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Direct involvement of p53 in programmed cell death of oligodendrocytes

Orly Eizenberg, Anat Faber-Elman, Eyal Gottlieb[1], Moshe Oren[1], Varda Rotter[2] and Michal Schwartz[3]

Departments of Neurobiology, [1]Chemical Immunology and [2]Cell Biology, The Weizmann Institute of Science, 76100 Rehovot, Israel

[3]Corresponding author

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A covalent dimer of interleukin (IL)-2, produced *in vitro* by the action of a nerve-derived transglutaminase, has been shown previously to be cytotoxic to mature rat brain oligodendrocytes. Here we report that this cytotoxic effect operates via programmed cell death (apoptosis) and that the p53 tumor suppressor gene is involved directly in the process. The apoptotic death of mature rat brain oligodendrocytes in culture following treatment with dimeric IL-2 was demonstrated by chromatin condensation and internucleosomal DNA fragmentation. The peak of apoptosis was observed 16–24 h after treatment, while the commitment to death was already observed after 3–4 h. An involvement of p53 in this process was indicated by the shift in location of constitutively expressed endogenous p53 from the cytoplasm to the nucleus, as early as 15 min after exposure to dimeric IL-2. Moreover, infection with a recombinant retrovirus encoding a C-terminal p53 miniprotein, shown previously to act as a dominant negative inhibitor of endogenous wild-type p53 activity, protected these cells from apoptosis.

Key words: apoptosis/dimeric interleukin-2/p53

Introduction

Oligodendrocytes are post-mitotic cells that develop from rapidly dividing precursor cells (Temple and Raff, 1986; Gard and Pfeiffer, 1990; Hardy and Reynolds, 1991). The survival of oligodendrocytes depends on exogenous signaling molecules, such as growth factors, cytokines and neurotrophic factors (Noble *et al.*, 1988; Richardson *et al.*, 1988; Arakawa *et al.*, 1990; McKinnon *et al.*, 1990; Barres *et al.*, 1992, 1993; Buchman and Davies, 1993; Louis *et al.*, 1993). The death of oligodendrocytes due to insufficiency of survival factors was shown to be apoptotic in nature (Barres *et al.*, 1992).

Recent studies in our laboratory have shown that dimeric interleukin (IL)-2, produced enzymatically by nerve-derived transglutaminase (TG_N; Eitan and Schwartz, 1993), is cytotoxic *in vitro* to mature oligodendrocytes (galactocerebroside-stained cells; Cohen *et al.*, 1990; Sivron *et al.*, 1991; Eitan *et al.*, 1992), known to inhibit axonal growth (Schnell and Schwab, 1990; Sivron *et al.*, 1994). In general, cytotoxicity may lead to cell death either by necrosis (as a result of injury, infection or other external stress) or through a more specific process of apoptosis (programmed cell death; Duvall and Wyllie, 1986). The latter is thought to be involved, among other functions, in restricting the sizes of immature progenitor cell pools in the absence of appropriate growth-promoting stimuli (Duke and Cohen, 1986; Rodríguez-Tarducy *et al.*, 1990; Williams *et al.*, 1990). Apoptotic cells exhibit a distinct morphology and often also a characteristic pattern of DNA fragmentation resulting from cleavage of nuclear DNA in internucleosomal regions (Duvall and Wyllie, 1986).

The aim of our study was to determine whether the death of oligodendrocytes induced by dimeric IL-2 is apoptotic and to identify signaling molecules involved in this process. Specifically, we examined whether p53, known to be involved in apoptotic death in other systems (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Clarke *et al.*, 1993; Debbas and White, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993; Gottlieb *et al.*, 1994), is also involved in the cell suicide program of oligodendrocytes.

Results

Dimeric IL-2 causes apoptosis in oligodendrocytes

Cultures enriched in mature rat brain oligodendrocytes were treated with a preparation containing dimeric IL-2, produced *in vitro* using recombinant human IL-2 and a fish optic nerve-derived preparation containing TG_N (Eitan and Schwartz, 1993). The treated cells exhibited a typical apoptotic morphology, visualized by fluorescent staining with 4,6-diamidino-2-phenylindole (DAPI). Control cultures were: (i) treated with only the enzyme in the presence or absence of Ca^{2+}, (ii) treated with only monomeric IL-2, or (iii) left untreated. Figure 1 shows an example of the results of one such experiment photographed 16 h after the initiation of treatment with dimeric IL-2. Cells with fragmented nuclei, indicative of an apoptotic process, are clearly visible. Apoptotic cells with fragmented nuclei and condensed chromatin were seen as early as 12 h after treatment with dimeric IL-2.

To analyze the cultures quantitatively, the DAPI-stained apoptotic cells were counted at different times after treatment and their percentages relative to the total cell population were calculated at each time point. Figure 2 depicts a representative experiment in which the peak incidence of apoptotic cells was observed 16 h after the initiation of treatment, whereas significant cell death (30–40%, average of three experiments), measured metabolically by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Eitan *et al.*, 1992), was seen after 40–48 h (data not shown). In control cultures (untreated cells and cells

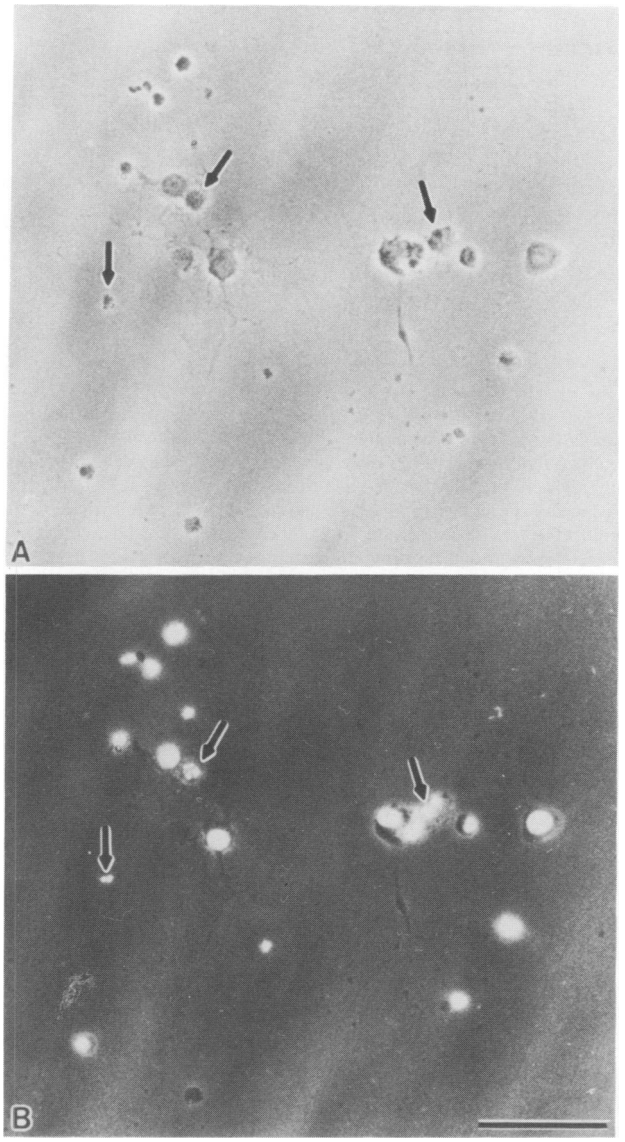

Fig. 1. Micrographs showing apoptotic cells in oligodendrocyte culture treated with dimeric IL-2. Enriched mature rat brain oligodendrocyte cultures were pretreated with dimeric IL-2. Cells were fixed, stained with DAPI and photographed at a magnification of ×1600. Phase-contrast micrograph shows the morphology of the cells (**A**). DAPI-stained oligodendrocytes, same field as in (A), were photographed using a UV filter (**B**). Arrows point to cells with patched nuclei.

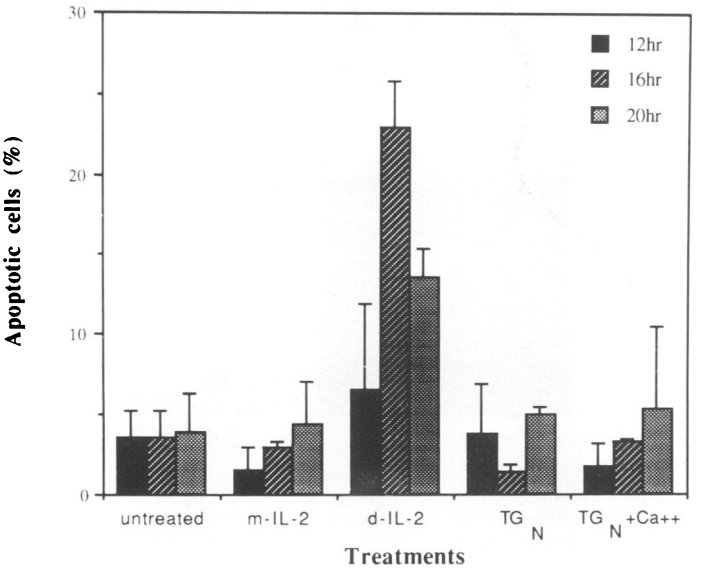

Fig. 2. Kinetics of apoptosis in dimeric IL-2-treated oligodendrocyte cultures. Apoptotic cells in the cultures described in Figure 1 were counted and their percentages calculated in relation to the total numbers of oligodendrocytes. The extent of cell death was determined in parallel cultures by the colorimetric assessment of the numbers of surviving cells (Eitan *et al.*, 1992). The experiment was performed in duplicate and was repeated three times. For each type of treatment, 400–1100 cells were counted in each experiment. One-factor analysis of variance (ANOVA; $DF = 3$, $F = 14.979$, $P = 0.0002$) revealed, according to Fisher's test, a significant difference (at 95%) in the number of apoptotic cells at the peak time period in the dimeric IL-2-treated cultures relative to untreated or other control cultures (results of two experiments were combined). m-IL-2, monomeric IL-2; d-IL-2, dimeric IL-2 produced *in vitro* (Eitan and Schwartz, 1993).

treated with monomeric IL-2 or with TG_N, in the presence or absence of Ca^{2+}), the percentage of apoptotic cells occurring spontaneously was much lower than in the dimeric IL-2-treated cultures at all time points analyzed (Figure 2). Of the total amount of IL-2 applied under these conditions, dimeric IL-2 comprised ~25% (Eitan and Schwartz, 1993). As apoptosis above background levels was observed only in the presence of the preparation containing dimeric IL-2, it is concluded that this apoptosis was attributable to the dimeric IL-2.

An analysis of DNA fragmentation in oligodendrocytes treated with dimeric IL-2 revealed a characteristic DNA ladder (Figure 3). The extent of DNA fragmentation detected 3 h after treatment (the first time point examined; data not shown) was similar to that in untreated cells. After 16 h of exposure to dimeric IL-2, the ladder became prominent (Figure 3, lane 2). At 20 h of exposure to dimeric IL-2 an intensive ladder appeared (Figure 3, lane 3). At this time point, cell death measured colorimetrically was barely detectable. It thus seems that DNA fragmentation precedes cell death by a significant time interval. This is in agreement with observations in a variety of cell types undergoing induced apoptosis (Arends and Wyllie, 1991; Tomey and Cope, 1991).

Commitment to dimeric IL-2-induced apoptosis occurs within a few hours

The time course of commitment to cell death was examined by treating mature rat brain oligodendrocytes with dimeric IL-2, and at the indicated time points (Figure 4) washing and further incubating them in dimeric IL-2-free medium for up to 16 h after treatment (the time of peak apoptosis). Apoptotic cells were visualized by DAPI staining. For up to 2 h of incubation with dimeric IL-2, we could not discern a commitment of the cells to programmed death. However, from 3 to 4 h onwards (varying slightly among different experiments) the cells gradually became committed, and by 5 h the percentage of apoptotic cells was already similar to that in cultures treated continuously for 16 h (Figure 4).

p53 in oligodendrocytes treated with dimeric IL-2

At different times after treatment with the preparation containing dimeric IL-2, oligodendrocytes were fixed and fluorescently stained with PAb421, a p53-specific

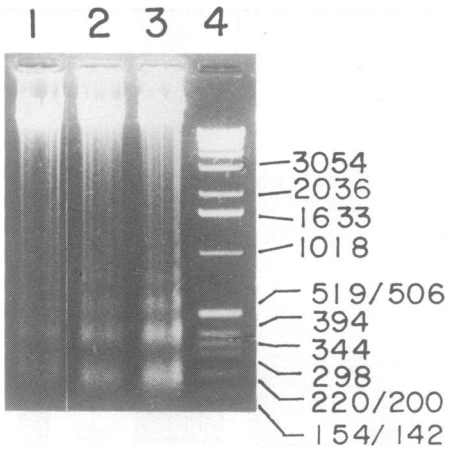

Fig. 3. DNA fragmentation in dimeric IL-2-treated oligodendrocytes. Enriched mature rat brain oligodendrocyte cultures were prepared, seeded and treated with dimeric IL-2, produced as described in Materials and methods. At various time points after treatment, cells were collected for either DNA fragmentation analysis or MTT analysis for determining cell death, as described in Figure 2. Lane 1, untreated oligodendrocytes, collected at the same time as treated samples; lanes 2 and 3, oligodendrocytes treated for 16 and 20 h, respectively, with dimeric IL-2; lane 4, molecular size markers (1 kb ladder; BRL). The experiment was repeated three times. Sizes in bp are indicated on the right.

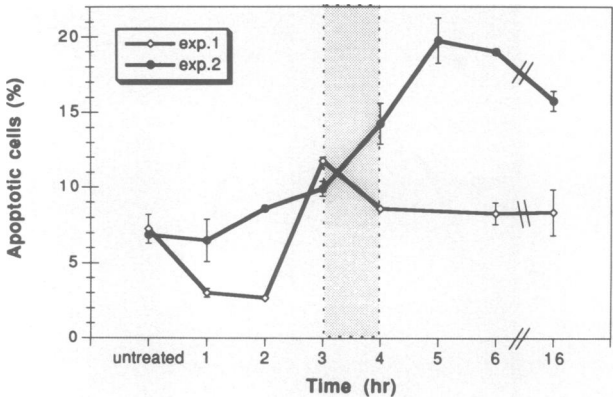

Fig. 4. Time course of commitment to cell death in dimeric IL-2-treated oligodendrocytes. Enriched mature rat brain oligodendrocyte cultures were prepared, seeded and treated with dimeric IL-2, produced as described in Materials and methods. At various time points after treatment, the dimeric IL-2 was washed off the cultures. Cells were incubated for up to 16 h after treatment, and were then fixed and stained with DAPI (as described in Materials and methods). The experiment was repeated twice. For each time point, 400–1300 cells were counted in each experiment.

monoclonal antibody (Harlow *et al.*, 1981) which recognizes rat wild-type (wt) p53, and with DAPI, which allows visualization of apoptotic morphology. In untreated cultures, specific p53 immunoreactivity was evident predominantly in the cytoplasm (Figure 5B). In contrast, in cultures treated with dimeric IL-2 the nucleus became strongly immunostained in ~80% of the cells as early as 15 min after treatment, while the cytoplasm was only weakly stained. Figure 5D demonstrates the nuclear localization of p53 at 2 h of treatment with dimeric IL-2. No changes in the subcellular localization of p53 immunoreactivity could be observed following treatment with monomeric IL-2. It thus appears that treatment with dimeric IL-2 induced a shift in the location of p53 from the cytoplasm to the nucleus. This shift occurred well before the appearance of apoptotic signs, such as chromatin condensation and DNA fragmentation (as described above).

PAb421 is known to react in the nucleus exclusively with p53. In the cytoplasm, however, it may crossreact with intermediate filament proteins such as certain keratins (Bartek *et al.*, 1990). Accordingly, one may argue that the high levels of immunostaining detected in the cytoplasm of untreated cells do not necessarily represent p53. This possibility is, however, ruled out by the fact that Western blot analysis revealed the presence of a p53 immunoreactive band in untreated oligodendrocytes. Furthermore, following treatment with dimeric IL-2, no change in the level of the p53 immunoreactive band was seen before and after the appearance of p53 in the nucleus (data not shown). These findings strongly suggest that the immunostaining detected in the cytoplasm prior to treatment with dimeric IL-2 represents wt p53 rather than an intermediate filament, and that the change in localization is probably the result of translocation of the p53 protein from the cytoplasm to the nucleus.

Protection from dimeric IL-2-induced apoptosis and from spontaneous apoptotic death in cells infected with a dominant negative mutant of p53

The nuclear localization of p53 observed in oligodendrocytes is in line with the reported correlation between the localization of p53 and its antiproliferative effects (Shaulsky *et al.*, 1990a,b, 1991; Gannon and Lane, 1991; Ginsberg *et al.*, 1991; Martínez *et al.*, 1991; Yonish-Rouach *et al.*, 1993), and thus suggests that p53 might participate in the apoptosis of oligodendrocytes induced by dimeric IL-2. To verify this possibility, we employed a dominant-negative mutant of p53, the DD p53 miniprotein (Shaulian *et al.*, 1992; Gottlieb *et al.*, 1994), to interfere with the function of endogenous wt p53 in dimeric IL-2-treated oligodendrocytes. The DD p53 miniprotein comprises the last 89 residues of mouse wt p53, including the dimerization domain, and acts as an inhibitor of co-expressed wt p53 (Shaulian *et al.*, 1992). The underlying mechanism appears to be due, at least in part, 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). For the infection of oligodendrocyte cultures we used a retroviral vector, LXSN, expressing the DD miniprotein (LXSNp53DD; Gottlieb *et al.*, 1994). As a control we used a similar retroviral vector, LXSN, encoding G418 resistance only. Because retroviruses can infect only proliferating cells, the cultures used for infection were enriched in rat brain oligodendrocyte progenitors, which continued to divide throughout viral infection and G418 selection, and then differentiated (see Materials and methods). On the fourth day of infection, the G418-resistant cells infected with either the control or the DD retrovirus were treated with dimeric IL-2 for 16 h, and were then fixed and analyzed. Cells were fluorescently stained with the p53 monoclonal antibody PAb421 and with DAPI to visualize p53 immunoreactivity and apoptotic morphology, respectively. In all cultures infected with LXSNp53DD, extensive p53 staining was detected in the nucleus (Figure 6C-2 and D-2), as expected (Shaulian *et al.*, 1992). In preparations infected

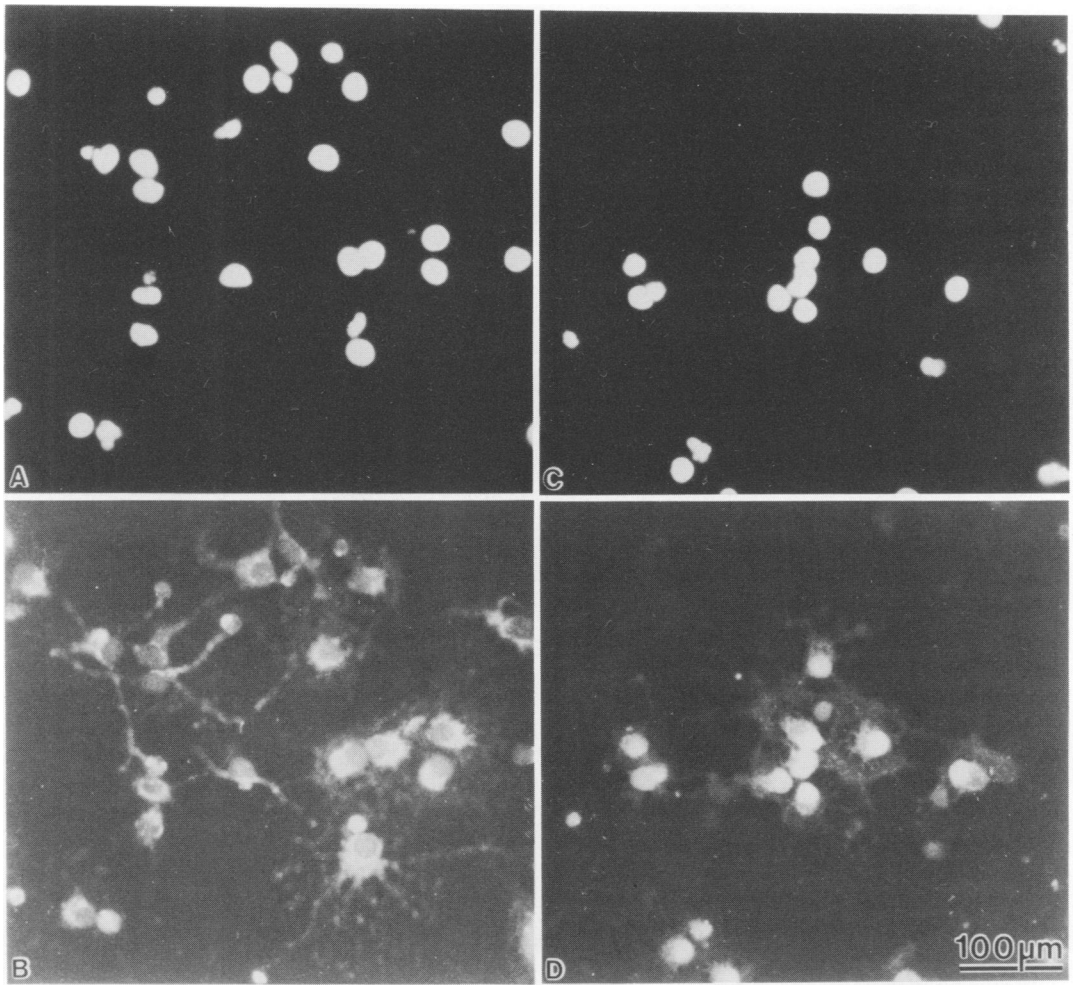

Fig. 5. Localization of p53 immunoreactivity in the nuclei of oligodendrocytes treated with dimeric IL-2. Enriched mature rat brain oligodendrocyte cultures were pretreated with dimeric IL-2 for 2 h. Cells were fixed, stained with monoclonal antibody PAb421 (directed against p53) and DAPI, and photographed at a magnification of ×1600. (**A** and **C**) DAPI-stained oligodendrocytes photographed using a UV filter. (**B** and **D**) PAb421-stained oligodendrocytes; the fields are the same as in (A) and (C), respectively. (A and B) Control untreated cultures. (C and D) Cultures treated with dimeric IL-2. The experiment was performed in duplicate and repeated three times.

with the control retrovirus LXSN, the p53 immunoreactivity in the nucleus was much lower (Figure 6A-2 and B-2), as in non-infected untreated cells (Figure 5B).

DAPI staining revealed no difference in the extent of apoptotic death between untreated cultures infected with the control retrovirus LXSN (Figure 6A-1) and with LXSNp53DD (Figure 6C-1). Treatment with dimeric IL-2 resulted in a higher incidence of apoptosis in cultures infected with the control retrovirus (Figure 6B-1) than in cultures that were infected with the DD-expressing retrovirus, indicating that the cells were protected from apoptosis (Figure 6D-1). When visualized immunocytochemically, the absence of apoptosis was found to be fully correlated with the expression of LXSNp53DD, i.e. none of the cells successfully infected with the DD showed apoptotic morphology. Quantitative analysis revealed that in cultures which were not treated with dimeric IL-2 and were infected with either LXSN or LXSNp53DD, ~20% of the total cell population underwent apoptosis (Figure 7). Following treatment with dimeric IL-2, the extent of apoptosis in cultures infected with only the control vector was 37%, whereas in the DD-infected cultures the percentage of apoptotic cells was equal to or even lower than in infected untreated cultures (Figure 7). From these results it appears that the DD protected the cells from apoptosis induced by dimeric IL-2 but not from spontaneous apoptosis. Accordingly, it is possible that spontaneous and induced apoptosis, observed in Figure 7, may occur via two different pathways, or that the spontaneous death observed in the infected cultures is mainly a reflection of the technical procedures related to the introduction of the retrovirus constructs and the use of neomycin, rather than of physiological spontaneous death such as that observed in Figure 2. Alternatively, the apoptotic cells observed despite the infection with the DD might be from cells that were committed to death at the time they were infected. In an attempt to resolve this issue, we assessed *in vitro*, the spontaneous apoptosis in retrovirus-infected oligodendrocytes at two different time periods post-infection. Cells were infected with the retrovirus constructs and kept in proliferation medium for 3 days. Apoptosis was monitored on days 4 and 7. As shown in Figure 8, the percentage of spontaneous apoptosis in these cells (kept in proliferation medium for 3 days) at days 4 and 7 was higher than that observed in less mature cells, i.e. those kept in proliferation medium for 5 days (Figure 7). Apoptosis on day 7, but not on day 4, could be partially blocked by the DD miniprotein (Figure 8). This may

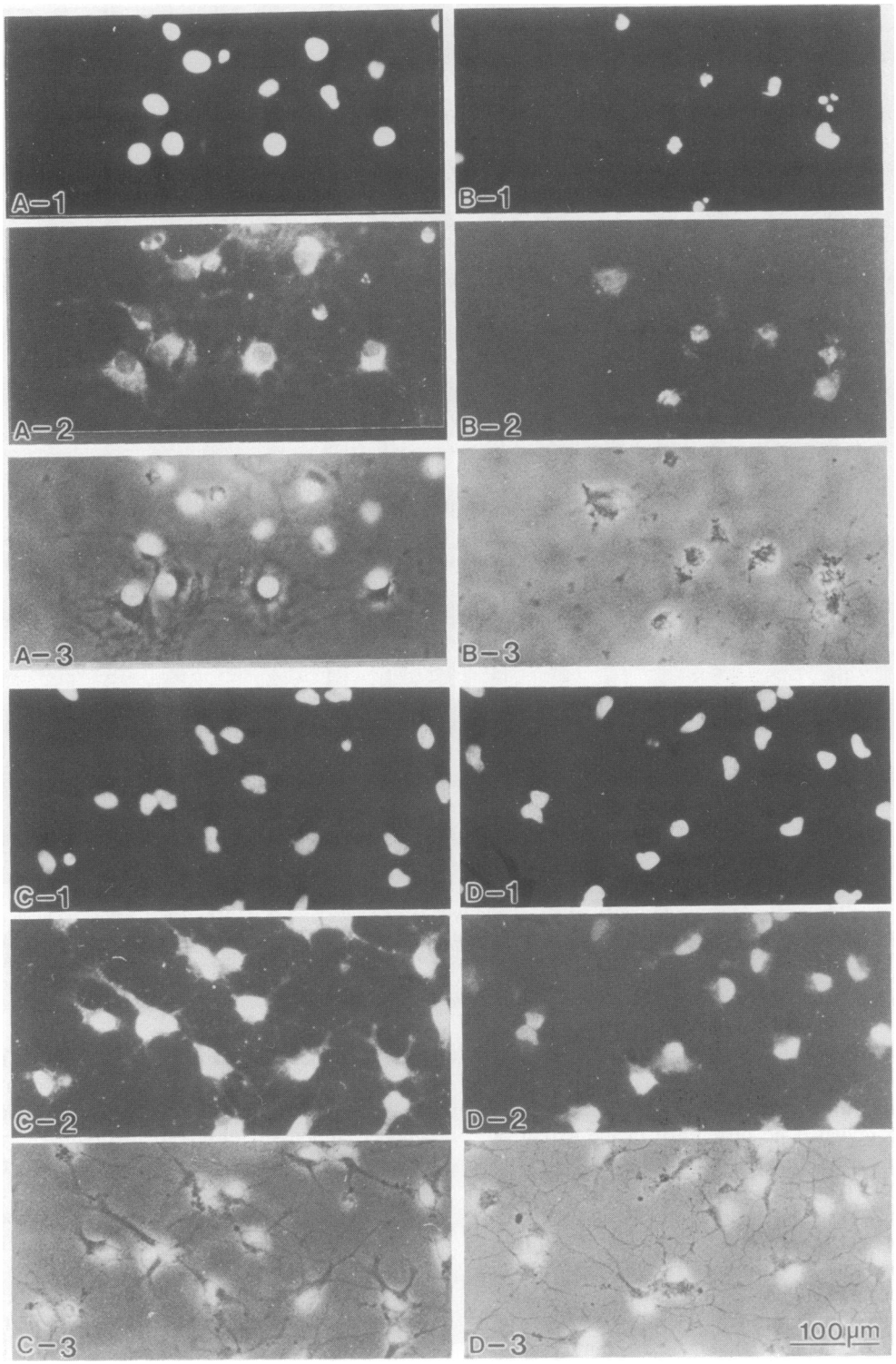

Fig. 6. Interference with dimeric IL-2-induced apoptosis by infection with a retrovirus encoding the DD p53 miniprotein. Enriched progenitor rat brain oligodendrocyte cultures were infected with LXSN (**A-1–3** and **B-1–3**) or LXSNp53DD (**C-1–3** and **D-1–3**). Following selection with G418, surviving cells were treated for 16 h with dimeric IL-2. Cells were then fixed, stained with monoclonal antibody PAb421 and DAPI, and photographed at a magnification of ×1600. The following description applies to all parts of the figure: Panel 1, DAPI-stained oligodendrocytes photographed using a UV filter. Panel 2, PAb421-stained oligodendrocytes. Panel 3, phase-contrast photographs (fields 1–3 are the same in each panel). (A and B) Cells infected with LXSN. (C and D) Cells infected with LXSNp53DD. (A and C) Untreated cultures. (B and D) Cultures treated with dimeric IL-2. The experiment was performed in duplicate and repeated three times. Note that the phase photographs were taken through the UV filter, which also enables the visualization of DAPI staining.

imply that the non-protectable cells observed on day 4 were the G418-sensitive cells which were still in the process of dying, whereas on day 7 most of the neomycin-sensitive cells had already been killed and the main death at this stage in culture was due to spontaneous apoptosis which could be blocked by the DD. Taken together, the

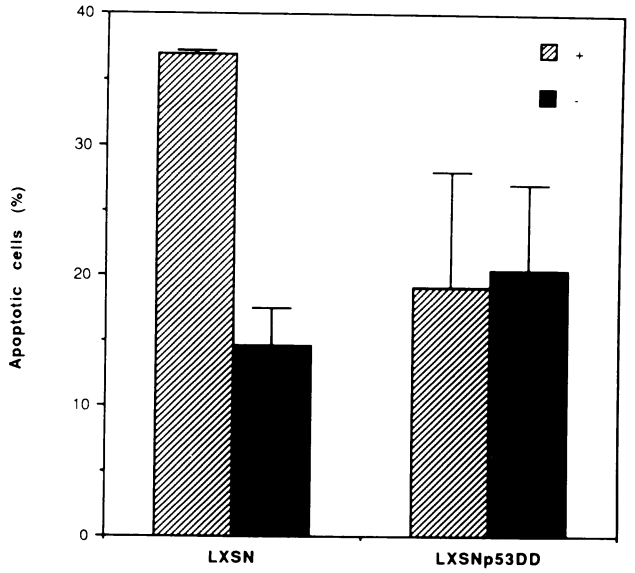

Fig. 7. Kinetics of cell death of LXSN- or LXSNp53DD-infected oligodendrocytes untreated or treated with dimeric IL-2. Apoptotic cells in the cultures described in Figure 6 were counted and their percentages calculated relative to the total numbers of oligodendrocytes. The experiment was performed in duplicate and repeated three times. The graph describes the averages of two such experiments. For each type of treatment, 400–1100 cells were counted in each experiment. Repeated measures ANOVA ($DF = 3$; $F = 6.5$; $P = 0.02$) revealed, according to Fisher's test, a significant reduction in the percentage of apoptotic cells in dimeric IL-2-treated cultures infected with the LXSNp53DD retrovirus relative to those infected with the LXSN retrovirus or to untreated controls (results of three experiments were combined). (−) Untreated cells; (+) cells treated with dimeric IL-2 produced *in vitro* (Eitan and Schwartz, 1993).

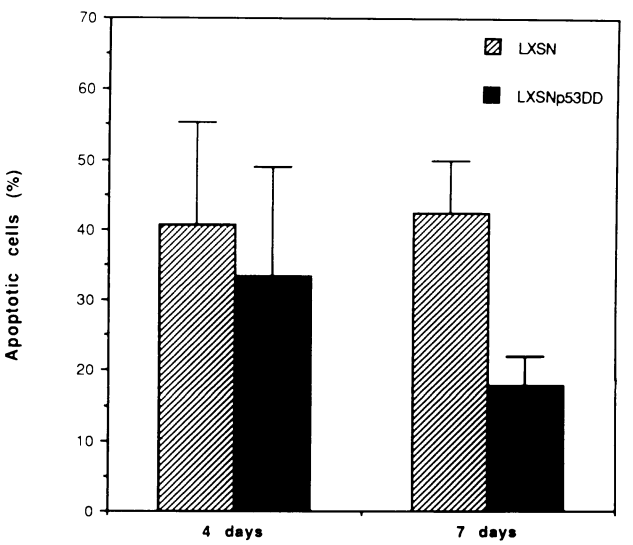

Fig. 8. Kinetics of spontaneous cell death of LXSN- or LXSNp53DD-infected oligodendrocytes. Apoptotic cells in culture, prepared as described in the legend to Figure 6, were analyzed 4 and 7 days after seeding. Apoptotic cells were counted and their percentages calculated relative to the total numbers of oligodendrocytes. The experiment was performed in duplicate and repeated three times. For each type of treatment, 300–1000 cells were counted in each experiment.

results depicted in Figures 7 and 8 suggest that (i) wt p53 is involved in the spontaneous apoptosis of oligodendrocytes, and (ii) the high percentage of cell death in the non-IL-2-treated cultures infected with the control retrovirus reflects both the physiological death of maturing cultures and the death caused by technical manipulations related to retroviral infection and neomycin selection; the latter death could not be blocked by the inactivation of wt p53. It should also be noted that we counted the number of apoptotic cells as a fraction of the total number of cells, rather than as a fraction of the successfully infected cells. It is possible that protection, if calculated on the latter basis, would reach 100%. Obviously, the stages of differentiation and maturation of the cells have an important effect on the inactivation of wt p53 in the cells. The results suggest that the stage in the cell cycle, together with the nuclear location of wt p53, influences the fate of the cell, i.e. its death by apoptosis or its continued differentiation and maturation.

Discussion

This study demonstrates that the treatment of mature oligodendrocytes with IL-2 in its dimeric form, shown previously to be cytotoxic to these cells, leads to their apoptotic death, and that p53 is involved in this process. Apoptotic death was demonstrated by chromatin condensation and DNA fragmentation. Some fragmentation of the DNA was also detected in untreated cells (Figure 3), indicating that cultured oligodendrocytes undergo spontaneous apoptosis. After exposure of the cells to dimeric IL-2 for 16 h, the ladder of DNA fragmentation became more prominent due to the addition of apoptotic cells that were induced by the treatment. The ladder signal, however, was not very intensive, probably because at the examined time only 20–25% of the cells showed apoptotic morphology. In CTLL-2, an IL-2-dependent cell line, low concentrations of IL-2 in its monomeric form suppress the apoptotic process by the induction of the *bcl-2* proto-oncogene (Deng and Podack, 1993) or via activation of either protein kinase C (PKC), which represses cell death, or a tyrosine kinase, which confers glucocorticoid resistance and permits cell cycle progression (Walker *et al.*, 1993). On the other hand, high concentrations of monomeric IL-2 (>100 U/ml) were involved in the induction of apoptosis in mouse thymocytes (Lenardo, 1991; Migliorati *et al.*, 1993).

Our results show that it takes ~3–4 h of treatment with dimeric IL-2 for the oligodendrocytes to become committed to programmed cell death. The delay in commitment may reflect a time-consuming activation of specific proteins or genes (apoptosis-inducing genes; Buttyan *et al.*, 1989; Iton *et al.*, 1991; Owens *et al.*, 1991; Oltvai *et al.*, 1993) and/or the repression of proteins or genes (apoptosis-suppressing genes) such as *bcl-2* (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990, 1991; Henderson *et al.*, 1991; Sentman *et al.*, 1991; Strasser *et al.*, 1991; Boise *et al.*, 1993; Oltvai *et al.*, 1993).

Here we demonstrate that p53 plays a role in oligodendrocyte death induced by dimeric IL-2. This conclusion is inferred from the appearance of wt p53 in the nucleus following treatment with dimeric IL-2, and is substantiated further by the protection from apoptosis provided by interference with endogenous wt p53 activity via infection with the DD-expressing retrovirus. The nuclear localization of p53 is a result of either its translocation from the cytoplasm to the nucleus or *de novo* synthesis of the

protein followed by its translocation to the nucleus coupled with degradation of the older protein in the cytoplasm. Western blot analysis supports the presence and cytoplasmic localization of constitutively expressed p53, and thus rules out the possibility that the immunoreactive p53 observed in the cytoplasm is due to a non-p53 epitope. This is substantiated further by our recent immunocytochemical localization of p53 by electron microscopy (data not shown), which showed that the cytoplasmic p53 immunoreactivity is not associated with cytoskeletal proteins. The relatively high constitutive level of wt p53 in mature oligodendrocytes in culture does not necessarily imply the presence of a high level of wt p53 in mature brain, as other glial cells might not express p53 at all (preliminary results indicate an absence of p53 immunoreactivity in rat astrocyte cultures).

At the peak time (16–20 h of treatment), only 20–25% of the cells in cultures treated with dimeric IL-2 showed apoptotic morphology (Figures 2 and 4). In contrast, ~80% of the cells exhibited nuclear localization of p53 as early as 15 min after the initiation of treatment with the dimer (Figure 6). The fact that only a relatively small fraction of the cells eventually showed apoptotic morphology suggests that p53 is important but not sufficient for the induction of apoptosis. The experiments in which endogenous wt p53 activity was blocked further support the involvement of p53 in apoptotic death. It thus seems likely that additional factors associated with the cellular context determine whether or not the nuclear localization of p53 will lead to death. The DD-expressing retrovirus interferes not only with apoptosis induced by dimeric IL-2 but also with spontaneous apoptosis, as shown by a comparison between the number of apoptotic cells in untreated oligodendrocyte cultures in the presence of LXSN and LXSNp53DD (Figure 8). It should be borne in mind that spontaneous apoptosis in oligodendrocytes results from the deprivation of trophic factors, in contrast to the induced cytotoxicity and apoptosis brought about in this study by the application of a cytokine. Nevertheless, in both cases the process seems to involve wt p53. The fact that the inactivation of endogenous wt p53 caused similar and only partial blocking of the spontaneous and dimeric IL-2-induced apoptosis suggests that the observed death of the remaining cells in both cultures can be attributed to either non-physiological factors such as virus manipulation or the fact that some of the cells were already committed to death prior to infection. It is also possible that the cells affected by dimeric IL-2 represent a subpopulation of oligodendrocytes that were susceptible at the time of treatment. Susceptibility to treatment with dimeric IL-2 may be related to cell cycle signaling and/or IL-2 receptor levels.

Several experimental models indicate that p53 can be regulated by differential subcellular localization. This notion is strongly supported by recent observations showing that p53 indeed acts as a cell cycle regulatory protein, and as such may require a well-controlled nuclear translocation mechanism. Overexpression of exogenous wt p53 activity in wt p53-deficient cells can induce apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Johnson *et al.*, 1993; Ramqvist *et al.*, 1993; Ryan *et al.*, 1993; Wang *et al.*, 1993). The need for functional wt p53 in mediating apoptosis has been discerned in other systems (Clarke *et al.*, 1993; Debbas and White, 1993; Lowe and Ruley, 1993; Lowe *et al.*, 1993). In addition, p53 can also modulate differentiation (Aloni-Grinstein *et al.*, 1993). The relatively high constitutive levels of p53 found in normal differentiated oligodendrocytes suggest a probable involvement of p53 in the normal physiological growth arrest of oligodendrocytes. The fact that the introduction of the DD p53 miniprotein did not interfere with the normal differentiation of the oligodendrocytes (infected cells were fully mature and differentiated when analyzed) could imply that at the time of infection the cells were already committed to differentiation. This finding also suggests that although p53 seems to be involved in both differentiation and apoptosis, it might operate through separate pathways, possibly mediated by different genes. This might explain why the inactivation of endogenous wt p53 by infection with the DD miniprotein prevents only apoptosis and does not interfere with the differentiation pathway.

Certain cells appear to have a salient commitment to apoptosis which needs to be continuously overridden by survival signals (Arends and Wyllie, 1991; Raff, 1992). Such cells probably possess a functional molecular circuitry poised to implement the apoptotic process. The fact that oligodendrocytes undergo apoptosis spontaneously, as indicated earlier (Raff, 1992), suggests that they belong to this category. Dimeric IL-2 could conceivably be a component of the putative molecular circuitry. The propensity of a cell to undergo apoptosis is often dictated by its state of differentiation and maturation (Arends and Wyllie, 1991; Golstein *et al.*, 1991). Programmed cell death is a fundamental feature of development and tissue survival in adulthood (Raff, 1992). In this study, we found that dimeric IL-2 is cytotoxic to oligodendrocytes and leads to their apoptotic death, and that wt p53 is involved in this process. We also found that wt p53 is involved in the spontaneous death of oligodendrocytes. Dimeric IL-2 has no known role in the central nervous system (CNS) in general or in development in particular. However, even if dimeric IL-2 does have a role in CNS regeneration, this does not necessarily imply its role in development. Nevertheless, it is conceivable that the p53-related molecular mechanism observed in this study might represent a more general pathway in the control of cell fate in the CNS. Moreover, as the elimination of oligodendrocytes can facilitate post-traumatic axonal regeneration (Schnell and Schwab, 1990; Sivron and Schwartz, 1994), this study suggests that p53 may play a role in the response of the nerve to trauma.

Materials and methods

Preparation of mature rat brain oligodendrocyte cultures

Highly enriched mature rat brain oligodendrocyte cultures were prepared by a modification of the procedure of McCarthy and de Vellis (1980). Cells dissociated from the cerebral cortex of 2 day-old Wistar rats were cultured for 8 days in poly-D-lysine (PDL)-coated Nunc tissue culture flasks (two brains per flask, 85 cm^2 surface area) in Dulbecco's modified Eagle's medium (DMEM) plus 7.5% fetal calf serum (FCS), 2 mM glutamine and antibiotics. The medium was changed every 2 days. After 8 days, flasks were shaken for 8 h at 37°C on a rotary platform and the medium, containing mostly macrophages, was discarded. Fresh medium was added to the flasks, which were then shaken for an additional 16 h at 37°C. The cells shaken off, containing mostly progenitor cells for oligodendrocytes and type-2 astrocytes (O-2A), were collected;

6×10^5 cells were seeded on three 2.4 cm^2 glass coverslips in a 5 cm plate. To encourage oligodendrocyte development, seeding was carried out in Raff's modification of Bottenstein and Sato's defined medium (Bottenstein and Sato, 1979; Raff *et al.*, 1983).

Production of dimeric IL-2

Cells were treated with dimeric IL-2 produced by overnight incubation at 37°C with a TG_N-containing preparation of soluble substances, obtained from regenerating fish optic nerve (Eitan and Schwartz, 1993) in the presence of Ca^{2+}. Recombinant human IL-2 (100 U/ml) was used as the monomer and as a source for preparing the dimer.

Immunostaining of oligodendrocytes with p53 antibodies

Highly enriched mature rat brain oligodendrocyte cultures were prepared as described above. Cells were collected and seeded (6×10^5 cells/5 cm plate containing nine 13 mm glass coverslips). After treatment with dimeric IL-2, produced as described above, cells were fixed in methanol for 5 min at −20°C, treated with cold acetone at 4°C for 2 min and washed in PBS. Monoclonal antibody PAb421, specific for p53 (Harlow *et al.*, 1981), was placed on the coverslips for 30 min in a moist chamber at 37°C. The coverslips were washed with PBS, stained for 30 min at room temperature by a specific second antibody [fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG + IgM; Jackson Immuno-Research Laboratories], diluted 1:30 in 1% bovine serum albumin (BSA)/PBS and then fluorescently stained with DAPI (5 mg/ml) diluted 1:10 000 in PBS for 15 min at room temperature. The cells were washed three times with PBS, mounted in a solution of glycerol containing 22 mM 1,4-diazobicyclo(2,2,2)octaine (Sigma) to prevent fading (Johnson *et al.*, 1982) and photographed.

DNA fragmentation analysis

DNA fragmentation analysis was performed essentially as described by Rodríguez-Tarducy *et al.* (1990). Oligodendrocyte cultures (1.5×10^6 cells/5 cm plate) were treated with dimeric IL-2. At the indicated time points, cells were washed twice in a solution of 140 mM NaCl and 20 mM ethylenediaminetetraacetic acid (EDTA), and incubated at 37°C for 4 h in 400 µl lysis buffer (200 mM Tris−HCl, pH 8.3, 100 mM EDTA, 50 µg/ml proteinase K and 1% SDS). The DNA was extracted twice with an equal volume of phenol/chloroform followed by two extractions with chloroform; the aqueous phase was dialyzed overnight against a solution of 10 mM Tris−HCl, pH 7.5, and 1 mM EDTA. The DNA solution was incubated for 3 h at 37°C with RNase A (50 µg/ml). Proteinase K (120 µg/ml) was added, and incubation was continued for another 3 h. DNA was extracted twice with phenol/chloroform, followed by two extractions with chloroform, and precipitated in ethanol. Pellets were resuspended in 50 µl H_2O; DNA concentrations were determined from the absorbance at 260 nm. Each DNA sample (3 µg) was electrophoresed through a 1.5% agarose gel. DNA bands were visualized by staining with ethidium bromide.

Infection with retroviral vectors

Highly enriched rat brain oligodendrocyte cultures were prepared and seeded as described above. To encourage oligodendrocyte proliferation, cells (2×10^6 cells/9 cm plate containing nine 24 mm glass coverslips) were seeded in an appropriate medium in the logarithmic phase of growth [B104 cells maintained in DGA medium (10% FCS, 2 mM L-glutamine and antibiotics in DMEM)]. [For preparation of the proliferation medium, DGA was removed, cells were washed twice with DMEM and Raff's modification of Bottenstein and Sato's defined medium (McCarthy and de Vellis, 1980) was added to the cells in the logarithmic phase of growth for 2–3 days, after which the medium was collected, filtered and diluted 1:3 in Raff's modification of Bottenstein and Sato's defined medium. This enabled the cells to continue proliferating without differentiating.] Two days after seeding, cells were infected with retroviruses expressing either LXSNp53DD or G418 resistance only (LXSN). The generation of these retroviral vectors has been described elsewhere (Gottlieb *et al.*, 1994). Infection was allowed to proceed for 2.5 h, after which the virus was washed out and fresh proliferation medium added. Selection with G418 was performed for 3 days (starting on the day after infection) by the daily addition of 1 mg/ml G418. At the end of the third day of selection, cells were treated with dimeric IL-2 for 16 h, fixed and stained as described above.

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