The Journal of Neuroscience, February 15, 1997, 17(4):1397–1405

p53 Expression Induces Apoptosis in Hippocampal Pyramidal Neuron Cultures

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The tumor suppressor gene p53 has been implicated in the induction of apoptosis in dividing cells. We now show that overexpression of p53 using an adenoviral vector in cultured rat hippocampal pyramidal neurons causes widespread neuronal death with features typical of apoptosis. p53 overexpression did not induce p21, bax, or mdm2 in neurons. X-irradiation of hippocampal neurons induced p53 immunoreactivity and cell death associated with features typical of apoptosis. Overexpression of a constitutively active nonphosphorylatable form of the retinoblastoma gene product blocked x-irradiation-induced neuronal death. However, overexpression of the cyclin-dependent kinase inhibitor p21 did not. Treatment of neurons with transforming growth factor-β1 protected them from x-irradiation. These results are consistent with a role for p53 in nerve cell death that is distinct from its actions relating to cell cycle arrest.

Key words: adenovirus; irradiation; retinoblastoma; tumor suppressor genes; overexpression; p21; transforming growth factor-β1

The death of populations of neurons occurs naturally by programmed cell death or apoptosis during the development of the nervous system (Oppenheim, 1991; Driscoll and Chalfie, 1992; Truman et al., 1992). In certain diseases, including stroke (Linnik et al., 1993; Charriaut-Marlangue et al., 1996a,b; Du et al., 1996), Alzheimer's disease (La Ferla et al., 1995; Prehn et al., 1996), and AIDS-associated dementia (Charriaut-Marlangue et al., 1996b; Meucci and Miller, 1996), the death of neurons may also involve events related to or identical to those occurring during development. Evidence suggests that the death of neurons after stroke, trauma, and seizure activity is attributable to "excitotoxicity," a process involving the overactivation of neuronal glutamate receptors (Choi, 1988). The molecular events that underlie excitotoxic neuronal death are believed to include large increases in [Ca]i and toxic free radicals (Choi, 1988; Bindokas and Miller, 1995; Bindokas et al., 1996). It was originally believed that neurons died by necrosis under excitotoxic conditions. However, more recent evidence suggests that many cells may also die by apoptosis (Linnik et al., 1993; Crumrine et al., 1994; Sakhi et al., 1994; Rink et al., 1995; Morrison et al., 1996), depending on the severity of the stimulus (Bonfoco et al., 1995). It is clearly important to understand the molecular basis of apoptosis in neurons and how these events can be manipulated.

Studies on a variety of cells have started to define the steps that are involved in apoptosis under different conditions (Kroemer et al., 1995). The final common pathway that produces programmed cell death is believed to operate in the cytoplasm and is probably common to most, if not all, cells (Jacobson et al., 1994; Kroemer et al., 1995). These events can be triggered by a variety of factors and modulated by several classes of proteins. Prominent among these proteins are a group of "tumor suppressors" that include the p53 gene product, the retinoblastoma gene product (pRb), and several inhibitors of the cyclin-dependent kinases, particularly the $p21^{WAF1/CIP1}$ protein (p21) (Cox and Lane, 1995; Katayose et al., 1995; Kouzarides, 1995). The p53 protein plays a central role in the cellular response to DNA damage (Elledge and Lee, 1995; Enoch and Norbury, 1995). p53 expression leads to arrest of the cell cycle so that DNA repair can occur or can activate apoptotic pathways when repair seems impossible (Lane et al., 1994). Consistent with this idea are observations that increases in p53 after x-irradiation (Clarke et al., 1993; Lowe et al., 1993) or DNA-damaging ("genotoxic") drugs (Wood and Youle, 1995) or p53 overexpression (Wu and Levine, 1994) cause cell cycle arrest and/or apoptosis in a variety of cell types. In addition, excitotoxic stimulation has been shown to stimulate p53 production by neurons, suggesting that p53 may also be responsible for triggering apoptosis under these circumstances (Sakhi et al., 1994). Although this seems to be an interesting possibility, it should be noted that virtually all studies on the role of tumor suppressor genes in apoptosis have been carried out on dividing cells. It is not known whether p53 actually causes highly differentiated, postmitotic cells such as neurons to die and if so, precisely how this process is manifest in such cells (but see Sadoul et al., 1996) (Eizenberg et al., 1996). The present series of studies explores the role of p53 in neuronal apoptosis. Our studies provide support for a potential role for p53 in neurodegenerative disease.

MATERIALS AND METHODS

Materials. Recombinant human TGF-β1 was obtained from R & D systems (Minneapolis, MN) and was prepared as a 1000 ng/ml stock in PBS containing 1 mg/ml ovalbumin and 4 mM HCl.

Hippocampal pyramidal neuron culture. Pyramidal neurons were pre-

Received Oct. 1, 1996; revised Nov. 27, 1996; accepted Dec. 4, 1996.

This study was supported by U.S. Public Health Service Grants DA02121, DA02575, and MH40165 (R.J.M.) and A 42596-09 (R.R.W.); Amyotrophic Lateral Sclerosis Association (R.P.R.) and AR42885, DK48987 (J.M.L.); German Research Foundation Grant Pr 338/2-1 (J.H.M.P.); and a grant from Ministerio de Educación y Ciencia of Spain (J.J.). We thank Dr. Ning-Sheng Wang for her technical help. We are grateful to Dr. J. Kokontis for advice and generous gifts of PC-3 cell line and to Dr. J. Tang of Schering-Plough Research Institute for providing Adp53.

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pared from the hippocampi of fetal rats at 17 d of gestation (E17) as described by Scholz and Palfrey (1991). These neurons are highly differentiated and establish functional synaptic connections (Scholz and Miller, 1995, 1996). Hippocampi were dissected in Ca^{2+}/Mg^{2+}-free HBSS (Cellgro) and incubated in 0.1% trypsin (Worthington) for 15 min. The hippocampi were triturated by aspirating 7 to 10 times using a normal-bore Pasteur pipette with a flame-narrowed Pasteur pipette. Cells were plated in DMEM (Life Technologies, Grand Island, NY) plus 10% horse serum (Life Technologies) on poly-L-lysine- (Sigma, St. Louis, MO; 0.5 mg/ml in borate buffer, pH 8.0) coated 15 mm round glass coverslips and allowed to adhere for 2–4 hr. The coverslips were then transferred to 60 mm dishes containing supporting astrocytes attached to the bottom of the culture dish. Astrocytes were prepared from the cerebral hemispheres of newborn rats.

For biochemistry experiments, the layer of glial and neuronal cells were inverted. After 4 d in culture, glial cells were removed from the dishes with trypsin and plated on 30 mm Thermanoxe coverslips equipped with paraplast feet. Neurons were plated in DMEM (Life Technologies) plus 10% horse serum (Life Technologies) on poly-L-lysine- (Sigma; 0.5 mg/ml in borate buffer, pH 8.0) coated 35 mm tissue culture dishes at 3.5 10^5 cell/Petri dish. After 2–4 hr, the medium was replaced with a serum-free defined medium (N2), and the coverslips containing the feeder glial cells were placed on top of each dish of pyramidal neurons. Cytosine-β-D-arabinofuranoside (5 μM) was added to each plate 2 d later to inhibit non-neuronal cell proliferation.

X-irradiation protocol. X-Irradiation was performed using a GE Maxitron 250 X-Ray Generator operating at 250 kV and 26 mA with a dose rate of 114 cGy/min. Cells received a single dose of 200, 500, or 1000 cGy at 22–25°C. Control dishes were sham-irradiated under identical conditions.

Cell viability assay. Cell death was determined using fluorescein diacetate/propidium iodide double-staining procedure (Favaron et al., 1988). The cells were incubated for 45 sec at 22–25°C with 15 μg/ml fluorescein diacetate (Sigma) and 4.6 μg/ml propidium iodide (Molecular Probes, Eugene, OR) in PBS, pH 7.4. The stained cells were examined immediately with a standard epi-illumination fluorescence microscope (Olympus, 450 excitation, 520 barrier). Cells stained with propidium iodide represent dead cells, whereas cells stained with fluorescein represent live cells. A total of ~300–400 cells (viable plus nonviable) were counted in random fields of each coverslip, and the percentage of cells surviving was then determined above the total cell number. The percentage of neurons surviving was determined on three or four coverslips for each condition in each experiment and normalized to controls examined in parallel under the same conditions. The average relative percent survival from at least three separate experiments for each condition is expressed in the text and figures as the mean ± SEM.

Analysis of DNA fragmentation. For evaluation of cellular DNA fragmentation, both the TUNEL (Gavrieli et al., 1992) and Hoechst 33342 (Earnshaw, 1995) stains were used. For the TUNEL method, we used the Apoptag kit (Oncor, Gaithersburg, MD). Briefly, cultures were fixed in Bouin's solution (Sigma) at room temperature for 20 min. After rinsing three times using PBS, cultures were incubated with terminal deoxynucleotidyl transferase and digoxigenin-dUTP at 37°C for 1 hr. After labeling, the DNA breaks were visualized with an anti-digoxigenin antibody coupled to peroxidase. diaminobenzidine (Sigma) and hydrogen peroxide were used to develop the stain.

For staining with Hoechst 33342, cultures were rinsed three times using PBS, fixed with 4% paraformaldehyde for 10 min at 37°C, permeabilized in ethanol/acetic acid (19:1 v/v) for 15 min at −20°C, washed three times in PBS, and then incubated with 1 ng/ml Hoechst 33342 (Molecular Probes) for 20 min at room temperature. After two rinses with PBS, the cell staining was analyzed using a fluorescent microscope.

Immunoblotting. Pyramidal neuron cultures were washed with cold PBS twice and then collected by mechanical scraping with 100 μl of PBS per tissue culture dish. Phenylmethylsulfonyl fluoride (10 μM) was added to halt further protease activity. The protein suspension was centrifuged at 12,000–14,000 rpm for 5 min. The supernatant was discarded, and the protein pellet brought up in 40 μl of sample buffer. The protein from each condition was quantified spectophotometrically (Micro BCA Protein Reagent Kit, Pierce, Rockford, IL), and an equal amount of protein (~30 μg) was loaded onto each lane of the SDS-PAGE (7.5% SDS-PAGE for p53, 12.5% SDS-PAGE for p21, and 10% SDS-PAGE for HΔpRb), which was then run at 30 mA. After electrophoresis, proteins were transferred to Immobilon PVDF membranes overnight at 110 mA. Nonspecific protein binding was blocked with Blotto [4% w/v nonfat dried milk, 4% bovine serum albumin (Sigma) and 0.1% Tween 20 (Calbiochem-Novabiochem, La Jolla, CA)] in PBS for 1 hr. The membranes were incubated with one of the following antibodies for 1 hr: anti-p53(Ab-1) and anti-mdm-2 (1:100 and 1:1000 dilution of mouse monoclonal, Oncogene Science); anti-p21 (1:1000 dilution of rabbit serum, PharMingen); anti-hemagglutinin protein of human influenza virus (1:1000 dilution of mouse monoclonal antibody HA-(12CA5), Boehringer Mannheim Corporation); anti-bax(N-20) (1:200 dilution of rabbit serum, Santa Cruz). After washing with Blotto, the membranes were incubated with a secondary antibody (1:5000 dilution of peroxidase-labeled anti-mouse or goat anti-rabbit IgG, Promega, Madison, WI) in Blotto. The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit). Immunoblots were developed by exposure to x-ray film (Eastman-Kodak, Rochester, NY).

Construction of replication-defective adenoviruses and infection protocol. The different viruses were constructed as described previously. Adp53 recombinant adenovirus (Wills et al., 1994) is based on Ad 5 with a deletion of E1 region of nucleotides 360–3325 replaced with a 1.4 kb full-length p53 cDNA driven by the CMV (A/M/53) promoter followed by Ad 2 tripartite leader cDNA. Adp21 and AdHΔpRb are described in Chang et al. (1995a,b), and AdBacLacZ (Barr et al., 1994) was used as a control virus vector. The enzymatic activity and high efficiency of AdBacLacZ have been shown previously. Under our conditions, AdBacLacZ was observed in >90% of cultured cells (Chard et al., 1995; Jordán et al., 1995). Similar results have been obtained with other viruses (Chard et al., 1995; Jordán et al., 1995; Prehn et al., 1996).

Cultured rat hippocampal neurons were infected using methods described previously (Chard et al., 1995). In brief, the coverslips were removed from the astrocyte feeder layer and placed in a 60 mm tissue dish in astrocyte-conditioned, N2.1 supplemented MEM culture medium. An aliquot of high-titer virus was then added to the culture medium to give a multiplicity of infection (MOI) of 100. The dish was agitated gently and placed in an incubator for 2 hr. The coverslips were then returned to the original tissue culture dish containing the astrocyte feeder layer. In each toxicity experiment, successful expression of the respective proteins was verified by immunocytochemistry (one coverslip per experimental condition).

Immunocytochemistry. Cultures were fixed by incubating at 37°C for 15 min with 4% paraformaldehyde in culture medium. After washing three times in 0.1 M PBS, pH 7.4, cells were permeabilized using 0.1% Triton X-100 (Eastman Kodak) in PBS, for 2.5 min. The coverslips were then incubated for 1 hr in blocking media [0.1% Tween 20 (Sigma), 4% BSA (Sigma), in 0.1 M PBS] at room temperature. Incubations with primary antibodies were performed overnight at 4°C using monoclonal mouse antibodies for anti-β-galactosidase (1:1000, Sigma), p53 (Ab-4, Oncogene Science, 1:100) diluted in blocking media. Monoclonal antibodies were detected using either Cy3-conjugated streptadivin or anti-mouse IgG (Jackson ImmunoResearch Lab, West Grove, PA) diluted 1:200 in blocking solution. The latter followed by Vectastain ABC Kit (Vector Labs, Burlingame, CA). The peroxidase was visualized using 3,3′-diaminobenzidine (Sigma) as a chromogenic substrate. Cultures were mounted in 90% glycerol with 0.1 phosphate buffer and 0.01% NaN_3.

RESULTS

Overexpression of p53 induces apoptosis in hippocampal pyramidal neurons

Previous investigations on dividing cells, including neuronal precursors, have demonstrated the importance of p53 in mediating apoptosis caused by x-irradiation or genotoxic agents (Kameyama and Inouye, 1994; Wood and Youle, 1995). Therefore, we investigated the effects of p53 on hippocampal pyramidal neurons in culture by using an adenovirus that expresses p53 (Adp53) (Wills et al., 1994). The neurons used in these studies are postmitotic and exhibit extensive functional synaptic connections (Scholz and Miller, 1995, 1996). p53 protein was undetectable in these neurons under control conditions. However, 48 hr after infection of the neurons with Adp53, expression of human p53 protein was detectable using Western blot analysis (Fig. 1*A*). Approximately half of the neurons (43%, $n = 4$) was clearly immunoreactive for human p53 at the same time. Neuronal death increased greatly during the 72 hr period after infection, with the death of ~75% of

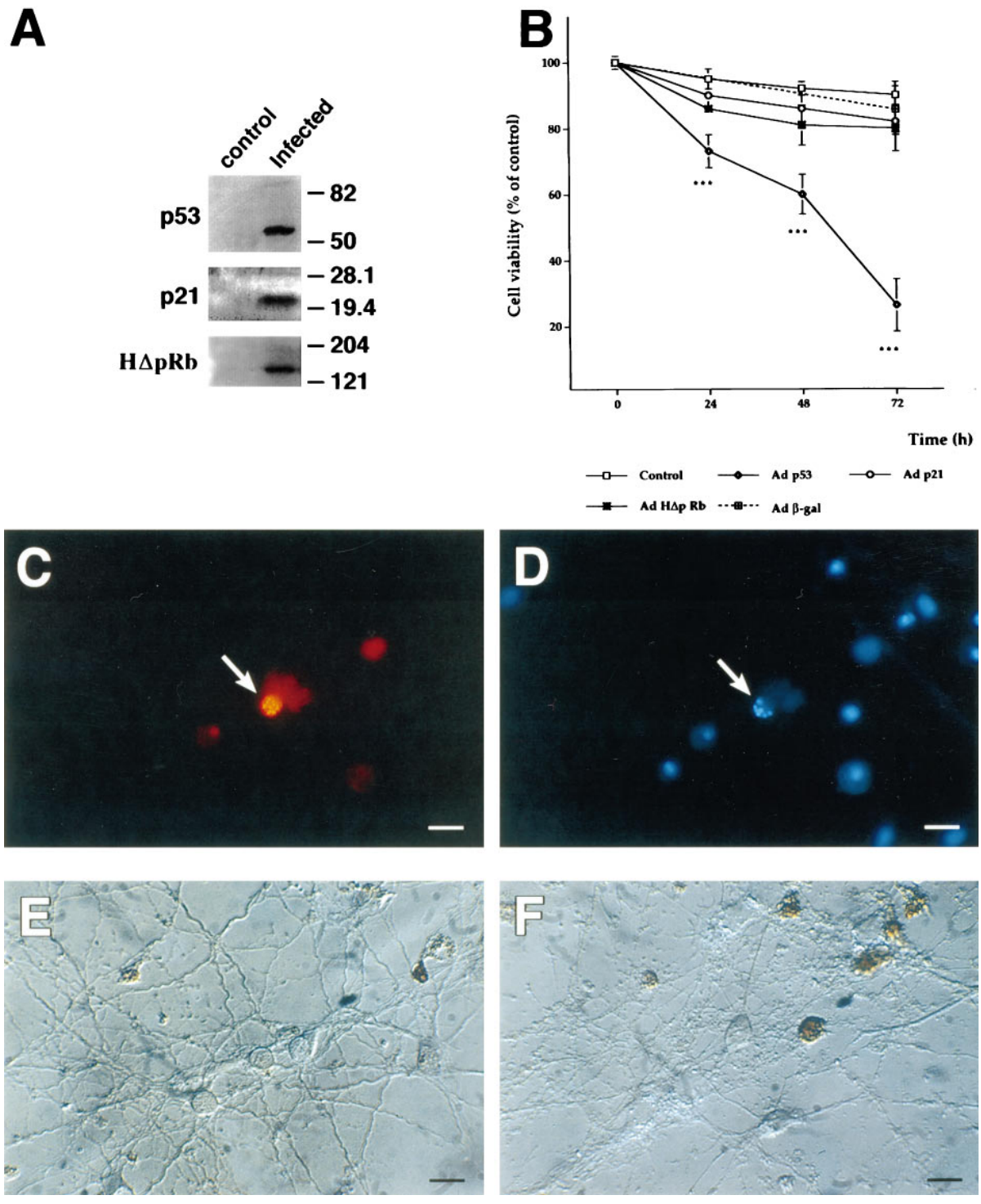

Figure 1. Overexpression of p53 induces neurotoxicity in rat hippocampal pyramidal neurons. Hippocampal cultures were infected at 7 DIV, using 100 MOI of each virus. *A*, Western blots showing overexpression of p53, p21, and HΔpRb after 48 hr of infection. Hippocampal cultures were infected on 7 DIV, using 100 MOI of each virus. Similar results were found in three separate experiments. *B*, Time course plot of cell viability after Adp53, Adp21, Adβgal, or AdHΔpRb infection of hippocampal pyramidal neuron cultures. Results represent the mean ± SEM of 12 coverslips. $^{***}p < 0.001$ versus control conditions (no virus); ANOVA and Tukey's test. *C*, *D*, Pictures showing colocalization of human p53 expression (*C*) and chromatin fragmentation measured using Hoechst 33342 (*D*, $n > 200$ cells). *E*, *F*, Cultures stained using the TUNEL technique illustrating the degree of double-stranded DNA breaks in control cultures (*E*) and 48 hr after Adp53 infection (*F*). Scale bar, 20 μm.

the neurons after 3 d. Neurons dying after the expression of p53 displayed many of the features typical of apoptosis, including cell shrinkage, nuclear condensation, and membrane blebbing. We observed that cells staining for p53 also invariably exhibited chromatin condensation as shown using Hoechst 33342 (Fig. 1*C*,*D*, $n > 200$ cells). p53 expression also produced a large increase in TUNEL staining, indicative of double stranded DNA breaks (Fig. 1*E*,*F*). After Adp53 infection (48 hr), ~80% of the neurons were

positive for TUNEL staining, whereas <5% were positive in cultures infected with a β-galactosidase-expressing control adenovirus. These results indicate that p53 expression causes postmitotic neurons to undergo apoptosis, consistent with its effects in a variety of dividing cells.

We also examined the effects of expressing two other tumor suppressor genes, the cyclin kinase inhibitor p21 and a constitutively active nonphosphorylatable form of the retinoblastoma gene product carrying an N-terminal epitope tag taken from the influenza hemagglutinin molecule (HΔpRb) (Chang et al., 1995b). The expression of p21 is of interest owing to the fact that many of the effects of p53, particularly those on the cell cycle, have been shown to be mediated by p21 (Cox and Lane, 1995; Kouzarides, 1995). In addition, pRb has also been shown to impact p53-mediated events in several circumstances (Kouzarides, 1995). A Western blot confirmed expression of both the p21 and HΔpRb proteins 48 hr after infection with adenoviruses that contained the respective cDNAs (Adp21, AdHΔpRb) (Chang et al., 1995 a,b) (Fig. 1*A*). Only a small increase in cell death was noted 72 hr after infection after expression of these two proteins (Fig. 1*B*). This small decrease in viability was comparable with that seen after overexpression of β-galactosidase (Fig. 1*B*) (Chard et al., 1995).

p53-Induced neuronal cell death uses a different pathway from that used after cell cycle arrest

The above studies demonstrate that p53 overexpression was able to induce apoptosis in postmitotic neurons. To characterize the pathway involved in this process, we compared the effects of infection with Adp53 on gene expression in two different cell types: hippocampal neurons and PC-3, a prostate carcinoma cell, (a mitotic cell line) (Kaighn et al., 1979). These latter cells do not expresses p53 normally (Isaacs et al., 1991). The levels of bax, mdm2, and p21 proteins were measured at 8, 12, and 24 hr after infection of the two cell types, using a Western blot technique. In addition, we measured bax at 48 hr in neuronal cultures. Figure 2 shows that neuronal cultures failed to show any increase in the proteins assayed at 12 hr (Fig. 2*A*) or at other time points (data not shown). mdm2 was undetectable before and after treatment. In contrast, PC-3 cells showed an increase in both p21 and mdm2, although not of bax, after p53 expression (Fig. 2*B*).

Effect of x-irradiation on hippocampal neurons in culture

X-irradiation is a commonly used mechanism for triggering the death of dividing cells by apoptosis. These effects are frequently, but not always, mediated by p53 (Strasser et al., 1996). Although it has been shown that x-irradiation can kill neuronal precursor cells, including those in the hippocampus and the external granule layer of the cerebellum (Kameyana and Inouye, 1994; Wood and Youle, 1995), little is known about the effect of x-irradiation on postmitotic neurons. Therefore, we examined the effects of x-irradiation on hippocampal pyramidal neurons in culture. Cultures at 11 d *in vitro* (11 DIV) were exposed to different doses of x-irradiation (200, 500, or 1000 cGy), and cell viability was subsequently measured at different time points (Fig. 3). There was a marked increase in cell death 6–48 hr after x-irradiation. The rate at which this occurred depended on the dose of x-irradiation used. However, 75–80% of the cells died 48 hr after x-irradiation, irrespective of dose (Fig. 3).

Dying neurons showed many of the hallmarks of apoptosis, including shrunken, irregularly shaped cell bodies and nuclear condensation. Double-stranded DNA breaks, measured by the TUNEL staining method, increased greatly after x-irradiation (Fig. 4*C*), with >80% of cells staining 24 hr after x-irradiation. Staining with the fluorescent dyes Hoechst 33342 and propidium iodide showed changes in the state of the chromatin, with many

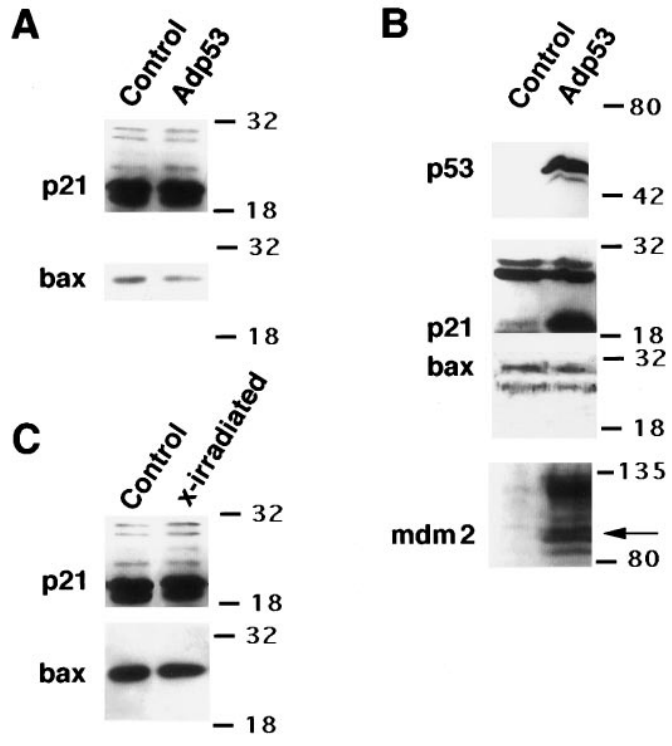

Figure 2. p53-Induced gene expression. Hippocampal pyramidal neurons (7 DIV) (*A*) or PC-3 cells (*B*) were infected with Adp53 at 100 MOI. Later (8, 12, or 24 hr), the cells were harvested. Result shown illustrate effects at 12 hr, but similar patterns were seen at other time points. Similar results were found in three separate experiments. In addition, no increase in bax was observed at a 48 hr time point in hippocampal cultures. *C*, No changes in levels of the proteins were found in extracts from x-irradiated hippocampal neurons (500 cGy; immunoblots show the 24 hr time point).

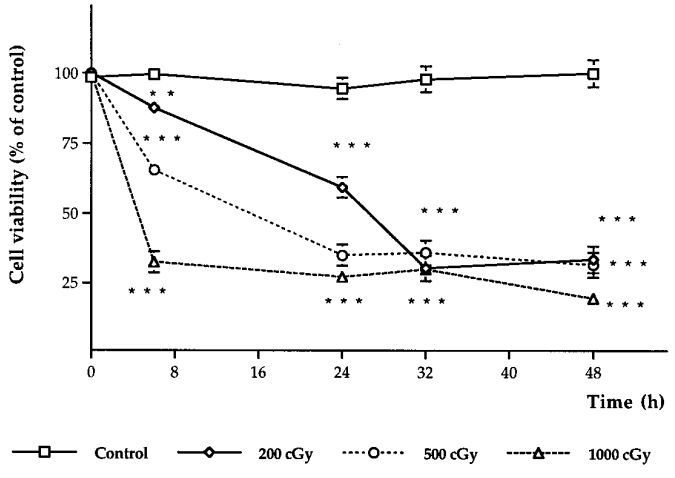

Figure 3. Time course plot of cell viability after exposure of the hippocampal pyramidal neuron cultures to 200, 500, and 1000 cGy total doses of x-irradiation. Cultures were irradiated at 11 DIV. Each point represents the mean ± SEM of 9 coverslips. $^{**}p < 0.01$; $^{***}p < 0.001$ versus non-x-irradiated control conditions; ANOVA and Tukey's test.

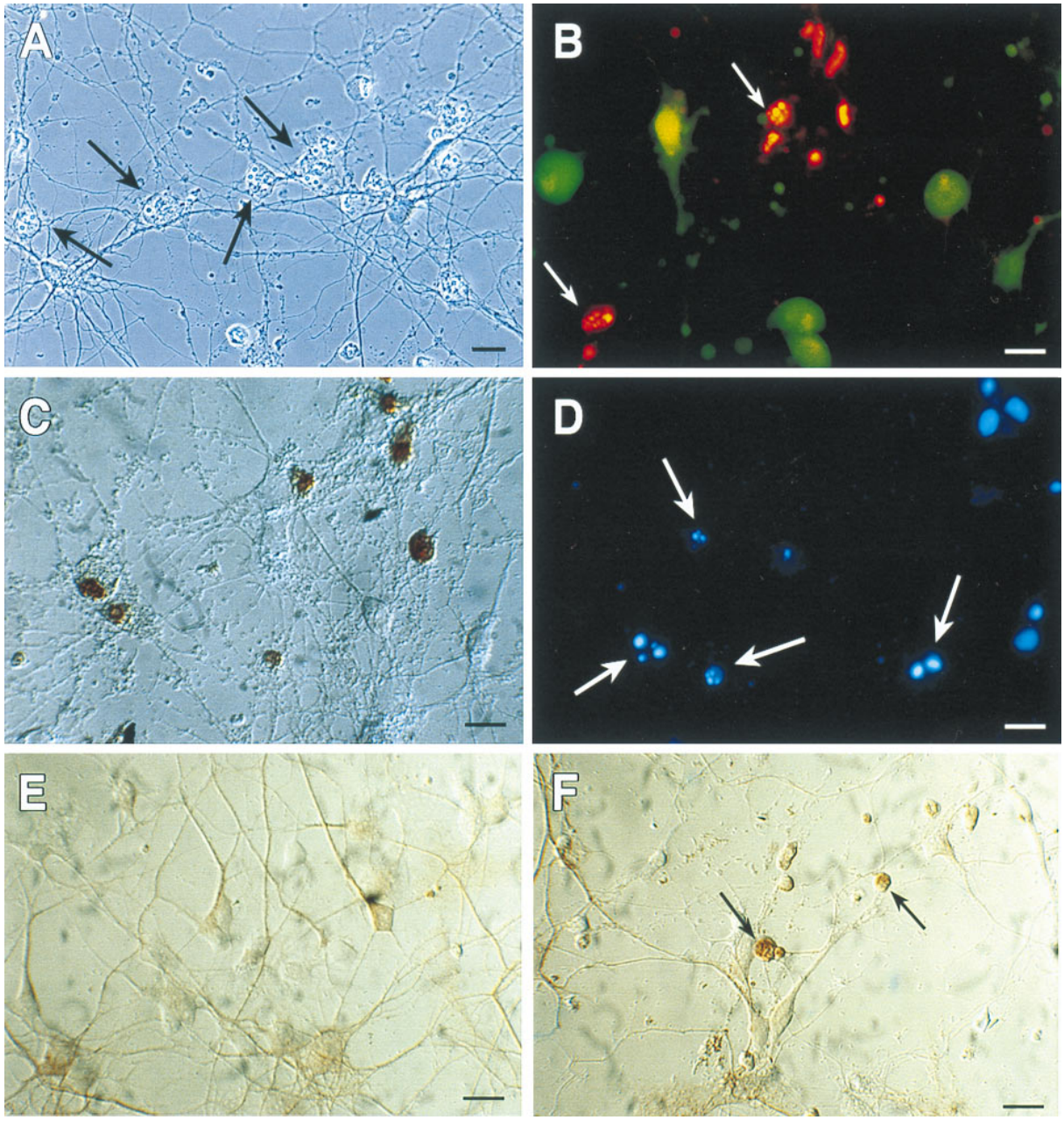

Figure 4. Apoptosis after x-irradiation of hippocampal pyramidal neuron cultures. The cultures were exposed to a 500 cGy dose of x-irradiation at 11 DIV and analyzed 24 hr later. *A*, Nomarski picture showing morphological changes ($n = 8$). *B*, Propidium iodide and fluorescein diacetate staining ($n = 8$). *C*, TUNEL staining illustrating the degree of double-stranded DNA breaks ($n = 3$). *D*, Chromatin of hippocampal nuclei from x-irradiated cultures stained with Hoechst 33342. *Arrows* in *A–D* illustrate examples of nuclei with condensed chromatin. *E*, *F*, Control (*E*) and 500 cGy irradiated hippocampal cultures (*F*) stained for p53 5 hr after x-irradiation ($n = 2$). Scale bar, 20 μm.

cells exhibiting chromatin condensation and nuclear fragmentation (Fig. 4*D*). Immunostaining for p53 was clearly observed in a small number of cells 5 hr after x-irradiation (−5%) (Fig. 4*F*), although staining was never observed in control cells (Fig. 4*E*). A correlation between p53 expression and morphological features typical of apoptosis was found in all of the p53 positive cells (Fig. 4*F*, *arrows*; n > 200). Nevertheless, and in agreement with that reported recently by Arai et al. (1996), p53 protein was not detected in the fragmented nuclei of most apoptotic cells of bodies. These observations show that x-irradiation kills hippocampal pyramidal neurons by apoptosis.

Effect of tumor suppressor genes on neuronal apoptosis

We investigated the possibility that p21 or HΔpRb expression could modify apoptosis induced by either x-irradiation or p53 overexpression. Cultures were infected 3 d before x-irradiation or Adp53 infection to allow sufficient time for maximal HΔpRb or p21 expression to occur. Overexpression of HΔpRb protected neurons from x-irradiation-induced damage (Fig. 5*B*). HΔpRb expression, however, was unable to prevent death induced by the expression of p53 when we reinfected the cultures with Adp53 (Fig. 5*A*). Death of neurons under these circumstances was not

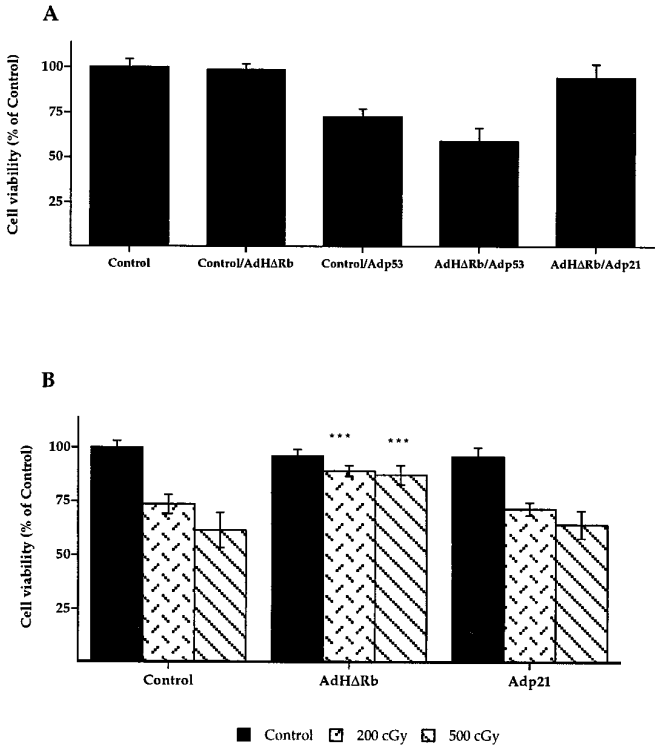

Figure 5. Effects of the overexpression of HΔpRb on p53- and x-irradiation-induced cell death. Cultures were infected with 100 MOI AdHΔpRb at DIV 7, and 3 d later were again infected with Adp53 or exposed to x-irradiation. Cell viability was analyzed 48 hr (Adp53) or 24 hr (X-irradiation) later. *A*, Overexpression of HΔpRb failed to protect the cultures against cell death induced by p53 expression (AdHΔpRb/Adp53). *B*, Overexpression of HΔpRb was able to block x-irradiation-induced death of hippocampal neurons (AdHΔpRb), whereas p21 overexpression did not (Adp21). Data represent mean ± SEM of 9 coverslips. $^{***}p < 0.01$, versus Adp53 or irradiated control conditions; ANOVA and Tukey's test.

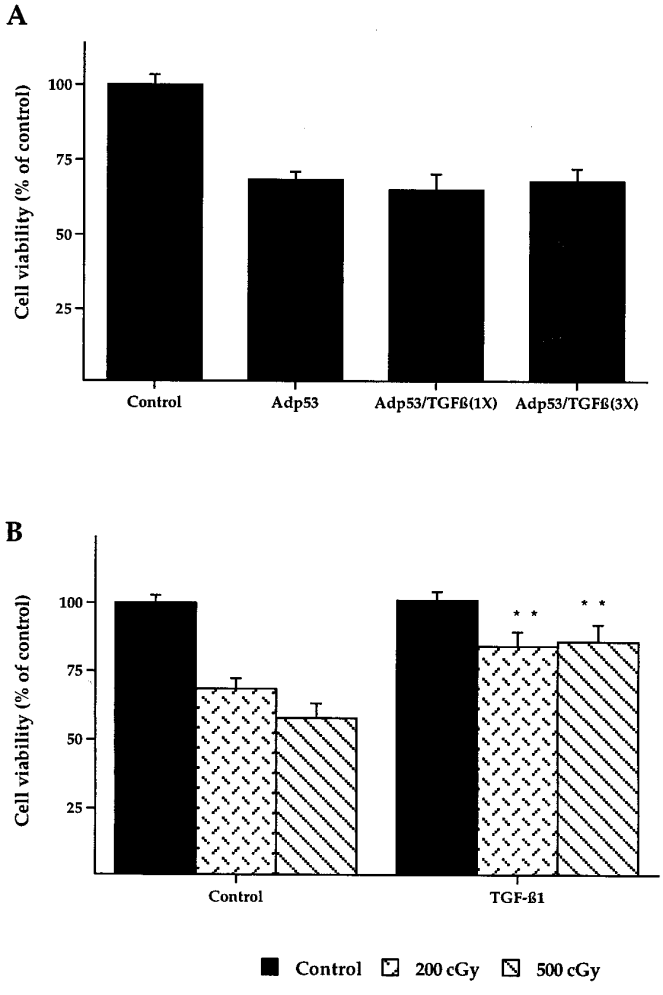

Figure 6. Effect of TGF-β1 (1 ng/ml) on neuronal death induced by either p53 overexpression or different doses of x-irradiation. *A*, 24 Hr pretreatment with TGF-β1 (Adp53/TGFβ(1X)) failed to protect the cultures 48 hr after 100 MOI Adp53 infection. Even the daily addition of the TGF-β1 for 3 consecutive days (Adp53/TGFβ(3X)) did not protect neurons 48 hr after Adp53 infection. Cultures were infected at 100 MOI, at 7 DIV. *B*, The 4 hr previous addition to the culture media of TGF-β1 (1 ng/ml) protected hippocampal pyramidal neuronal cultures against x-irradiation-induced neurotoxicity. Data represent mean ± SEM of 9 coverslips. $^{**}p < 0.01$ versus Adp53 or irradiated control conditions; ANOVA and Tukey's test.

attributable to the fact that we infected them with viruses twice. For example, if the same experiment was performed with AdHΔRb followed by Adp21, significant death did not occur (Fig. 5*B*). The overexpression of p21 was unable to protect cells from death caused by x-irradiation (Fig. 5*B*). Protein immunoblots performed with extracts from irradiated neuronal cultures did not show any increase in the levels of p21, bax, or mdm2, 8, 12 (data not shown), or 24 hr after 500 cGy (Fig. 2*C*).

Effect of TGF-β1 on x-irradiation-induced apoptosis

We have demonstrated previously that the multifunctional cytokine transforming growth factor-β1 (TGF-β1) can protect neurons from a variety of insults. In hippocampal pyramidal neurons, these effects are associated with the ability of this cytokine to upregulate the synthesis of the proteins Bcl-2 and Bcl-xL, both of which are known to oppose apoptotic death induced in many instances (Prehn et al., 1995, 1996). Treatment with TGF-β1 protected neurons against x-irradiation-induced cell death (Fig. 6*B*). However, TGF-β1 was ineffective in preventing death attributable to the overexpression of p53 (Fig. 6*A*).

DISCUSSION

It is well established that the p53 protein plays a central role in the death of many types of cells in response to DNA damage (Cox and Lane, 1995). The protein has frequently been shown to be induced in response to x-irradiation and genotoxic drugs and to produce either cell cycle arrest or apoptosis, depending on the situation (Lowe et al., 1993; Elledge and Lee, 1995; Enoch and Norbury, 1995). Both of these events can be viewed as ways of protecting cells from the consequences of faulty DNA replication.

p53-Induced cell cycle arrest and apoptosis are probably mediated by different pathways (Rowan et al., 1996). The arrest of the cell cycle appears to involve the ability of p53 to enhance the synthesis of the cyclin kinase inhibitor p21, leading to inhibition of cyclin-dependent kinases (Cox and Lane, 1995; Haffner and Oren, 1995; Kouzarides, 1995; Macleod et al., 1995). The mechanism by which p53 induces apoptosis has remained more elusive. Our data demonstrate that p53-induced apoptosis, in postmitotic neurons, does not involve p21 or require induction of the same set of target genes as those seen after cell cycle arrest, indicating that at least portions of these pathways are distinct. p53-Induced apoptosis has also been observed to occur in mice that are deficient in p21 (Brugarolas et al., 1995; Kouzarides, 1995; Attardi et al., 1996).

On the other hand, we found that p53 overexpression and x-irradiation were unable to induce other proteins such as bax, which have been widely shown to induce apoptosis in many cell types (Miyashita and Reed, 1995). This implies that p53-induced neuronal apoptosis is independent of bax, although corresponding changes in other key proteins such as bcl-2 or bcl_X could mask its effects. Notably, however, it has been reported that p53 can induce apoptosis without the transactivation of transcription (Caelles et al., 1994; Wagner et al., 1994). An important question is whether the function of p53 in the apoptosis of postmitotic cells, such as neurons, is similar to its functions on non-neuronal cells. It may be that p53 acts to ensure the fidelity of gene transcription in postmitotic cells, rather than to function as a response to replication errors as in mitotic cells.

The levels of p53 are known to increase in some neurons after a number of insults that can lead to neuronal death. These include ischemia (Chopp et al., 1992; Li et al., 1994), seizure activity (Sakhi et al., 1994; Morrison et al., 1996) and the death of dentate granule cells after adrenalectomy (Schreiber et al., 1994). p53 is also induced in replicating cells after hypoxia (Graeber et al., 1995, 1996). Because neuronal damage after ischemia or seizures is thought to involve a significant component of apoptotic death (Bonfoco et al., 1995; Charriaut-Marlangue et al., 1996a,b; Du et al., 1996), and glutamate-mediated excitotoxicity, a relationship between p53 and excitotoxicity has been suggested (Sakhi et al., 1994; Morrison et al., 1996). This relationship is supported by the observation that the AMPA/kainate receptor agonist kainic acid increases the level of p53 mRNA in certain neurons (Sakhi et al., 1994; Morrison et al., 1996). In addition, p53-deficient mice show reduced neuronal death after ischemia (Crumrine et al., 1994) and decreased neurotoxicity after kainate administration (Morrison et al., 1996). The hypothesis that p53 is active during insults to the brain such as those occurring during ischemia or treatment with certain kinds of genotoxic drugs (Wood and Youle, 1995; Enokido et al., 1996) depends on whether p53 actually produces apoptosis in postmitotic cells such as neurons. The results of the present series of experiments clearly show that this is the case, as do recent data by Eizenberg et al. (1996) using a p53 antagonist protein. Therefore, p53 may form an important link between toxic stimuli of varying types and the death of neurons.

The ability of x-irradiation to induce p53 and to produce apoptosis in hippocampal pyramidal neurons, as demonstrated in our studies, is also consistent with a role for this protein in some forms of neuronal apoptosis. This result is similar to results found in other studies that have demonstrated that x-irradiation induces apoptosis and the induction of p53 (Lowe et al., 1993). It should be noted, however, that not all types of x-irradiation-induced apoptosis involve p53 induction (Strasser et al., 1994). In addition, p53 is not involved in all forms of neuronal apoptosis. For example, the apoptosis of neurons after growth factor withdrawal or culture in low-K^+ medium presumably is not related to p53, because these phenomena occur in cells from p53-deficient mice (Davies and Rosenthal, 1994; Enokido et al., 1996). Furthermore, apoptosis during the development of cerebellar granule cells is normal in p53-deficient mice, even though these same neurons exhibit increased resistance to the effects of x-irradiation and genotoxic drugs such as methylazoxymethanol (Wood and Youle, 1995).

In our experiments, overexpression of a constitutively active nonphosphorylatable form of pRb (Chang et al., 1995b) in hippocampal cultures was found to protect against x-irradiation. This protective effect of HΔpRb is consistent with the idea that x-irradiation-induced apoptosis is a p53-mediated event, because pRb has been shown to act as an inhibitor of p53-induced cell death in several instances, giving rise to the idea that pRb has a generally antiapoptotic function (Haupt et al., 1995; Kouzarides et al., 1995; Slack and Miller, 1996). pRb-Deficient mice are not viable and die in mid- to late gestation, exhibiting defects in the hematopoietic system as well as the CNS and PNS. Massive amounts of cell death occur throughout the CNS as early as E11.5 (Lee et al., 1992). This cell death is dependent on p53 and is ameliorated in p53-deficient mice (Morgenbesser et al., 1994). pRb can overcome p53-induced apoptosis in cultured cells (Haupt et al., 1995). The mechanism by which pRb protects cells from apoptosis remains obscure. However, it may act though an interaction with other factors such as E2F1 (Wu and Levine, 1994).

The effects of TGF-β1 are consistent with a role for p53 in neuronal apoptosis. We have demonstrated previously that TGF-β1 upregulates the proteins Bcl-2 and Bcl-xL in hippocampal neuronal cultures (Prehn et al., 1995, 1996) and inhibits apoptosis in many different circumstances, including growth factor withdrawal, hypoxia, excitotoxicity, β-amyloid, and gp120 (Prehn et al., 1993, 1995, 1996; Jordán et al., 1995; Meucci and Miller, 1996). p53-Induced apoptosis can be blocked by increases in the levels of Bcl2 (Chiou et al., 1994).

It should be noted that although treatment with TGF-β1 or expression of HΔpRb effectively blocked apoptosis induced by x-irradiation, both agents were ineffective in blocking apoptosis induced by direct expression of p53. The reason for these different effects may be relate to different levels and kinetics of p53 in these two situations. Adenoviral expression of p53 produces continuous expression of the protein, and its effects may not be easy to inhibit compared with the transient expression achieved with stimuli such as x-irradiation. It is, of course, also possible that the effects of x-irradiation do not involve a p53-linked pathway.

Interestingly, some populations of neurons, such as cerebellar Purkinje cells and sympathetic neurons, constitutively express p53 at high levels (Wood and Youle, 1995; Sadoul et al., 1996). In sympathetic neurons and oligodendrocytes (Eizenberg et al., 1995), p53 is localized in the cytoplasm. Manipulations that cause p53 to be translocated to the nuclei of these cells produce apoptosis or at least chromatin condensation (Eizenberg et al., 1995; Sadoul et al., 1996). The cytoplasmic localization of p53 in these cells, together with the fact that they are normally viable, suggests additional functions for p53 in the nervous system.

Our results support the idea that p53 may act as a mediator of the apoptotic death of neurons under some conditions. If this is so, it is of interest to define the intracellular mechanism that lead to p53 induction. Traditionally, p53 induction has been associated with DNA damage; however, is this always the case? What is the link between p53 induction and ischemia, for example (Graeber et al., 1994, 1995)? One possibility is that this relates to changes in Ca^{2+} homeostasis. Indeed, large changes in Ca^{2+} homeostasis certainly occur under ischemic conditions and are believed to be important for cell death (Choi, 1988). Furthermore, Ca^{2+}-sensitive processes are believed to be involved in many instances of apoptosis, particularly those involving Ca^{2+}-dependent breakdown of DNA and proteins (Kroemer et al., 1995). Recently, a Ca^{2+} binding protein has been identified as a key element in the "programmed cell death" pathway (Vito et al., 1996). Another possibility is that some reactive oxygen radicals mediate p53-induced apoptosis; thus, increases in free radical production have been demonstrated after ischemia (Siesjo, 1989) or excitotoxic stimulation (Bindokas et al., 1996). The results reported here suggest that proteins such as p53 and pRb may be important

targets for novel therapeutic agents for combating neurodegenerative processes in several disease states.

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