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Down-regulation of wild-type p53 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal

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Overexpression of wild-type p53 in p53-deficient leukemic cells induces apoptosis, which can be inhibited by hematopoietic survival factors. This suggests that p53 may contribute to survival factor dependence. To assess the role of wild-type p53 in mediating apoptosis following survival factor withdrawal, we interfered with endogenous p53 activity in interleukin-3 (IL-3)-dependent cells. Extended survival without IL-3 was conferred by recombinant retroviruses encoding either a full-length p53 mutant or a C-terminal p53 miniprotein, both of which can act as negative-dominant inhibitors of wild-type p53. On the other hand, excess wild-type p53 activity failed to elicit apoptosis as long as IL-3 was present. We propose that p53 is a positive, though not exclusive, mediator of survival factor dependence in hematopoietic cells.

Key words: leukemia/survival factor/tumor suppressor

Introduction

The p53 protein is the product of a tumor suppressor gene (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Mercer, 1992; Oren, 1992b; Prives and Manfredi, 1993). Overexpression of exogenous wild-type (wt) p53 activity in wt p53-deficient cells can induce apoptosis (Yanish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Johnson *et al.*, 1993; Ramqvist *et al.*, 1993; Ryan *et al.*, 1993; Wang *et al.*, 1993). Moreover, thymocytes of p53-deficient mice are refractory to the induction of apoptosis by ionizing radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993). Recently, it has been shown that the ability of the adenovirus E1A oncogene to induce apoptosis is mediated through, and requires the presence of, functional wt p53 (Debbas and White, 1993; Lowe and Ruley, 1993). All these observations suggest that wt p53 may play a role in mediating apoptosis under a variety of conditions, and that this feature of p53 may provide part of the explanation for its tumor suppressor activity (Oren, 1992a; Sachs and Lotem, 1993).

In p53-deficient leukemic cells, the apoptotic response which results from the induced overexpression of exogenous wt p53 can be at least partially inhibited by the addition of specific hematopoietic survival factors, such as interleukin-6 (IL-6; Yonish-Rouach *et al.*, 1991) or interleukin-3 (IL-3; Yonish-Rouach *et al.*, 1993) for myeloid cells, and erythropoietin for erythroid cells (Johnson *et al.*, 1993). These data can be interpreted as implying that, in these leukemic cells, the experimental activation of wt p53 induces a state of survival factor dependence, which can be regulated by the addition of the appropriate cytokines. One could thus propose that wt p53 plays a role in survival factor dependence, and in mediating cell death once such factors become limiting. In this case, loss of wt p53 function may confer a reduced requirement for certain factors, whereas reconstitution of wt p53 activity in such cells may reconfer full dependence on these factors.

To test whether wt p53 indeed contributes to survival factor dependence in a more physiological context, we employed dominant negative mutants of p53 in order to interfere with the functions of endogenous p53 in an IL-3-dependent hematopoietic cell line. We report here that such interference with endogenous wt p53 activity significantly slowed down cell death following IL-3 withdrawal. Overexpression of wt p53 on its own did not elicit any cell death as long as IL-3 was available. On the other hand, excess wt p53 did slightly accelerate the death of IL-3-deprived cells. These findings support a role for p53 in mediating survival factor dependence in at least some hematopoietic cells.

Results

DA-1 cells, which undergo apoptosis upon IL-3 withdrawal, express apparently wild-type p53

Bone marrow-derived cells, as well as many IL-3-dependent cell lines, undergo apoptosis upon IL-3 withdrawal (Rodriguez-Tarduchy *et al.*, 1990; Williams *et al.*, 1990). DA-1 (Ihle, 1984) is an IL-3-dependent cell line originating from a murine lymphoma, which dies rapidly upon IL-3 withdrawal. The DA-1 p53 protein is reactive with the p53-specific monoclonal antibody PAb246, which recognizes selectively the wt conformation of the protein (Gannon *et al.*, 1990), but not with PAb240, which recognizes common mutant forms of p53 (see Figure 4, lanes 7 and 9). The DA-1 p53 protein is short-lived (see Figure 5A) and is capable of sequence-specific binding to p53 target DNA (Figure 1). Thus, DA-1 cells apparently express functional wt p53.

To determine whether DA-1 cells undergo apoptosis following IL-3 withdrawal, they were analyzed in a number of ways. DA-1 cells transfected with the *neo* gene only (DIN, see below) were stained at various times after IL-3 withdrawal by the DNA intercalating agent DAPI, to visualize condensed and fragmented nuclei, and by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) method, which scores for cells with DNA strand breaks (Gavrielli *et al.*, 1992). Each microscopic field was inspected sequentially for DAPI staining and TUNEL positivity, as described in Materials and methods. Fragmented nuclei, typical of apoptotic cells,

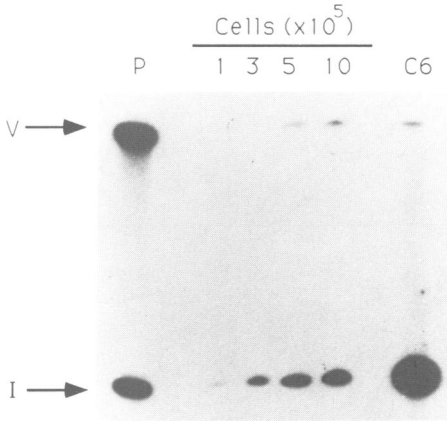

Fig. 1. Sequence-specific DNA binding by the p53 protein of DA-1 cells. Extracts were prepared from DA-1 cells and aliquots corresponding to the indicated numbers of cells were subjected to a sequence-specific DNA binding assay as described in Materials and methods. The autoradiogram of the bound DNA is shown. P, 2% of the radioactive probe, applied directly to the gel. V denotes the plasmid vector, indicative of non-specific DNA binding; I denotes the insert, containing the p53-binding site. C6, extract of Clone 6 cells, overexpressing p53 (Zauberman *et al.*, 1993).

were already visible shortly after IL-3 removal (Figure 2B). These nuclei were also significantly brighter, indicative of chromatin condensation (evident only on short photographic exposures). Moreover, apoptosis could be confirmed directly by the TUNEL method (Figure 2D). With very few exceptions, cells whose nuclei appeared fragmented by DAPI staining were also TUNEL positive. Thus, IL-3-deprived DA-1 cells exhibited distinct apoptotic features. Interestingly, cells with apoptotic nuclei did not accumulate in the culture. Instead, they were gradually replaced at later times with enucleated 'ghosts', which were barely visible by DAPI (Figure 2C; see also Figure 9). These 'ghosts' could be seen more clearly after longer photographic exposures (data not shown). In some 'ghosts' residual condensed chromatin could still be detected (straight arrowhead in Figure 2C). These observations suggest that apoptotic DA-1 cells retain their fragmented nucleus only transiently, and eventually release the condensed chromatin into the culture medium or else degrade the DNA altogether. Dying cells also displayed a DNA 'ladder' characteristic of apoptosis (data not shown). However, only a minority of the total cellular DNA appeared fragmented, probably owing to the failure to accumulate large numbers of highly apoptotic nuclei.

A temperature sensitive p53 mutant delays cell death following IL-3 withdrawal at the non-permissive temperature

We sought to manipulate the levels of wt p53 activity in DA-1 cells, in order to determine whether p53 contributed to their survival factor dependence. Mutant p53 can inhibit the activity of wt p53 in a dominant negative fashion (Farmer *et al.*, 1992; Kern *et al.*, 1992; Shaulian *et al.*, 1992). The temperature sensitive (ts) mutant p53Val135 behaves like other mutants at 37.5°C (Michalovitz *et al.*, 1990) but regains wt-like properties at 32°C (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991; Raycroft *et al.*, 1991). DNA encoding p53Val135 was introduced into the pLXSN vector (Figure 3) and the resultant plasmid was used to generate a high titer recombinant retrovirus. DA-1 cells were

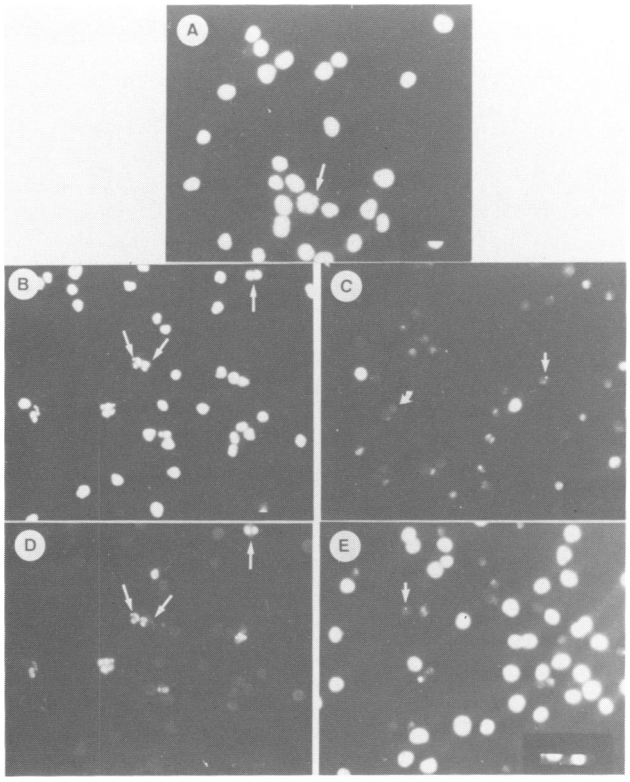

Fig. 2. Nuclear features of DA-1-derived cells undergoing cell death after IL-3 withdrawal. DA-1 cells expressing the *neo* selectable marker (DIN, see text) were deprived of IL-3, and samples were taken for analysis at various times thereafter (panels A–D). Each sample was stained simultaneously with DAPI, to visualize total nuclear DNA, and with the TUNEL method, to visualize nuclear DNA fragmentation *in situ*. (**A–C**) DAPI staining 2, 8 and 20 h, respectively, after IL-3 withdrawal. (**D**) Identical field to that in panel B (8 h), visualized for TUNEL staining. (**E**) DIDD1 cells expressing the DD negative dominant miniprotein (see text), stained with DAPI 20 h after IL-3 withdrawal. The arrow in panel A indicates a cell undergoing mitosis. Arrows in panel B indicate fragmented apoptotic nuclei; the positive TUNEL staining of the same nuclei is shown in D. Straight arrowheads in C and E indicate 'ghosts' (see text) with residual chromatin; bent arrowhead in C indicates a 'ghost' without visible residual DNA. Photographs were taken at a magnification of 400×.

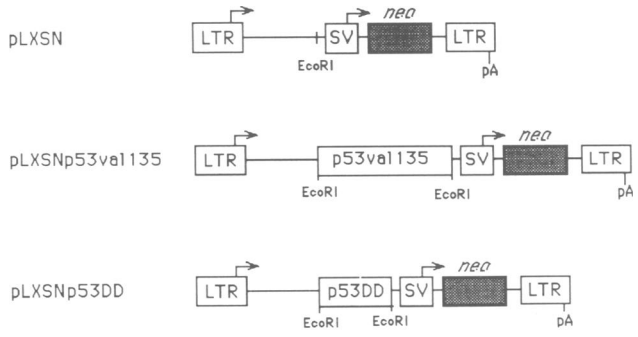

Fig. 3. Plasmids employed to generate retroviral vectors harboring mutant forms of p53. SV, SV40 early region promoter; pA, polyadenylation site. See Materials and methods for details.

infected by co-cultivation with virus-producing cells at 37.5°C. Many cells in the culture were successfully infected, giving rise to a G418-resistant population. Two pooled populations, generated through separate infections (DIV1 and DIV2, Figure 4), were subjected to biosynthetic labeling followed by immunoprecipitation with p53-specific

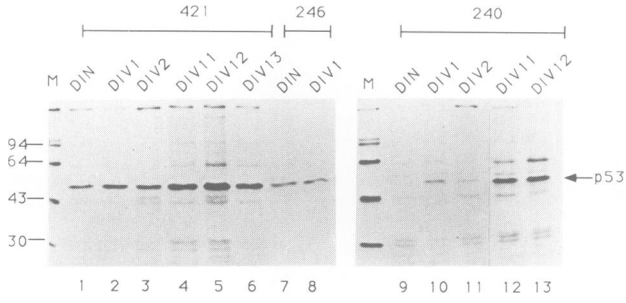

Fig. 4. Analysis of biosynthetically labeled p53 protein in DA-1 cells and their derivatives. G418-resistant pools were generated by infection with recombinant retroviruses (see text) encoding either the neomycin resistance gene alone (DIN) or the neomycin resistance gene plus p53Val135 (DIV1 and DIV2). DIV11 to DIV13 are clones generated from DIV1 through single cell cloning. Extracts were immunoprecipitated with PAb421, PAb246 or PAb240, as indicated above each lane. M, molecular weight markers.

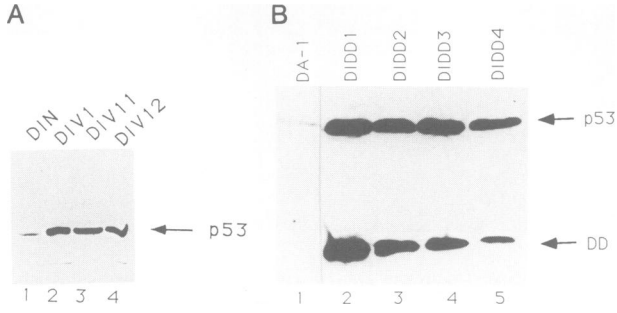

Fig. 5. Analysis of steady-state levels of p53 in p53Val135-expressing (**A**) and DD-expressing (**B**) derivatives of DA-1. Extracts of the indicated cell populations (5×10^5 cells per lane) were applied directly to an SDS–polyacrylamide gel and subjected to Western blotting. The positions of the p53 protein and the DD miniprotein are indicated.

antibodies. Immunoprecipitation with the monoclonal antibody PAb421, which recognizes both wt and mutant conformations of murine p53 (Gannon *et al.*, 1990), revealed a mild increase in the overall rate of p53 synthesis relative to DIN cells, infected with a retrovirus containing the *neo* gene only (Figure 4, compare lanes 2 and 3 with lane 1). In contrast to the rather mild increase in the rate of synthesis, steady-state levels of p53 in these pools were much higher than in DIN (Figure 5A, lanes 1 and 2). This probably reflects the augmented stability of p53Val135 when present in a mutant conformation at 37.5°C (Finlay *et al.*, 1988; Ginsberg *et al.*, 1991). The DIV pools contained PAb240-reactive p53, indicative of a mutant conformation (Figure 4, lanes 10 and 11), while no such reactivity was observable upon examination of the endogenous p53 in DIN (lane 9).

We next determined whether the overexpression of exogenous mutant p53 affected the ability of DA-1 cells to undergo apoptosis upon survival factor deprivation. When IL-3 was withdrawn from DIV1 (Figure 6A) at 37.5°C, the rate of cell death (as assessed by the inability to exclude the dye trypan blue) was moderately but reproducibly slower than that of DIN; a quantitatively similar result was obtained with DIV2 (data not shown). This observation was consistent with the notion that a reduction in wt p53 activity, brought about by a dominant negative mutant, can interfere with the

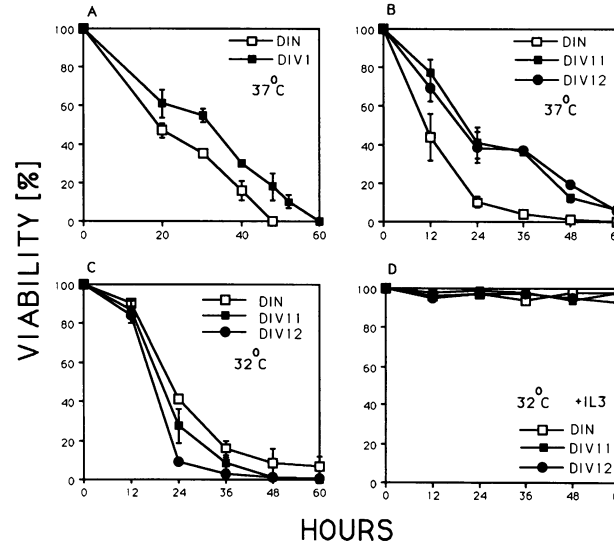

Fig. 6. Kinetics of cell death of p53Val135-expressing DA-1 cells in the absence or presence of IL-3. Viability was determined at various time points by the ability to exclude trypan blue. (**A**) Infected pools without IL-3, 37.5°C; (**B**) infected individual clones without IL-3, 37.5°C; (**C**) infected individual clones without IL-3, 32°C; (**D**) individual clones plus IL-3, 32°C.

apoptotic death of hematopoietic cells deprived of essential survival factors.

Even though the DIV pools were established by retroviral infection, the levels of the exogenous p53Val135 might have varied among individual cells; consequently, some cells probably still retained significant residual wt p53 activity. To maximize the inhibitory effect of the introduced mutant p53, single cell clones were established from DIV1. Three clones, DIV11 to DIV13, were found to express more p53 than the parental DIV1 pool (Figure 4, lanes 4–6); PAb240 reactivity was also proportionately increased (lanes 12 and 13). When the behavior of these clones was examined in the absence of IL-3 at 37.5°C, survival was indeed found to be more pronounced than in the parental DIV1 pool (Figure 6B).

The protective effect of p53Val135 on DA-1 was seen only at 37.5°C, where it can act as a dominant negative mutant. At 32°C, where the ts p53 exhibits wt-like p53 activity, its expression in fact led to a slightly accelerated rate of cell death in the absence of IL-3 (Figure 6C). Hence, on this particular cell background, increased wt p53 activity potentiated the apoptotic response to IL-3 withdrawal.

Importantly, excess wt p53 activity (p53Val135 at 32°C) did not elicit apoptosis as long as IL-3 was present (Figure 6D). Thus, the mere overexpression of active p53 is by itself not capable of inducing DA-1 cell death. Rather, the presence of active wt p53 appears to facilitate the propagation of the apoptotic signal generated by survival factor withdrawal.

A dominant negative p53 miniprotein inhibits apoptosis of DA-1 cells

The use of p53Val135 to inhibit endogenous wt p53 function has two potential drawbacks. Firstly, inhibition may be only partial. As in fibroblasts harboring the ts p53Val135 (Martinez *et al.*, 1991), a significant fraction of the p53Val135 expressed at 37.5°C in DIV cells retains a wt-

like conformation, as defined by PAb246 reactivity (Figure 4, lane 8). Furthermore, full-length mutant p53 can exhibit gain of function (Wolf *et al.*, 1984; Dittmer *et al.*, 1993); it is conceivable that such gain of function may sometimes result in a block to apoptosis. Evidence supporting this notion has been obtained in transfected leukemic M1 cells (Lotem and Sachs, 1993a).

The concerns about the validity of p53Val135 as a dominant negative inhibitor of endogenous wt p53 can be remedied by using the DD p53 miniprotein (Shaulian *et al.*, 1992). This miniprotein comprises the last 89 residues of wt mouse p53, encompassing the dimerization domain of the protein, and can act as an effective antagonist of co-expressed wt p53 (Shaulian *et al.*, 1992). The underlying mechanism appears to be at least partially due to the formation of functionally impaired mixed oligomers between the DD miniprotein and full-length wt p53 (Shaulian *et al.*, 1992; Reed *et al.*, 1993).

A retroviral vector, LXSNp53DD, expressing the DD miniprotein, was generated using the pLXSNp53DD plasmid (Figure 3). DA-1 cells were co-cultivated with a pool of virus-producing packaging cells, and a G418-resistant population (DIDD1) was isolated after 5 days of drug selection. In parallel, a similar infection and drug selection procedure was performed with three independent virus-producing clones, giving rise to populations DIDD2, DIDD3 and DIDD4, respectively. All DD-infected populations showed abundant expression of the corresponding miniprotein (Figure 5B, lanes 2–5). Moreover, the endogenous p53 of DIDD cells accumulated to very high levels, probably owing to its stabilization within the apparently non-functional mixed oligomers; a similar situation has been observed in fibroblasts overexpressing the DD miniprotein (Shaulian *et al.*, 1992). Consistent with observations made in such fibroblasts (Shaulian *et al.*, 1992), the endogenous p53 protein in DIDD cells displayed an altered conformation, reflected by a loss of PAb246 reactivity without acquisition of PAb240 positivity (not shown).

The effect of DD overexpression on the death of factor-deprived DA-1 cells was most pronounced. After 48 h in the absence of IL-3, 65% of DIDD1 cells were still viable, as measured by the ability of non-permeabilized cells to exclude propidium iodide, as compared with <5% of parental DA-1 cells (Figure 7). The kinetics of cell death of parental DA-1 and of the various DD-infected populations are shown in Figure 8A.

In primary thymocytes, wt p53 function was recently shown to be required for the induction of apoptosis in response to ionizing radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993). We therefore determined the effect of p53 inactivation on the viability of irradiated DA-1 cells, in the presence of IL-3. As seen in Figure 8B, DIDD cells indeed exhibited a significant degree of resistance to radiation-induced cell death. This attests strongly to the validity of the DD miniproteins as effective blockers of endogenous wt p53 function in DA-1 cells. Moreover, it extends the relationship between radiation-induced cell death and p53 function (Clarke *et al.*, 1993; Lowe *et al.*, 1993) to additional cell types.

In all the above experiments, loss of viability was scored on the basis of alterations in membrane permeability. To confirm that differences in the rate of actual cell death were being measured, DIN and DIDD cell pools were studied with

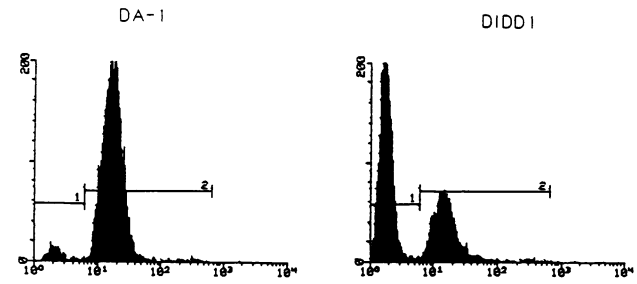

Fig. 7. Inhibition of DA-1 cell death by overexpression of the DD miniprotein. Cell viability was determined 48 h after IL-3 withdrawal, by propidium iodide staining followed by FACS analysis, as described in Materials and methods. The high intensity peak (fraction 2) represents dead cells, whereas the low intensity peak (fraction 1) represents viable cells capable of excluding the dye.

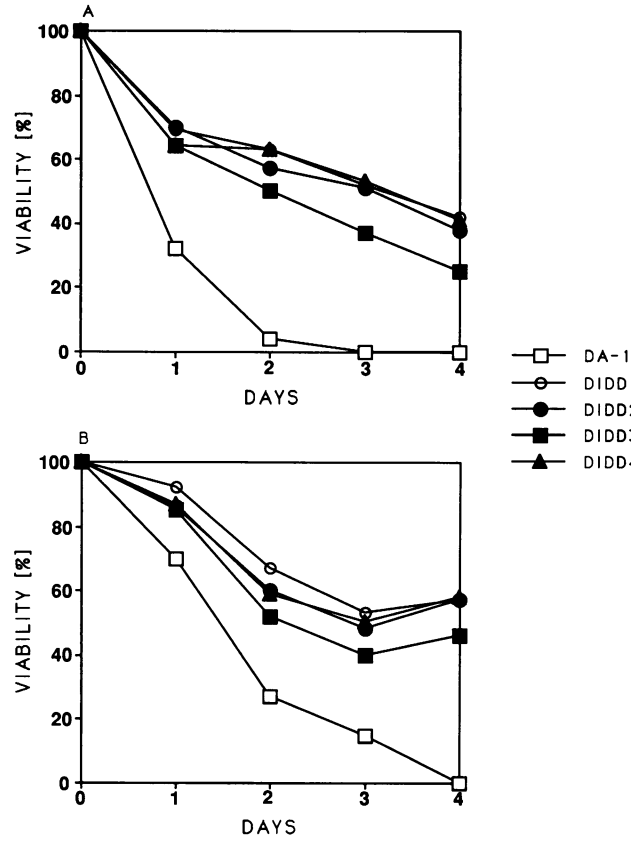

Fig. 8. Kinetics of cell death following IL-3 withdrawal (**A**) or exposure to ionizing radiation (**B**). Viability was scored as in Figure 7; the relative proportions of dead and viable cells were calculated by integrating the corresponding peak areas. In panel B, cells were exposed to gamma irradiation (500 rad) and maintained under standard growth conditions in the presence of IL-3.

regard to nuclear events occurring after IL-3 withdrawal. Cells of each type were analyzed simultaneously by staining with DAPI and by TUNEL analysis at various time points after IL-3 removal. The results are shown in Figure 9. As discussed earlier, the fragmented nucleus stage appears to be transient in DA-1 cells (Figure 2). Accordingly, the percentage of cells with typical apoptotic nuclei remained in both cell types at a constant level of ~10% (Figure 9B) until late times, when practically all DIN cells had been converted into enucleated 'ghosts' (data not shown). However, the rate of accumulation of such 'ghosts' was

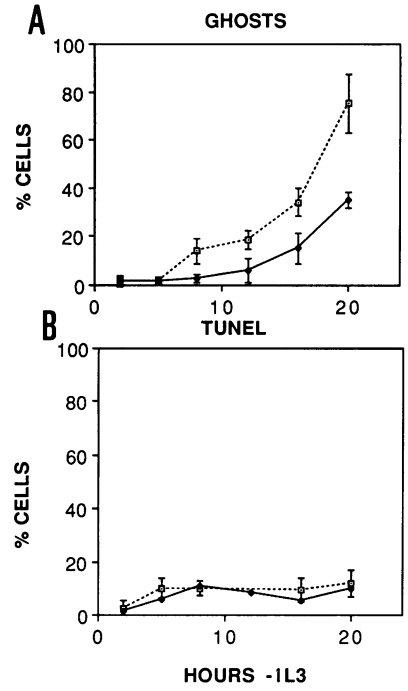

Fig. 9. Kinetics of apoptotic cell death following IL-3 withdrawal, as analyzed by DAPI and TUNEL. DIN (dotted line) and DIDD1 (solid line) cells were deprived of IL-3, and samples were stained simultaneously with DAPI and by the TUNEL method at various times after IL-3 withdrawal. At least three separate microscopic fields were scored for each time point. Percentages of enucleated 'ghosts' (**A**) and TUNEL positive cells (**B**) are displayed as mean plus standard deviation.

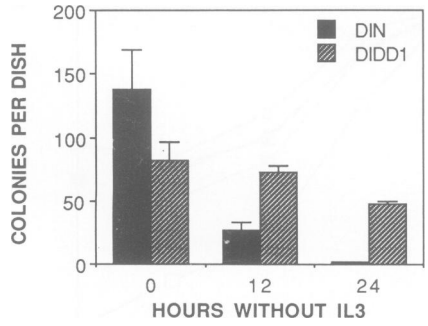

Fig. 10. Clonogenic survival of DIN and DIDD1 cells following IL-3 removal. Cells of each pool (4×10^3 per sample) were withdrawn from the culture at the indicated times after IL-3 removal, and seeded in methylcellulose in the presence of IL-3, as described in Materials and methods. Each time point was analyzed in triplicate dishes. Colonies were counted 7 days after seeding.

much slower in DIDD cells (Figure 9A). Representative photomicrographs are displayed in Figure 2 for the 20 h time point; compare panel C (DIN) and panel E (DIDD1). Hence, the rate of death of DA-1 cells deprived of IL-3 is greatly reduced when their wt p53 function is compromised.

An independent confirmation for this conclusion was provided by a clonogenic survival assay. Cells were transferred into medium without IL-3 for the indicated times (Figure 10), and then plated in methylcellulose in the presence of IL-3. Colonies were scored 7 days later. It is evident that the clonogenic survival of DIN cells dropped much more rapidly than that of DIDD1 (Figure 10) in response to IL-3 deprivation.

In conclusion, the above experiments demonstrate that overexpression of a negative-dominant p53 miniprotein renders DA-1 cells more refractory to apoptosis mediated by either survival factor deprivation or ionizing radiation.

Discussion

The results reported above demonstrate that reduction in the normal activity of wt p53 leads to enhanced viability upon IL-3 withdrawal. This suggests that p53 may play a role in mediating cell death in the absence of hematopoietic survival factors.

It should be noted that excess wt p53, on its own, does not lead to any measurable apoptotic response in DA-1 cells as long as IL-3 is maintained in the culture (Figure 6D). Hence it is likely that additional proteins, whose expression or function are presumably affected by the availability of survival factors, dictate the cellular outcome of p53 overexpression. Such putative proteins either may be components of a p53-dependent pathway, or may regulate the state of activity of p53 itself. Investigation of the molecular properties of the p53 protein in the presence and in the absence of IL-3 may help resolve this issue.

Recent studies have established that wt p53 function is required for the induction of apoptosis in thymocytes exposed to ionizing radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993). This observation is in line with the central role assigned to p53 in mediating the response to DNA damage and in maintaining genomic stability (Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992; Lane, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992). Thymocytes of p53-deficient mice remain viable following exposure to lethal doses of ionizing radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993). Thus, in the context of DNA damage, p53 appears to be absolutely essential for the apoptotic response of some cells. On the other hand, abrogation of wt p53 activity in DA-1 cells does not rescue them from death in the absence of IL-3, but only delays their death. The requirement for p53 in the factor withdrawal response is therefore not absolute; there must exist pathways which can partially bypass the need for wt p53 in apoptosis in cells deprived of hematopoietic survival factors. As long as these alternative pathways remain intact, the loss of wt p53 function is predicted to have only a limited effect on the ability of the cell to undergo apoptosis upon survival factor deprivation. In this regard, the relative contribution of p53 may be greater in DA-1 cells than in some other cell types. For instance, while introduction of the DD miniprotein into two other IL-3-dependent cell lines, 32D and FDCP1, also delayed apoptosis following IL-3 withdrawal, the delay was less pronounced than in DA-1 (data not shown). It is conceivable that DA-1 cells, which originate from a lymphoma (Ihle, 1984), may have already lost some of the alternative pathways, allowing the contribution of p53 to become more critical. Leukemic cell lines such as M1 (Yonish-Rouach *et al.*, 1991, 1993) or DP16 (Johnson *et al.*, 1993; Ryan *et al.*, 1993) may represent a case that has progressed even further. In these p53-deficient lines, the reintroduction of p53 appears to restore a strong dependence on survival factors, which does not exist in the non-transfected parental leukemic cells. It is therefore plausible that, in these parental cells, all the relevant alternative pathways may have already been inactivated.

Studies on p53-deficient mice are also consistent with the

notion that wt p53 contributes to, but is not absolutely required for, survival factor dependence. The apparently normal development of such mice (Donehower *et al.*, 1992) implies that p53 cannot be the only protein capable of mediating apoptosis in cells which encounter limiting concentrations of survival factors. Yet, p53-deficient mice are prone to developing malignant lymphoma (Donehower *et al.*, 1992). A recent study by Lotem and Sachs (1993b) demonstrates directly that hematopoietic progenitor cells from such p53-deficient mice indeed display extended survival in the presence of limiting concentrations of the appropriate cytokines. Reduced dependence on survival factors, probably in conjunction with additional oncogenic lesions, may thus constitute a mechanism whereby loss of wt p53 function can contribute to the induction of hematopoietic malignancies.

The involvement of p53 in the response to DNA damage, as well as in survival factor dependence, suggests a link between these two processes. Such a link is very much in line with the studies of Collins *et al.* (1992), who found that IL-3 can protect myeloid progenitor cells from apoptosis induced by DNA damage. In that case, however, IL-3 allowed the cells to overcome an apoptotic signal delivered at a G_2 checkpoint (Collins *et al.*, 1992). In contrast, the checkpoint at which p53 is believed to operate following DNA damage is in G_1 (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). Furthermore, the commitment to apoptosis following the activation of wt p53 in p53-deficient lines also takes place in G_1 rather than in G_2 (Ryan *et al.*, 1993; Yonish-Rouach *et al.*, 1993). Taken together, these data suggest that survival factors may uncouple apoptosis from DNA damage at more than one checkpoint, probably through operating on more than a single pathway.

Increased levels of survival factors are often induced when there is an acute need for recruitment of progenitor cells into a rapidly proliferating state. Under such emergency conditions, the requirement for the production of as many mature cells as possible, combined with the fact that the mature progeny are typically short-lived, may dictate that the strict maintenance of genomic stability be relaxed. This is probably achieved through the ability of survival factors to bypass the various checkpoints, p53-dependent as well as p53-independent, from which cells exit into apoptosis if their DNA is not perfectly intact. It is noteworthy, however, that in DA-1 cells IL-3 does not confer resistance to high doses of ionizing radiation, whereas inhibition of wt p53 function does (Figure 8B); this differs from the situation at the G_2 checkpoint, as described by Collins *et al.* (1992). Hence, survival factors may allow the bypass of p53-mediated apoptotic signals only in cells carrying mild DNA damage, of the sort likely to occur at an increased frequency in rapidly proliferating cells. Such differential effect of survival factors, which still allows the elimination of cells with extensive DNA damage, will probably be more advantageous under physiological conditions.

Materials and methods

Cells and plasmids

Cells were maintained routinely at 37.5°C in DMEM supplemented with 10% fetal calf serum (FCS). DA-1 cells and their derivatives were supplemented with 0.1% medium conditioned by X63/0 cells (Karasuyama and Melchers, 1988), serving as a source of IL-3. For factor withdrawal experiments, cells were washed twice by pelleting and resuspension in IL-3-free medium, and were finally resuspended in IL-3-free medium at a cell density of 2×10^5 per ml. Where appropriate, cells were transferred to 32°C immediately after being washed and resuspended. Viability was determined by the ability to exclude trypan blue or propidium iodide. Cells stained with trypan blue were counted in a cell counter chamber (at least 100 cells per determination). For propidium iodide exclusion analysis, cells were incubated for 1 min with 50 μg/ml propidium iodide, without prior permeabilization, and subjected to fluorescence-activated cell sorting (FACS) analysis (FACScan, Becton and Dickinson).

Plasmid pLXSNp53Val135 was generated by introduction of cDNA encoding the Val135 mutant of p53 into the *Eco*RI site of pLXSN (Miller and Rosman, 1989) in the sense orientation. Plasmid pLXSNp53DD was generated by replacing the *Xho*I−*Stu*I fragment of pLXSNp53Val135 with the *Xho*I−*Stu*I fragment of pCMVDD (Shaulian *et al.*, 1992). Recombinant retroviruses were generated by transfection of the appropriate plasmid into GP+E-86 packaging cells (Markowitz *et al.*, 1988), followed by selection of individual clones producing high titer virus. DA-1 cells were infected by co-cultivation with virus-producing cells; infection and subsequent selection with G418 were as described by Alexander *et al.* (1991), except that producer cells were treated with gamma irradiation (1500 rad) rather than with mitomycin C. G418-resistant pools were propagated as mass cultures and analyzed. Pools DIV1 and DIV2 were generated by infection with the p53Val135-encoding retrovirus, whereas DIN was generated by infection with the pLXSN virus, encoding G418 resistance only. To generate clones DIV11−13, cells of pool DIV1 were subjected to limiting dilutions in microtiter plates; and clones were established from a dilution at which fewer than 30% of the wells contained microscopically observable cells. DIDD1 was generated by co-cultivation with a pooled population of pLXSNp53DD-transfected GP+E-86 packaging cells. Three isolated pLXSNp53DD-transfected GP+E-86 colonies were expanded and each was co-cultivated separately with DA-1 cells, giving rise to infected pools DIDD2, DIDD3 and DIDD4, respectively. Infected, G418-resistant DA-1 populations were analyzed within 5−7 days of infection.

Protein analysis

Cellular proteins were biosynthetically labeled by incubation for 2 h in methionine-free medium, in the presence of [^{35}S]methionine; protein analysis was performed as described before (Maltzman *et al.*, 1981). For the determination of steady-state p53 levels, cell extracts were prepared and processed by Western blotting as described before (Shaulian *et al.*, 1992). The immobilized p53 was detected by probing with a mixture of the monoclonal antibodies PAb248 and PAb421, and the ECL (enhanced chemiluminescence) system (Amersham).

Analysis of apoptotic nuclei using DAPI and TUNEL

Cells were pelleted and fixed by resuspension in 3% paraformaldehyde for 30 min at room temperature. The cells were then pelleted, washed twice in PBS and resuspended in PBS at a concentration of 10^6 cells/ml, and stored at 4°C until required. 20 μl of the suspension were dropped onto slides precoated with 50 μg/ml poly-L-lysine and air dried. The slides were rinsed in double distilled water and incubated in terminal deoxynucleotidyl transferase (TDT) Co^{2+} reaction buffer (IBI Chemicals) for 10 min at room temperature. The cells were then incubated in reaction buffer containing 40 μM biotin-16-dUTP (Boehringer) and 0.3 U of TDT (IBI Chemicals) for 60 min at 37°C in a humid chamber. The reaction was terminated by washing in 2 × SSC for 15 min at room temperature. The slides were rinsed in PBS and blocked with 2% bovine serum albumin for 10 min, rinsed in PBS and then incubated for 30 min at 37°C in ExtrAvidin-TRITC (conjugated with tetramethyl rhodamine; Biomakor, Rehovot, Israel) diluted 1:50 in PBS, in a humid chamber. The cells were then washed in PBS and incubated in 4′,6′-diaminido-2-phenylindole (DAPI, Sigma) at a concentration of 0.5 μg/ml in PBS for 10 min at room temperature. Finally, the cells were rinsed twice in PBS, air dried and mounted for light microscopy. Red fluorescence at 570 nm (TRITC) and blue fluorescence at 460 nm (DAPI) were monitored sequentially on the same sample, using appropriate filters.

Sequence-specific DNA binding assay

Sequence-specific DNA binding was analyzed by the McKay assay, as described previously (Zauberman *et al.*, 1993). Briefly, pBlueScript DNA containing a p53 binding site derived from the GLN LTR (Zauberman *et al.*, 1993) was digested with *Hpa*I and *Hin*dIII and end-labeled with [^{32}P]dATP with the aid of the Klenow DNA polymerase. Extracts were prepared from DA-1 cells, and increasing amounts thereof were incubated with 2×10^5 c.p.m. of the radiolabeled probe. DNA−p53 complexes were collected with the aid of the p53-specific monoclonal antibody PAb248, and the bound DNA was resolved by polyacrylamide gel electrophoresis.

Clonogenicity assay

DIN and DIDD1 cells (4×10^3 cells per sample) were removed from the liquid culture at various points after IL-3 withdrawal, resuspended in 4 ml of semi-solid medium (20% FCS, 2% X63/0 conditioned medium, 10^{-4} M 2-mercaptoethanol and 1% methylcellulose in DMEM), and plated immediately in 35 mm dishes (1 ml per dish, in triplicate). The dishes were incubated at 37°C in a humid chamber, and colonies were counted 7 days later.

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