5°C and 32.5°C for 48 and 72 h, respectively, were comparable. To investigate whether the initial cell cycle regulatory differences of ptsp53 were reflected by an altered p53-mediated up-regulation of the cell cycle regulator p21, a Western blot was performed in parallel with the cell cycle analysis made after 24 h. No p21 was detected in a mock-transfected control clone or in the U-937/ptsp53/A2 clone incubated at 37°C (*i.e.,* the nonpermissive temperature; Fig. 2). However,

Table 2 Cell cycle distribution of ptsp53-expressing and mock-transfected U-937 cells

Cells were seeded in culture medium at 200,000 cells/ml and incubated at the indicated temperatures. On days 1, 2, and 3, cells were explored for cell cycle distribution by a FACS analysis. Values show percentage of viable cells (*i.e.*, cells with sub-G_1 DNA content are excluded). One representative experiment is shown.

	Temperature	Day 1			Day 2			Day 3		
		G_0–G_1	S-phase	G_2-M	G_0–G_1	S-phase	G_2-M	G_0–G_1	S-phase	G_2-M
U-937 M1	37°C	44.2	45.8	9.93	46.8	45.4	7.8	62.2	34.2	3.5
U-937/ptsp53/A2		55.2	31.6	13.2	53.2	41.3	5.5	55.5	39.8	4.7
U-937 M1	32.5°C	48.4	34.8	16.8	41.5	46.6	11.9	27.8	72.1	0.2
U-937/ptsp53/A2		76.6	16.2	7.2	87.2	5.1	7.7	96	4	0
U-937 M1	31.5°C	46.7	31.6	21.7	36.8	52.7	10.5	36.1	55.7	8.3
U-937/ptsp53/A2		76.1	6.55	19.4	85.4	6.5	8.1	86.5	11.7	1.8

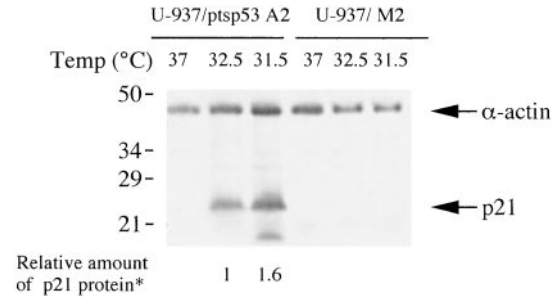

Fig. 2. Temperature dependence of the levels of ptsp53-induced p21. The U-937/ptsp53 clone A2 and the mock-transfected U-937 clone M2 were incubated at 31.5°C, 32.5°C (*i.e.,* temperatures permissive for wild-type p53 activity), and 37°C (*i.e.,* the nonpermissive temperature for wild-type p53 activity). After 24 h, cells were subjected to Western blot using the mouse moAb anti-p21 WAF-1 Ab-1 and an actin antibody, serving as a control for equal loading (described in "Materials and Methods"). *Arrows* on the *right,* positions of the p21 and actin proteins. *Left,* positions of molecular weight standards (in thousands). ∗, the relative amount of p21 protein was estimated by densitometry as described in "Materials and Methods" and normalized to the amount of actin in the corresponding lane.

when incubated at 32.5°C and 31.5°C, p21 was up-regulated at comparable levels in the U-937/ptsp53/A2 clone. Moreover, a slightly higher level of p21 protein in the 31.5°C incubation was observed on repeated Western blots, further supporting that ptsp53 has a higher wild-type p53 activity at 31.5°C than at 32.5°C. To investigate whether this elevated level of p21 protein reflected a higher transactivating capacity of p53 at the p21 promoter, luciferase reporter experiments were performed. Mock-transfected and ptsp53-expressing U-937 cells were transfected with the firefly luciferase gene under the control of the p21 promoter. The firefly luciferase gene under the control of the SV40 promoter was serving as a positive control of luciferase activity. After incubation at 32.5°C and 31.5°C for 16 h, the luciferase activity of the cells was determined (Table 3). As measured by luciferase activity, the p53-mediated transactivation of the p21 promoter was slightly higher at 31.5°C as compared with 32.5°C, possibly indicating a higher wild-type p53 activity at 31.5°C. Therefore, to obtain maximal wild-type p53 activity, all viability studies and transactivation studies in subsequent experiments were performed at 31.5°C. However, because pronounced cell death makes it difficult to perform differentiation experiments, all differentiation studies were performed at 32.5°C.

p53-mediated Cell Death Is Partially Overrun by High Levels of Bcl-2. To study the role of Bcl-2 in p53-mediated differentiation, stable Bcl-2 overexpression was established in the U-937/ptsp53/A2 clone (13). Transfection of the U-937/ptsp53/A2 clone with *bcl-2* resulted in 10 clones growing under selective conditions. When analyzed for expression of Bcl-2 protein by IP-Western, all of these clones showed a clear overexpression of Bcl-2 as compared with wild-type U-937 cells (Fig. 3). To ascertain that clones still expressed high levels of p53, they were analyzed for expression of p53 by biosynthetic labeling, IP, and fluorography (data not shown). On the basis of their high expression levels of both p53 and Bcl-2, clones A4, A8, and A9 were chosen for further examination.

To examine whether Bcl-2 can rescue U-937 cells from p53-mediated cell death, U-937/ptsp53/Bcl-2 clones and mock-transfected U-937/ptsp53 clones were incubated at the optimal temperature for the apoptosis-inducing activity of p53 (*i.e.,* 31.5°C) for 4 days. Each day, cells were counted, and the viability was assessed by trypan blue exclusion. As shown in Fig. 4, Bcl-2 conferred partial resistance to p53-mediated cell death. On day 2, most Bcl-2-expressing cells were alive, in contrast to control cells already showing pronounced p53-mediated cell death. On day 4, the viability of U937/ptsp53/bcl-2 cells was ~45%, still obviously higher than control cells. To determine whether the cell death was attributable to apoptosis, the cells were analyzed for expression of Annexin V by FACS analysis, concomitantly with propidium iodide staining (Table 4), providing a selective method for detection of apoptosis (46). As demonstrated, the cells showed characteristics of apoptosis, as measured by expression of Annexin V.

Overexpression of Bcl-2 Does Not Affect the Proliferation Rate of U-937 Cells. Because Bcl-2 has been shown previously to possess growth-arresting features (24–26), we asked whether U-937 cells transfected with Bcl-2 showed a decreased proliferation rate. U-937/ptsp53/Bcl-2 cells and mock-transfected U-937/ptsp53 cells were incubated at the temperature nonpermissive for wild-type p53 activity (*i.e.,* 37°C) for 4 days. Each day, cells were counted, and viability was assessed by trypan blue exclusion. No difference in proliferation rate was seen between Bcl-2-expressing and mock-transfected U-937/ptsp53 clones, indicating that Bcl-2 does not influence the proliferation rate of U-937 cells (Fig. 5).

Table 3 Temperature dependence of the ptsp53-induced transcriptional activation of the p21 promoter in U-937 cells

Mock-transfected and ptsp53-expressing U-937 cells were transfected with luciferase expression constructs driven by the p21 promoter. The SV40 promoter was serving as a control of luciferase activity. Cells were incubated at 32.5°C and 31.5°C, respectively, and after 16 h, the luciferase activity was determined as described in "Materials and Methods." Values represent mean values from three independent experiments, each consisting of a triplicate of samples.

U-937 clone	Promoter sequence driving luciferase expression	Incubation temperature	Luciferase activity ± SE (arbitrary units)
Mock 2	SV40	32.5°C	485 ± 52
	p21	32.5°C	229 ± 42
ptsp53 A2	SV40	32.5°C	395 ± 60
	p21	32.5°C	1425 ± 264
Mock 2	SV40	31.5°C	571 ± 70
	p21	31.5°C	313 ± 52
ptsp53 A2	SV40	31.5°C	554 ± 62
	p21	31.5°C	2290 ± 439

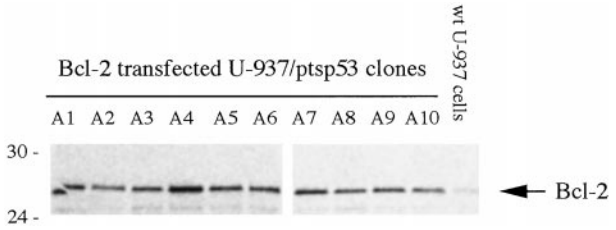

Fig. 3. Expression of Bcl-2 protein in transfected U-937/ptsp53 cells. Bcl-2-transfected U-937/ptsp53 clones and wild-type U-937 cells were subjected to IP, followed by Western Blot (IP-Western) using the mouse monoclonal anti-bcl-2-antibody 1550 as described in "Materials and Methods." *Arrow* on the *right,* position of Bcl-2 protein at M_r 26,000. Low amounts of Bcl-2 protein were detected in U-937 wild-type cells. *Left,* positions of molecular weight standards (in thousands).

Preserved p53-mediated Differentiation in the Presence of Bcl-2. U-937 cells are known to respond with signs of differentiation, when incubated with substances like Vit D3 (47). To investigate whether Bcl-2 interferes with the Vit D3-induced differentiation of U-937 cells, mock-transfected and Bcl-2-expressing U-937/ptsp53 clones were incubated with and without Vit D3 at the temperature nonpermissive for wild-type p53 activity (*i.e.,* 37°C). NBT reduction test provides a functional assessment of myeloid differentiation, reflecting the capacity of the cells for respiratory burst. No difference in NBT reduction was observed between mock-transfected and Bcl-2-expressing U-937/ptsp53 clones, indicating that Bcl-2 does not interfere with the induced differentiation of U-937 cells in the absence of wild-type p53 activity (Fig. 6). However, Bcl-2 can inhibit the apoptosis-inducing aspects of p53. Therefore, we asked whether Bcl-2 would inhibit p53-mediated differentiation as well. For this purpose, Bcl-2-expressing and mock-transfected U-937/ptsp53 clones, as well as mock-transfected wild-type U-937 cells, were incubated at the optimal temperature for the differentiation-inducing activity of ptsp53 (*i.e.,* 32.5°C). As measured by reduction of NBT, mock-transfected U-937 cells not expressing ptsp53 showed almost no signs of differentiation (Fig. 7). However, U-937 clones coexpressing ptsp53 and Bcl-2 responded with reduction of NBT at levels comparable with control clones expressing ptsp53 alone. This suggests that Bcl-2 does not interfere with differentiation induced by p53 *per se*. Furthermore, in experiments with different concentrations of Vit D3, the Bcl-2-expressing U-937/ptsp53 clones responded with reduction of NBT at levels comparable with the ptsp53-expressing control clones (Fig. 8), again supporting the conclusion that Bcl-2 does not interfere with the p53-mediated differentiation of U-937 cells.

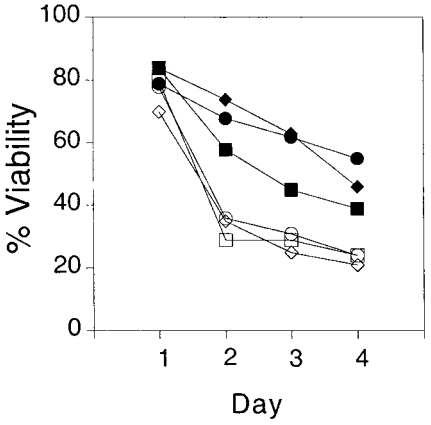

Fig. 4. Viability in suspension culture of Bcl-2-expressing U-937/ptsp53 cells. Mock-transfected and Bcl-2-expressing U-937/ptsp53 cells at an initial concentration of 0.2×10^6 cells/ml were grown in suspension culture at 31.5°C (*i.e.,* the permissive, apoptosis-inducing ptsp53 temperature) for 4 days. Viability as judged by trypan blue exclusion, and the total number of cells was determined daily. ●, Bcl-2/A4; ■, Bcl-2/A8; ♦, Bcl-2/A9; ○, Mock 2; □, Mock 5; ◇, Mock 6. Mean values are from at least three separate experiments. The total number of cells was always $0.2 \pm 0.05 \times 10^6$ cells/ml throughout the experiments.

p53 Transactivates p21 in U-937 Clones Overexpressing Bcl-2. Bcl-2 can inhibit the transcriptional activity of p53 in some cell lines (32, 40). Therefore, to determine whether Bcl-2 inhibits the transcriptional activity of p53 in U-937 cells, mock-transfected and Bcl-2-expressing U-937/ptsp53 clones were incubated at the permissive (*i.e.,* 31.5°C) and nonpermissive (*i.e.,* 37°C) temperature. After 24 h, cells were subjected to analysis of p53-mediated transactivation of p21 by biosynthetic labeling, IP, and fluorography (Fig. 9). As demonstrated, no p21 is expressed in mock-transfected or Bcl-2-expressing U-937/ptsp53 clones incubated at the nonpermissive temperature. However, at the permissive temperature, both mock-transfected and Bcl-2-expressing U-937/ptsp53 clones respond with up-regulation of p21 at comparable levels. The up-regulation of p21 is not attributable to the incubation at 31.5°C, because incubation of wild-type U-937 cells at the permissive temperature does not provoke expression of p21 (data not shown; Ref. 15). These data indicate that Bcl-2 does not interfere with the transcriptional activity of p53 in U-937 cells.

Characterization of Cell Clones Expressing a Transcriptionally Inactive p53-Mutant in U-937 Cells. To study the role of transcriptional activity of p53 for p53-mediated differentiation, TADp53, a transcriptionally inactive form of the murine temperature-sensitive ptsp53(Val135) (Ref. 41)

Table 4 Expression of the apoptosis-related cell surface antigen Annexin V in control cells and Bcl-2 expressing cells upon expression of wild-type p53 activity

U-937 cells were incubated at an initial concentration of 200,000 cells/ml in culture medium at 31.5°C. After 1 day, cells were subjected to analysis of Annexin V by flow cytometry. Values shown are the percentages of cells expressing Annexin V. One representative experiment of three is shown.

Control U-937 clones			Control U-937/ptsp53 clones			Bcl-2 expressing U-937/ptsp53 clones		
Mo1	Mo2	Mo4	p53 M2	p53 M5	p53 M6	A4	A8	A9
3.96	2.36	2.08	18.7	26.9	18.1	13.5	8.50	7.87

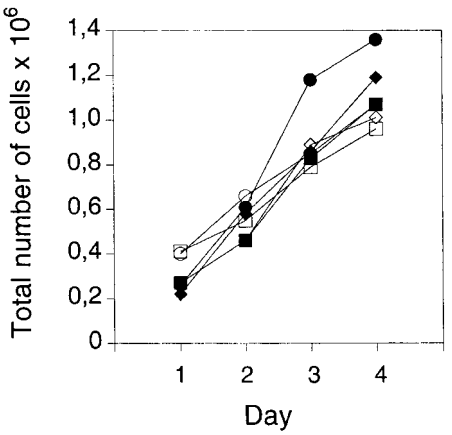

Fig. 5. Growth rate in suspension culture of Bcl-2-expressing cells. Mock-transfected and Bcl-2-expressing U-937/ptsp53 cells at an initial concentration of 0.2×10^6 cells/ml were grown in suspension culture at 37°C (*i.e.,* the temperature nonpermissive for wild-type p53 activity) for 4 days. The total number of cells and viability, as judged by trypan blue exclusion, was determined daily. ●, Bcl-2/A4; ■, Bcl-2/A8; ◆, Bcl-2/A9; ○, Mock 2; □, Mock 5; ◇, Mock 6. Mean values are from at least three separate experiments. Viability was always >90% throughout the experiments.

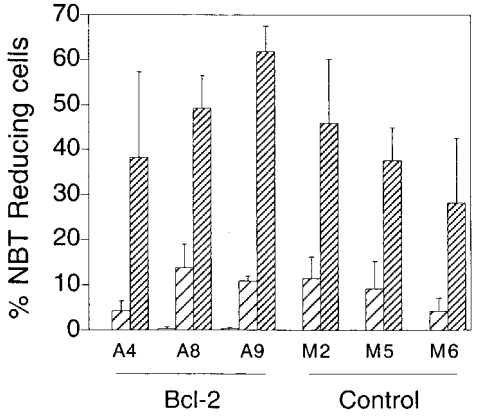

Fig. 6. Effects of Bcl-2 on the Vit D3-induced differentiation of U-937 cells in the absence of wild-type p53 activity assayed by the capacity to reduce NBT. Mock-transfected (*Control*) and Bcl-2-expressing (*Bcl-2*) U-937/ptsp53 cells at an initial concentration of 0.2×10^6 cells/ml were incubated with or without Vit D3 at different concentrations at 37°C (*i.e.,* the nonpermissive ptsp53 temperature). After 4 days, the cells were subjected to an NBT test. □, culture medium; ▨, Vit D3 1 nM; ▨, Vit D3 10 nM. Mean values from three separate experiments are shown; *bars,* SE.

was transfected into U-937 cells, resulting in 36 clones growing under selective conditions. Four of these clones were shown to express TADp53 by biosynthetic labeling, IP, and fluorography (Fig. 10) and also by repeated Western blots (data not shown). These four clones were chosen for further investigation and were designated U-937/TADp53 C5, C14, C31, and C35.

Because recent reports have shown that TADp53 causes a slight activation of reporter constructs carrying a p53 promoter sequence (48, 49), we analyzed the protein levels of the endogenous p53 target gene *p21* in response to expression of wild-type TADp53 by Western blot. As shown in Fig. 11, no p21 is expressed in U-937/ptsp53/A2 cells at the nonpermissive temperature (*i.e.,* 37°C), but when incubated at the permissive temperature (*i.e.,* 31.5°C), the cells respond with a pronounced up-regulation of p21. However, U-937 cells expressing TADp53 show no detectable up-regulation of p21 when incubated at the temperature permissive for wild-type p53-activity. Thus, TADp53 does not induce detectable expression of endogenous p21 protein in U-937 cells as estimated by Western blot, confirming its defective transactivating capacity.

The apoptosis-inducing capacity of p53 does not necessarily depend on the transcriptional activity of p53 (37–39). To determine whether transcriptionally deficient p53 can induce apoptosis in U-937 cells, ptsp53 and TADp53-expressing U-937 clones and mock-transfected control clones were incubated in culture medium at the optimal temperature for the apoptosis-inducing activity of ptsp53 (*i.e.,* 31.5°C). Each day, cells were counted, and viability was assessed by trypan blue exclusion (Fig. 12). As shown, expression of ptsp53 conferred a rapid cell death to U-937 cells. In contrast, both mock-transfected and TADp53 expressing U-937 clones were viable throughout the experiment, although U-937/TADp53 clones were viable to a slightly lesser extent. These data indicate that the main mechanism for p53-mediated apoptosis in U-937 cells depends on the transcription regulatory capacity of p53.

Transcriptionally Deficient p53 Does Not Induce Differentiation of U-937 Cells. To determine whether p53-mediated differentiation of U-937 cells is separable from the transcriptional activity of p53, TADp53-expressing, ptsp53 expressing, and mock-transfected U-937 clones were incubated at the optimal temperature for the differentiation-inducing activity of ptsp53 (*i.e.,* 32.5°C) with or without 1 nM of Vit D3. As expected, mock-transfected control U-937 clones showed no NBT reducing activity when incubated in culture medium alone, whereas the U-937/ptsp53/A2 clone showed an NBT reducing activity of 14 ± 3%, reflecting the differentiation inducing ca-

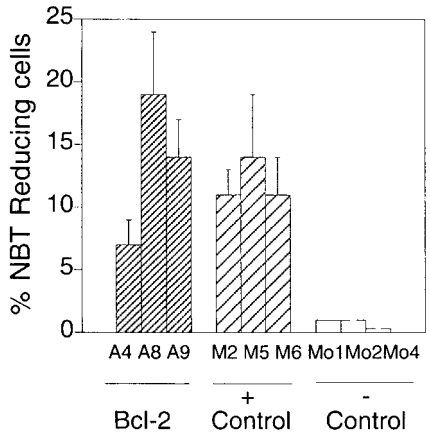

Fig. 7. Effects of Bcl-2 on p53-mediated differentiation, assayed by the capacity to reduce NBT. Mock-transfected wild type (− *Control*) and ptsp53-expressing (+ *Control*) U-937 cells as well as U-937 cells coexpressing ptsp53 and Bcl-2 (*Bcl-2*) were incubated at 32.5°C (*i.e.,* the permissive, differentiation-inducing ptsp53 temperature) at an initial concentration of 0.2×10^6 cells/ml. After 6 days of incubation, the cells were subjected to an NBT test. Mean values from at least four separate experiments are shown; *bars,* SE.

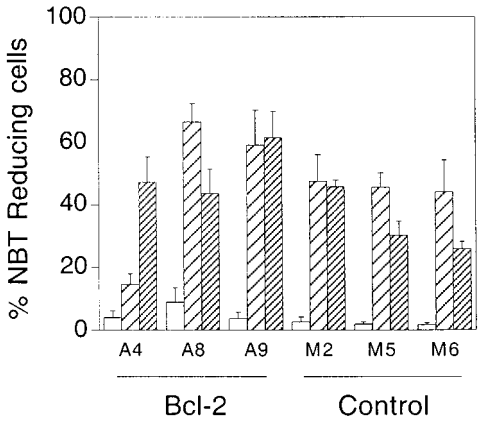

Fig. 8. Effects of Bcl-2 on the Vit D3-facilitated p53-induced differentiation, assayed by the capacity to reduce NBT. Mock-transfected (*Control*) and Bcl-2-expressing (*Bcl-2*) U-937/ptsp53 cells at an initial concentration of 0.2×10^6 cells/ml were incubated with or without Vit D3 at different concentrations at 32.5°C (*i.e.,* the temperature permissive for ptsp53-mediated differentiation). After 4 days, the cells were subjected to an NBT test. The percentage of NBT-reducing cells was determined by counting 200 cells. □, culture medium; ▨, Vit D3 1 nM; ▨, Vit D3 10 nM. Mean values from at least four separate experiments are shown; *bars,* SE.

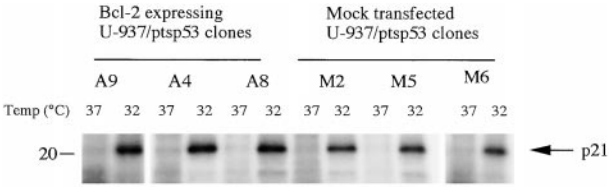

Fig. 9. Effects of Bcl-2 on p53-mediated transactivation of p21 in U-937 cells. Mock-transfected and Bcl-2-expressing U-937/ptsp53 clones at an initial concentration of 0.2×10^6 cells/ml were grown in suspension culture at 31.5°C (*i.e.,* the temperature permissive for wild-type p53 activity) and 37°C (*i.e.,* the nonpermissive temperature for wild-type p53 activity). After 24 h, cells were biosynthetically labeled with [^{35}S]methionine/cysteine and subjected to IP with the anti-p21 mouse moAb sc-817. The IP was followed by fluorography, as described in "Materials and Methods." *Arrow* on the *right,* position of p21 protein. *Left,* positions of molecular weight standards (in thousands).

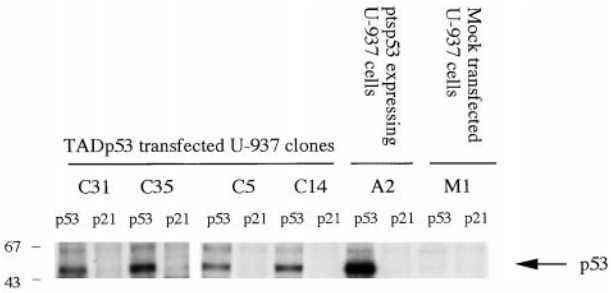

Fig. 10. Expression of transfected TADp53 protein in U-937 cells. Control and TADp53-transfected clones were biosynthetically labeled with [^{35}S]methionine/cysteine and subjected to IP with the anti-p53 mouse moAb Ab-1 and the anti-p21 mouse moAb WAF-1 Ab-1, serving as a negative control. The IP was followed by fluorography, as described in "Materials and Methods." TADp53-expressing clones C5, C14, C31, and C35, the ptsp53-expressing clone A2, and the mock-transfected clone M1 are shown. *Arrow* on the *right,* position of p53 protein. *Left,* positions of molecular weight standards (in thousands).

pacity of wild-type p53 *per se* (Fig. 13) and consistent with previous results (13). However, expression of TADp53 induced almost no signs of NBT reduction (<2%) of the U-937 cells. The discrepancy between U-937 clones expressing wild-type p53 and transcriptionally deficient p53 was even more striking when incubated with Vit D3. Although the U-937/ptsp53/A2 clone had a pronounced differentiation response as measured by reduction of NBT, the differentiation response of U-937/TADp53 clones was comparable with the mock-transfected control clones, showing a low degree of NBT reduction. When incubated at 31.5°C, the differentiation response of U-937/TADp53 clones was still comparable with the mock-transfected control clones (data not shown).

To extend the analysis of the differentiation-inducing capacity of transcriptionally inactive p53, TADp53 expressing U-937 clones and positive and negative control clones incubated at the differentiation permissive temperature (*i.e.,* 32.5°C) with or without Vit D3 were screened for expression of the granulocyte/monocyte-related cell surface antigen CD11c (50) by a FACS analysis. As shown in Table 5, the differentiation response of the U-937 clones expressing transcriptionally deficient p53 was comparable with mock-transfected control clones both with and without Vit D3. In contrast, wild-type p53 expressing U-937 cells responded with up-regulation of CD11c, which was even more pronounced when incubated with 1 nM Vit D3.

Thus, as judged by reduction of NBT and expression of the differentiation-related cell surface antigen CD11c, transcriptionally deficient p53 is incapable of inducing or facilitating differentiation of U-937 cells. This indicates that the transcription regulatory potential of p53 is essential for p53-mediated differentiation.

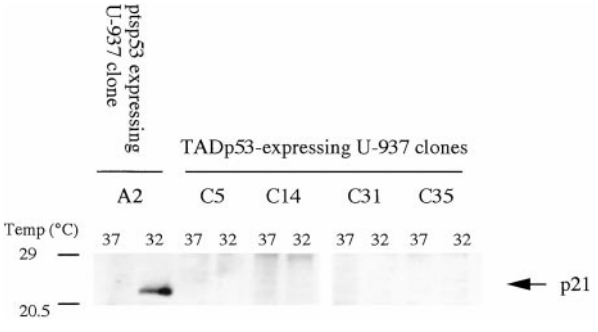

Fig. 11. Expression of p21 in response to TADp53. TADp53- and ptsp53-expressing cells were incubated at the permissive (*i.e.,* 31.5°C) and the nonpermissive (*i.e.,* 37°C) temperature. After 24 h, cells were subjected to Western blot using the mouse monoclonal anti-p21 antibody WAF-1 Ab-1, as described in "Materials and Methods." *Arrow* on the *right,* position of p21 protein. *Left,* positions of molecular weight standards (in thousands).

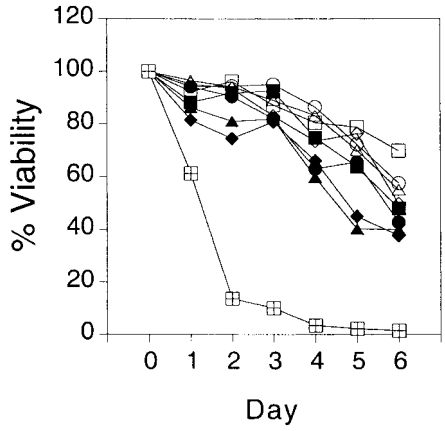

Fig. 12. Viability in suspension culture of TADp53-expressing cells. Positive and negative control cells and TADp53-expressing cells at an initial concentration of 0.2×10^6 cells/ml were grown in suspension culture at 31.5°C (*i.e.,* the permissive, apoptosis-inducing ptsp53 temperature) for 6 days. Viability, as judged by trypan blue exclusion, was determined daily. The cell number was always $(0.2 \pm 0.05) \times 10^6$ cells/ml throughout the experiments. ●, TADp53/C5; ■, TADp53/C14; ◆, TADp53/C31; ▲, TADp53/C35; ○, Mock 1; □, Mock 2; ◇, Mock 3; △, Mock 4; ⊞, ptsp53/A2. Mean values are from four separate experiments.

Discussion

The tumor suppressor protein p53 has the potential to counteract all three features characterizing acute leukemia: the differentiation block, the growth advantage, and the inhibition of apoptosis. However, the mechanisms for p53-induced differentiation are largely unknown. The proto-oncogene *Bcl-2* inhibits a number of p53-dependent activities. We demonstrate that although Bcl-2 partially inhibits p53-mediated cell death, it does not interfere with p53-mediated differentiation, suggesting that p53-induced apoptosis and differentiation rely on, at least partly, separable molecular mechanisms. Results from others indicate separable pathways for p53-mediated cell cycle arrest and apoptosis (3, 51–53). Hence, although both p53-mediated differentiation and cell cycle arrest can be separated from p53-mediated apoptosis, the question as to whether p53-mediated differentiation can be separated from p53-mediated cell cycle arrest still remains to be answered.

Bcl-2 does not inhibit the induced differentiation of U-937 cells, regardless of expression of p53, in concert with earlier work suggesting that Bcl-2 does not interfere with differentiation induced by other means (19, 28, 29, 54). Moreover, although Bcl-2 has been shown to inhibit cell proliferation (24–26), we did not observe any effects of Bcl-2 on the proliferation rate of U-937 cells. It is possible that a selection against the G_1 arresting aspects of Bcl-2 took place during the initial stages of establishment of the clones, rendering cell clones unresponsive to the cell cycle regulatory properties of Bcl-2.

Although we show that Bcl-2 inhibits p53-mediated apoptosis, the protection against p53-mediated apoptosis is not complete. This might be explained by distinct pathways from p53 leading to cell death, in line with previous reports indicating multiple pathways for p53-mediated apoptosis (38). Accordingly, it has been shown that p53 can induce apoptosis in the absence of bax (55). Moreover, the high background levels of bax in U-937 cells (data not shown) might neutralize some of the overexpressed Bcl-2 protein, by these means reducing the levels of active Bcl-2.

The levels of p21 protein in response to wild-type p53 induction were unaltered regardless of Bcl-2 expression, suggesting that Bcl-2 does not inhibit p53-mediated transactivation in U-937 cells. Moreover, the absence of Bcl-2-mediated inhibition of the transcriptional activity of p53 indicates that Bcl-2 allows at least some nuclear import of p53 in U-937 cells, in contrast with previous results (31).

The transcriptional activity of p53 has for long been the main explanatory mechanism for the cellular effects of p53. However, recently several reports have demonstrated that p53 can mediate both apoptosis and cell cycle arrest independently of p53-mediated transcriptional activation (35–38, 56), bringing up a role for p53 not only as a transcription factor but also in direct protein-protein signaling. Consequently, a p53-mediated, differentiation-inducing route independent from its transactivating properties might well exist. For this purpose, we overexpressed TADp53 (41), a transcriptionally inactive p53 mutant in monoblastic U-937 cells. Although the capacity of TADp53 to activate and repress transcription of genes is reduced severely (38, 41, 57), recent studies have demonstrated a weak ability to activate reporter constructs (48, 49). Our observation that TADp53 did neither mediate differentiation nor apoptosis in U-937 cells indicates that the transcription regulatory potential of p53 is essential both for induction of differentiation and of apoptosis in U-937 cells. Furthermore, these data are consistent with our finding that Bcl-2 does not interfere with either the p53-mediated differentiation induction or the transcriptional activity of p53 in U-937 cells. However, because the TADp53 used in this study has been shown defective for p53-mediated repression as well as activation (57), we cannot exclude that p53-mediated differentiation and apoptosis rely on p53-mediated transcriptional repression.

Interestingly, the characteristics of the ptsp53(Val135) provide further evidence for distinct pathways in p53-mediated

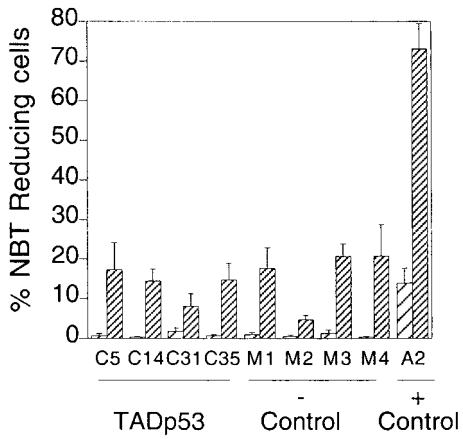

Fig. 13. Effects of TADp53 on induced differentiation, assayed by the capacity to reduce NBT. Mock transfected (− *Control*) and ptsp53-expressing (+ *Control*) U-937 cells as well as TADp53-expressing U-937 cells (*TADp53*) at an initial concentration of 0.2×10^6 cells/ml were incubated at 32.5°C (*i.e.,* the permissive, differentiation-inducing ptsp53 temperature) with or without 1 nM Vit D3. After 4 days of incubation, the cells were subjected to NBT test. ▨, culture medium; ▨, Vit D3 1 nM. Mean values from three separate experiments are shown; *bars,* SE.

cell death *versus* differentiation. When incubated at 31.5°C, the cell death-inducing and cell cycle-arresting features of ptsp53 dominate, causing an almost complete cell death after 4 days. However, when incubated at 32.5°C, cell death is not observed. Instead, the differentiation-related properties of ptsp53 appear. These functional differences may reflect changes in the levels of wild-type conformation p53, in that high levels of active p53 are required for apoptosis induction, whereas lower levels might suffice for induction of differentiation. This is in concordance with previous data showing high levels of p53 to induce apoptosis whereas low levels induce differentiation of monoblastic HL-60 cells (58). Accordingly, our own data demonstrate a slight but reproducible reduction in the levels of p53-induced p21 when U-937/ptsp53(Val135) cells are incubated at 32.5°C as compared with 31.5°C. We also show that the transactivating capacity of ptsp53 is higher at 31.5°C as compared with 32.5°C, as measured by luciferase reporter assays. However, because the luminescence in control incubations performed at 31.5°C was slightly elevated as compared with the luminescence in control incubations performed at 32.5°C throughout the experiments, unspecific temperature-related effects, possibly influencing the luminescence, cannot be excluded. It has been shown that ptsp53(Val135) is located predominantly in the cytoplasm at 37°C (*i.e.,* the mutant conformation) but is imported into the nucleus at 32°C (*i.e.,* the wild-type conformation; Ref. 59). It may well be that slight temperature shifts around 32°C modulate the precise amounts of active nuclear p53 protein.

In conclusion, Bcl-2 does not interfere with the p53-facilitated differentiation, although it does inhibit p53-mediated apoptosis, indicating separate molecular mechanisms in p53-mediated apoptosis *versus* differentiation. Furthermore, our results indicate that p53-mediated differentiation relies on the transcriptional activity of p53 in U-937 cells.

Table 5 Expression of CD11c

U-937 cells expressing transcriptionally deficient p53 and positive and negative control U-937 cells were incubated at 32.5°C with or without 1 nM Vit D3. After 3 days, cells were analyzed for the monocyte-related cell surface antigen CD11c by a FACS analysis. The percentage of gated cells in one representative experiment is shown.

	Culture medium	Vit D3, 1 nM
Mock 1	17.0	18.6
Mock 2	4.10	4.48
Mock 3	5.28	12.9
Mock 4	13.0	14.3
TADp53/C5	16.8	17.8
TADp53/C14	4.46	5.29
TADp53/C31	4.21	7.63
TADp53/C35	10.3	13.7
ptsp53/A2	33.9	66.2

Materials and Methods

Cells and Culture Conditions. The human monoblastic cell line U-937-4 (60) and the subclone U-937ptsp53/A2 (13), expressing a murine temperature-sensitive form of p53 [*i.e.*, ptsp53(Val135)] (Refs. 61 and 62), was cultured in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom), supplemented with 10% heat-inactivated FCS (Life Technologies) in a humidified CO_2 atmosphere at 37°C. For wild-type activity of p53, cells were incubated at 31.5°C–32.5°C. The number of cells and viability, as judged by trypan blue exclusion, were determined by counting in a Bürker chamber. Exponentially growing cells were used for all experiments.

Vector Constructs. The pGL3/Waf1/Luc vector (51), carrying 2.3 kb of the p21 promoter sequence in control of the firefly luciferase gene, was kindly provided by professor Moshe Oren (Weizmann Institute of Science, Rehovot, Israel). The pGL3/SV40/Luc vector, having the firefly luciferase gene under the control of the SV40 promoter, was from Promega Corp. (Madison, WI). The cDNA for human *bcl-2* was generously provided by Dr. Klas Wiman (Karolinska Institute, Stockholm) and was cloned into the eukaryotic expression vector pCEP4. pCEP4 provides a CMV promoter-driven expression of Bcl-2 and confers resistance to hygromycin B, allowing for selection of recombinant cells. The eukaryotic expression vector pMSVCl/p53(25,26,Val135) (Ref. 56), carrying the cDNA for a murine transcriptionally inactive double mutant form of ptsp53(Val135) (Ref. 41), driven by the long terminal repeat from Harvey murine sarcoma virus, was generously provided by Professor Arnold Levine (Rockefeller Institute, NY). p53(25,26,Val135) (TADp53) shows temperature-sensitive DNA binding (41) as well as the original ptsp53(Val135), but the residues at amino acid positions 25 and 26 (corresponding to human Leu-22 and Trp-23, which bind to the TATA-associated factors $TAF_{II}70$ and $TAF_{II}31$ of the transcriptional machinery) have been mutated, abolishing the transactivating effects (1, 56). PMSVCl confers resistance to geneticin, allowing for selection of recombinant cells (63). To obtain control clones, U-937 cells and U-937/ptsp53/A2 cells were transfected with pMSVCl and pCEP4, respectively.

Reporter Assays. For transient transfection, cells were resuspended in 37°C culture medium (RPMI 1640 + 10% FCS) to a concentration of 20×10^6 cells/ml. The plasmid was introduced into the cells by electroporation using the

Bio-Rad gene-pulser (Bio-Rad, Melville, NY) with electrical settings of 280 V and 960 μF, after which cells were incubated at 32.5°C or 31.5°C. After 16 h, the luminescence of transfected cells was determined using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI), according to the manufacturer's instructions. Briefly, transfected cells were washed in PBS and lysed in Passive Lysis Buffer (Promega) under constant agitation for at least 20 min. Twenty μl of lysate were mixed with 100 μl of LARII (Promega), after which the luminescence of the firefly luciferase was measured in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA).

Transfection Procedure. The transfection for constitutive protein expression was performed as described previously (64). Cells were resuspended in 37°C culture medium (RPMI 1640 + 10% FCS) to a concentration of 5×10^6 cells/ml. The plasmid was introduced into the cells by electroporation using the Bio-Rad gene-pulser (Bio-Rad) with electrical settings of 270 V and 960 μF. After 2 days, cells were seeded with Geneticin at 1.5 mg/ml (Boehringer Mannheim, Mannheim, Germany) or hygromycin B at 1.4 mg/ml (Calbiochem-Novabiochem Corp., La Jolla, CA) in 96-well plates to allow for selection of transfected clones. After 2–3 weeks, individual cell clones were expanded to mass cultures and assayed for expression of TADp53 by biosynthetic labeling, IP, and fluorography and for expression of Bcl-2 by IP-Western blot.

Biosynthetic Labeling, Immunoprecipitation, Electrophoresis, and Fluorography. Biosynthetic labeling of newly synthesized proteins was obtained by incubation of cells with 15–30 μCi/ml [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S Label; ICN Biomedicals, Inc., Costa Mesa, CA) as described previously (65). Briefly, cells were harvested and incubated for 30 min at 37°C in methionine- and cysteine-free RPMI 1640 (Life Technologies) supplemented with 1% dialyzed FCS (Life Technologies) to deplete the intracellular pools of methionine and cysteine. Subsequently, the cells were incubated for 1 h at 37°C in the same medium supplemented with [^{35}S]methionine/[^{35}S]cysteine. Labeled p53 and p21 protein was immunoprecipitated with 1 μg of mouse moAbs anti p53 Ab-1 PAb421, anti-p21 WAF-1 Ab-1 (both from Oncogene Research Products, Cambridge, MA), and anti-p21 (187) sc-817 (Santa Cruz Biotechnology Inc., CA). The immunoprecipitates were run on a pre-cast 10–20% Tris-glycine gel (Novex, San Diego, CA), followed by fluorography as described (65).

IP-Western Blot Analysis. Cell lysates were prepared by suspension of 20×10^6 cells in lysis buffer [1 M NaCl, 50 mM Tris-HCl (pH 8.0), and 0.5% Triton X-100], followed by two to four repeated exposures for freezing and thawing. DNA was removed by centrifugation at 38,000 × *g* at 4°C for 1 h, after which cell lysates were subjected to IP using 1 μg of mouse moAb anti-Bcl-2 (Serotec MCA 1550). The immunoprecipitates were run on a pre-cast 10–20% Tris-glycine gel (Novex, San Diego, CA). Proteins were electrophoretically transferred to Hybond-P polyvinylidene difluoride membranes (Amersham, Life Sciences International) in blotting buffer (39 mM glycin, 48 mM Tris, 1.3 mM SDS, and 20% methanol) using a Graphite Electroblotter I (Milliblot; WEP Co., Seattle, WA) at 20 V for 1 h. After electroblotting, the blotted membrane was blocked in TBS, 2% BSA and probed for Bcl-2 with the mouse moAb anti-Bcl-2 (Serotec MCA 1550) in TBS 0.2% BSA. Bands were visualized through an alkaline-phosphatase conjugated goat-antimouse antibody (Promega, Madison, WI) as described previously (66).

ECL-Western Blotting. Expression of p21 was detected with the anti-p21 WAF-1 Ab-1 mouse moAb (Oncogene Research Products, Cambridge, MA). For control of equal loading, the mouse moAb actin antibody C-1 sc-8432 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the incubation with the p21 antibody. The ECL Western blotting kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) was used according to the manufacturer's instruction. Briefly, 5×10^6 cells were washed once in PBS and then frozen at −80°C for at least 20 min. The cell pellet was diluted in 75 μl lysis buffer [92 mM Tris (pH 6.8), 12.1% glycerol, 2.4% SDS, 1.4% β-mercaptoethanol, and 2.9% bromphenol blue), after which the cells were lysed by sonication for ~10 s with a Dr. Hielsher sonicator (B. Braun Biotech International, Melsungen, Germany). Samples were boiled for 5 min and subsequently spun down in a table top centrifuge at 14,000 × *g* at 4°C for 10 min. Lysate from 0.5×10^6 cells was loaded in each lane of a precast 10–20% Tris-glycine gel (Novex). Proteins were electrophoretically transferred to Hybond-P polyvinylidene difluoride membranes (Amersham, Life Sciences International) in blotting buffer as described above. Detection was performed according to the manufacturer's instructions, and the membranes were exposed to ECL hyper film (Amersham, Life Sciences International) for 5–15 s. The relative amount of protein in each lane was estimated with a LAS 1000 Plus chilled CCD camera (Fuji, Tokyo, Japan).

Assessment of Differentiation by NBT Reduction Test. The NBT reduction test was performed as described previously (64, 66). Briefly, cells (0.2×10^6 cells/ml) were incubated with Vit D3 (a generous gift from Roche, Basel, Switzerland) for 4 days. At harvest, cells were incubated with 0.075% (w/v) NBT and 0.15 mg/ml phorbol 12-myristate 13-acetate (both from Sigma Biochemical Co. St. Louis, MO) for 25 min at 37°C. Cells were stained with May-Grünwald-Giemsa, and the percentage of cells containing formazan deposits, thus reducing NBT, was determined by counting at least 200 cells.

Assessment of Cell Surface Antigens by Flow Cytometric Analysis. Cells were washed in PBS and resuspended to $5–10 \times 10^6$ cells/ml 50 μl of the cell suspension was incubated with 5 μl of the following moAbs in microtiter wells: control IgG1-FITC/IgG1-PE, cd 11c-PE (Becton Dickinson, San José, CA), Annexin V-FITC (PharMingen, San Diego, CA), and propidium iodide (Sigma), for 10 min at room temperature under constant agitation. The cells were then washed three times and fixed in 1% paraformaldehyde before flow cytometric analyses (FACSscan; Becton Dickinson). Ten thousand cells were collected for each antibody. Dead cells and debris were excluded from analysis by gating prior to the calculation of the percentage of positive cells, using the control incubation with IgG1-FITC/IgG1-PE for marker settings. Cells were analyzed for expression of An-

nexin V in parallel with staining with propidium iodide, which makes it possible to exclude necrotic cells. This provides a selective method for detection of apoptosis (46).

Determination of Cell Cycle Distribution by Flow Cytometric Analysis. Staining of nuclei and flow cytometric analysis were performed as follows. Cells were washed in Dulbecco's PBS, after which 0.2 ml of a nuclear isolation medium containing propidium iodide was added (50 μg/ml propidium iodide, 0.6% NP40, 100 μg/ml RNase, DNase-free, in PBS; all reagents from Sigma). The cells were then incubated at room temperature in the dark for 60 min before the addition of 0.4 ml of PBS and taken to flow cytometric analysis in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Up to 20,000 nuclei were analyzed per sample. Using the electronic peak and area detectors, processor signals from nuclei doublets were rejected. Cell cycle phase distribution, *i.e.,* the percentages of $G_0 + G_1$, S, and G_2 nuclei of the analyzed cell population, was determined by applying ModFit LT cell cycle analysis software (Verity Software House, Inc., Topsham, ME) on the DNA histograms. The DNA histogram was corrected for contribution of nucleic debris.

Acknowledgments

We thank Tor Olofsson and the staff at the ImmunoCytoHematology Laboratory for valuable discussions and for skillfully performing the analysis of cell cycle distribution and cell surface antigens by flow cytometry. We also thank Olga Göransson for help with densitometric analyses.

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