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-β 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-β
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 Ca2+-Mg2+-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 Thermanox coverslips equipped with parafilm septa. 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 × 10^6 cells/dish. After 2–4 h, the medium was replaced with a serum-free defined medium (N2), and the coverslips containing the feeder glial cells were transferred to each dish of pyramidal neurons. Cytosine-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 Gy/min. Cells received a single dose of 200, 500, or 1000 Gy at 22–25°C. 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 4.8 μg/ml propidium iodide (Molecular Probes, Eugene, OR) in PBS, pH 7.4. The stained cells were examined immediately under a fluorescence microscope (Olympus, Nomarski diaphragm, 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) diluted 1:200 in blocking solution. 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 strepavidin or anti-mouse IgG (Jackson ImmunoResearchLab, West Grove, PA) diluted 1:500 and then detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit). Immuno blotso were developed by exposure to x-ray film (Eastman Kodak, Rochester, NY).

**Immunocytochemistry.** Cultured hippocampal neurons were infected using methods described previously (Chard et al., 1995). 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. A total of 100 neurons were stained per coverslip. Neurons were probed with either anti-mdm-2 (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 mouse IgG) for 1 hr. The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit). Immunoblotso were developed by exposure to x-ray film (Eastman Kodak, Rochester, NY).

**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. 1A). 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...
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. 1C,D, n > 200 cells). p53 expression also produced a large increase in TUNEL staining, indicative of double-stranded DNA breaks (Fig. 1E,F). After Adp53 infection (48 hr), ~80% of the neurons were

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.
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 (HA[pRb]) (Chan 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 HA[pRb] proteins 48 hr after infection with adenoviruses that contained the respective cDNAs (Adp21, AdHA[pRb]) (Chan et al., 1995a, b) (Fig. 1A). Only a small increase in cell death was noted 72 hr after infection after expression of these two proteins (Fig. 1B). This small decrease in viability was comparable with that seen after overexpression of β-galactosidase (Fig. 1B) (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 express 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. 2A) 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. 2B).

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 (Kameyama 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. 4C), 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.
cells exhibiting chromatin condensation and nuclear fragmentation (Fig. 4D). Immunostaining for p53 was clearly observed in a small number of cells 5 hr after x-irradiation (−5%) (Fig. 4F), although staining was never observed in control cells (Fig. 4E). A correlation between p53 expression and morphological features typical of apoptosis was found in all of the p53 positive cells (Fig. 4F, 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 H↓pRb 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 H↓pRb or p21 expression to occur. Overexpression of H↓pRb protected neurons from x-irradiation-induced damage (Fig. 5B). H↓pRb expression, however, was unable to prevent death induced by the expression of p53 when we reinjected the cultures with Adp53 (Fig. 5A). Death of neurons under these circumstances was not

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.
attributable to the fact that we infected them with viruses twice. For example, if the same experiment was performed with AdHΔpRb followed by Adp21, significant death did not occur (Fig. 5B). 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.

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. 6B). However, TGF-β1 was ineffective in preventing death attributable to the overexpression of p53 (Fig. 6A).

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 (Low 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; Haßner 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 bclX 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 adenalecctomy (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 through 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 (Chiu 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 Ca2+-homeostasis. Indeed, large changes in Ca2+-homeostasis certainly occur under ischemic conditions and are believed to be important for cell death (Choi, 1988). Furthermore, Ca2+-sensitive processes are believed to be involved in many instances of apoptosis, particularly those involving Ca2+-dependent breakdown of DNA and proteins (Kroemer et al., 1995). Recently, a Ca2+ 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
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