

Adenovirus-mediated Gene Transfer of the Tumor Suppressor, p53, Induces Apoptosis in Postmitotic Neurons

Ruth S. Slack,* Daniel J. Belliveau,* Madelaine Rosenberg,[§] Jasvinder Atwal,* Hanns Lochmüller,[‡] Raquel Aloyz,* Ali Haghghi,[§] B. Lach,^{||} Prem Seth,[¶] Ellis Cooper,[§] and F.D. Miller*

*Centre for Neuronal Survival, [‡]The Neuromuscular Research Group, Montreal Neurological Institute, and [§]Department of Physiology, McGill University, Montreal, Canada H3A 2B4; ^{||}Department of Pathology, University of Ottawa, Health Science Centre, Ottawa, Ontario K1H 8M5; and [¶]Medical Breast Cancer Section, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Programmed cell death is an ongoing process in both the developing and the mature nervous system. The tumor suppressor gene, p53, can induce apoptosis in a number of different cell types. Recently, the enhanced expression of p53 has been observed during acute neurological disease. To determine whether p53 overexpression could influence neuronal survival, we used a recombinant adenovirus vector carrying wild type p53 to transduce postmitotic neurons. A control consisting of the same adenovirus vector background but carrying the lacZ reporter expression cassette was used to establish working parameters for the effective

genetic manipulation of sympathetic neurons. We have found that recombinant adenovirus can be used at titers sufficiently high (10 to 50 multiplicity of infection) to transduce the majority of the neuronal population without perturbing survival, electrophysiological function, or cytoarchitecture. Moreover, we demonstrate that overexpression of wild type p53 is sufficient to induce programmed cell death in neurons. The observation that p53 is capable of inducing apoptosis in postmitotic neurons has major implications for the mechanisms of cell death in the traumatized mature nervous system.

PROGRAMMED cell death is an ongoing process in both the developing and the mature nervous system (for review see 38). Developing neurons have a default suicide program that predominates in the absence of target-derived neurotrophins (6, 31). In the mature nervous system, inappropriate apoptosis has been implicated in neurodegenerative diseases as well as acute neurological insults (22, 38, 42). Defining the molecular mechanisms regulating apoptosis is the first step toward effective treatment strategies for such neurological diseases.

The p53 tumor suppressor gene can induce apoptosis in a number of different cell types (for review see 12, 41). As a tumor suppressor, the primary role for p53 when induced after DNA damage is to mediate cell cycle arrest. p53-mediated growth arrest appears to be dependent on its transcriptional activation function whereby it binds to p53 consensus sequences on a number of promoters, such as that for the cyclin kinase inhibitor, p21 (WAF-1) (10, 11, 33). p21 can then mediate cell cycle arrest and prevent the propagation of damaged DNA (15, 43). The cellular response to enhanced expression of p53 varies depending on

the cell type and may lead to either growth arrest or apoptosis (1, 18, 19, 33, 41). The precise mechanism by which p53 mediates apoptosis is not well understood, but it is believed to proceed by a number of mechanisms including direct transactivation, transcriptional repression, and direct involvement in DNA cleavage (4, 12, 19, 28).

While p53 has been shown to regulate apoptosis in a number of systems involving tumor cells, we questioned whether the p53 pathway could play any role in neuronal cell death. Studies with p53-deficient transgenic mice suggest that p53 is not required for apoptosis after neurotrophin withdrawal (5, 7). Recently, however, p53 levels have been shown to be induced after acute neuronal disease (22, 36, 42). For example, enhanced expression of p53 has been observed before neuronal cell death after kainic acid treatment (36) and after focal ischemia (22). Radiation damage in cerebellar granule cells has also been shown to involve p53 mediated apoptosis (42). In light of the fact that neurons are terminally differentiated cells, a role for p53 in neuronal cell death seems inconsistent with its function as a tumor suppressor. To determine directly if p53 induction is sufficient to trigger the onset of apoptosis in postmitotic neurons, we have used a recombinant adenovirus vector to deliver p53 to cultured sympathetic neurons.

Recently, a number of studies have demonstrated that adenovirus-based vectors can effectively transduce postmitotic neurons of the central nervous system (CNS) in

Address all correspondence to F.D. Miller, Associate Professor and Coordinator, Centre for Neuronal Survival, Montreal Neurological Institute, McGill University, 3801 University, Montreal, Canada H3A 2B4. Tel.: (514) 398-4261. Fax: (514) 398-1319. e-mail: MDFM@musica.mcgill.ca

vivo, and cells derived from the CNS in vitro (20; for review see 39). While these viral vectors appear to successfully deliver reporter genes to postmitotic neurons, little is known as to how these agents may impact on the function of the recipient cell. If adenovirus-derived vectors are to be used to define the molecular pathways regulating apoptosis, their influence on the biochemistry and physiology of the neuron must first be understood. In the present study we evaluate the efficacy of adenoviral vectors as gene transfer agents to sympathetic neurons, as well as define the parameters within which they can be effectively used. To this end, a recombinant adenovirus carrying the lacZ reporter gene inserted in the deleted E1 region (2) was used to transduce neurons from the superior cervical ganglia in vitro. Examination of infectivity and cytotoxicity, as well as cell physiology and cytoarchitecture, indicated that such adenovirus recombinants have the potential to serve as effective gene transfer agents to sympathetic neurons. Then, working within the parameters we have defined, we introduced a wild-type human p53 expression cassette using the same vector backbone. These studies indicate that overexpression of p53 mediates apoptosis in cultured sympathetic neurons. The demonstration that p53 is sufficient to induce apoptosis in postmitotic neurons has major implications for the mechanisms of cell death in the traumatized mature nervous system.

Materials and Methods

Viral Vectors

The adenoviral recombinant carrying an *Escherichia coli* β -galactosidase expression cassette with a cytomegalovirus promoter (Ad5CA17LacZ) was kindly provided by Dr. Frank Graham (McMaster University, Hamilton, Ontario). The recombinant virus was generated by the cotransfection of PCA14(lacZ) and pJM17 as previously described (2). This E1- and E3-deleted virus contained the reporter gene in the E1 region. The recombinant adenovirus carrying wild-type human p53 was constructed according to the method of Graham (2) and has been recently described (18). Results obtained were verified with a second preparation of adenovirus vector conferring p53 expression (Adwtp53), kindly provided by Dr. F. Graham (1). Recombinant adenoviruses were amplified on 293 cells, a human embryo kidney cell line, expressing the adenovirus type 5 E1A and E1B proteins (14). These vectors were harvested from cell lysates and used directly or further purified on CsCl gradients according to Graham and Prevec (14). Infectious titer was determined by plaque assay on 293 cells as previously described (14). When comparing the effects of adenovirus-mediated p53 vs β -galactosidase overexpression, we ensured that observations were not attributable to differences in viral preparations by verifying that: (a) virus preparations being compared were purified in an identical manner; (b) several preparations of each virus were examined; (c) particle content (a potential source of cytotoxicity) was in a similar range; and (d) results were reproduced with two different adenovirus recombinants for both lacZ and p53. Particle content was determined by optical density at 260 nm according to standard procedures (14). The ratio of infectious titer to particle content routinely obtained in our laboratory is $\sim 1/100$. The measurements for the lacZ and p53 adenovirus recombinants were found to be in the same range, at 1/110 and 1/120, respectively.

Previous results have indicated the emergence of E1-containing, replication-competent virus contamination in stocks of replication-defective adenovirus after serial passage (24). To verify the purity of virus stocks, both PCR and Southern blot analysis for the detection of any contaminating E1-containing virus were carried out. For PCR analysis, recombinant viral DNA was extracted and PCR amplification was carried out with primers specific for the E1 region (24). DNA derived from wild-type virus was used as a positive control. When wild-type contamination was detected, results were confirmed by Southern blots in which the wild-type and recombinant viral DNAs were probed with fragments corresponding to the E1 and E2 region of the viral genome (24). For pure preparations,

hybridization with a probe for the E1 region should reveal a positive signal in wild-type only, while the probe for E2, a region present in both recombinant and wild-type virus, should produce a single band indicating a pure population of virus in both DNA preparations. If traces of wild-type contamination were detected, recombinant viruses were further plaque purified according to Graham and Prevec (14). All vector preparations used for experiments were free of wild-type contamination.

The HSV vector (RH105) was constructed by Ho and Mocarski (16). This recombinant virus carries the *E. coli lacZ gene* inserted in the *TK gene* upstream of the immediate early promoter ICP4. The disrupted *TK gene* renders the virus replication incompetent in postmitotic cells such as neurons (3, 8, 23, 37). The virus was propagated on Vero cells until a 100% cytopathic effect was observed, after which time cells were freeze thawed and sonicated on ice to release virus particles. Large cell debris was removed by centrifugation at 1,800 g for 10 min. The resulting supernatant was then layered on a 25% sucrose cushion in PBS and centrifuged at 70,000 g for 18 hours. The pellet containing recombinant herpes virus was resuspended in PBS and titered on Vero cells. The absence of wild-type virus was confirmed by X-gal staining of plaques generated on Vero cells.

Multiplicity of infection (MOI)¹ was calculated based on titration on 293 cells for adenovirus-based vectors and on Vero cells for the HSV RH105 vector, and represents the number of plaque-forming units added per cell.

Cell Culture

Mass cultures of purified sympathetic neurons were prepared according to the procedure of Ma et al. (25). Superior cervical ganglia were removed from newborn Sprague-Dawley rat pups (Charles River Breeding Laboratories, Inc., Wilmington, MA) and collected in L15 medium without sodium bicarbonate. The ganglia were washed in PBS (pH 7.4) and treated with 0.1% trypsin (Calbiochem-Behring Corp., San Diego, CA) at 37°C for 20 min, followed by treatment with DNase (10 μ g/ml; Sigma Chemical Co., St. Louis, MO) for 2 min. Ganglia were triturated and passed through a 40- μ m mesh (Falcon Labware, Oxnard, CA) to yield a single cell suspension. After centrifugation in a clinical centrifuge, the pellet was resuspended in L15 medium supplemented with sodium bicarbonate (30 mM), vitamin C (1 μ g/ml), cytosine arabinoside (10 μ M), 3% rat serum, and 50 ng/ml NGF (Cedarlane Laboratories, Ontario, Canada). Cells were plated at a density of 100,000 cells per ml medium on rat tail collagen-coated tissue-culture dishes. As previously stated (25), these cells are essentially free of nonneuronal cells. Cultured neurons were allowed to mature and to extend processes for 3 to 5 d before viral infection. For viral infection, medium was removed and replaced with 25% of the usual volume containing the appropriate titer of virus. Cells were incubated for 1 h and dishes were rocked every 15 min, after which time the remaining 75% vol of medium was added to each dish. For long-term cultures, medium was changed every 3 d.

Detection of β -Galactosidase-positive Cells

Staining for expression of the β -galactosidase marker gene was performed for the times described in the figure legends. Cells were fixed with 0.2% glutaraldehyde in PBS (pH 7.4) for 15 min at 4°C. After two washes with PBS, cells were incubated for 18 h in X-gal stain (2 mM MgCl₂, 1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆ in PBS (pH 7.4). To estimate the percentage of cells that were infected, the total cell number and lacZ-positive cells were counted in five random fields. The data were expressed as the average of two separate experiments with error bars representing the range. The number of cells per field was 125 ± 50 for experiment 1, and 200 ± 59 for experiment 2.

Cell Survival Assay

Neurons were seeded at a density of 5,000 cells per well in 48-well tissue-culture dishes. Neurons were infected with varying titers of either adenovirus or HSV-1 vectors and maintained for 2 to 10 d, as indicated in the text, for the assessment of cell survival. Cell viability was measured by the metabolic conversion of a tetrazolium salt into formazan salt according to the CellTiter 96 Assay Kit (Promega, Madison, WI).

1. *Abbreviations used in this paper:* β -gal, β -galactosidase; HSV, herpes simplex virus; MOI, multiplicity of infection; MTT, 3-[4,5-dimethylthiozole-2-yl]-2,5-diphenyl tetrazolium bromide; SCG, superior cervical ganglia.

Electron Microscopy

Sympathetic neurons were infected with the recombinant adenovirus Ad5CA17LacZ at titers of 0, 10, 50, 100 and 500 MOI. After 7 d in culture, cells were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h. In some experiments, neurons were infected with Adwtp53 at 50 MOI for 2 d, and fixed in the same manner. After initial fixation, samples were washed three times in 0.1 M sodium cacodylate buffer, for 15 min each wash. Specimens were postfixed on ice in 1% osmium tetroxide for 90 min, dehydrated in ascending concentrations of ethanol and acetone, and embedded in Epon-Araldite. Thin-sections were cut, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7100; Hitachi Ltd., Tokyo, Japan). Three separate grids containing 40 to 60 cells were examined for each specimen.

Electrophysiology

Recombinant adenovirus-infected and control SCG neurons were voltage clamped using whole-cell patch recording technique to measure outward potassium (K) currents (27). Briefly, postnatal day 1 superior cervical ganglia (SCG) neurons cultured for 3 d, were infected with Ad5CA17LacZ at an MOI of 50 for 24 h, and allowed to survive for an additional 6 d (a total of 7 d) before electrophysiological recordings. The cells were fixed (2% paraformaldehyde/2% glutaraldehyde) immediately after recording and stained with X-gal to detect β -galactosidase (β -gal) activity. Only cells that exhibited β -galactosidase activity were considered to be infected neurons. Results presented in Fig. 5 are the average of recordings obtained from six cells for the adenovirus-infected samples and five cells for controls.

The total outward current in SCG neurons is made up of three voltage-gated currents that differ in their kinetic and voltage-dependent properties: a non-inactivating current (IK); a fast transient A-type current (IA); and a small slow transient A-type current (IAs) (26). By holding the membrane at different potentials, we were able to selectively activate one or two of the currents, and thus characterize individual currents by subtraction techniques. Briefly, the membrane was held at a potential (-10 or -20 mV) where depolarizing steps evoked only IK. The IK currents were subtracted from total currents (IA+IK+IAs) evoked by steps to the same depolarizing potentials from a more negative potential, -90 mV, to isolate the A currents. For measurement of the current density (pA/pF), IA and IK current amplitudes were determined from the current evoked by a voltage step to +30 mV after each current was isolated from the other two, and divided by the cell capacitance (pF). Cell capacitance (pF) was obtained by integrating the capacity current evoked by a 10-mV hyperpolarizing voltage step, and then dividing this current by the voltage step.

Voltage steps were delivered by a computer-controlled stimulator. The software for stimulation, data acquisition, and analysis was written by Mr. A. Sherman (Alembic Inc., Montreal, Quebec). Membrane currents were filtered with an amplifier (EPC-7; List Biological Laboratories, Inc., Campbell, CA), sampled at 5 kHz, displayed, and stored online. For all experiments, the durations of the voltage steps were 125-ms steps.

All experiments were done at room temperature (21–24°C). The pipettes were filled with intracellular media (5 mM NaCl, 50 mM potassium acetate, 65 mM KF, 1 mM MgCl₂, 10 mM Hepes, (pH 7.4, adjusted with KOH), 10 mM EGTA, 0.5 mM CaCl₂); the pipette current was balanced to zero with the pipette immersed in the bathing solution. The neurons were continuously superfused with extracellular solution (140 mM choline Cl, 2 mM NaCl, 5.4 mM KCl, 0.4 mM CaCl₂, 0.18 mM MgCl₂, 10 mM Hepes, pH 7.4, adjusted with NaOH, 5.6 mM glucose, 0.5 mM tetrodotoxin [TTX; Sigma Chemical Co.], 1.5 mM CoCl₂, and pH 7.3–7.4) at a rate of 0.5 ml/min during the recording session. The extracellular solution included pharmacological agents to block inward sodium and calcium currents, and calcium-dependent currents as previously described (26).

Western Analysis

For the detection of p53, cells were harvested in lysis buffer (40) 30 and 48 h after infection with a titer of 50 MOI. Protein was separated on a 10% acrylamide gel and transferred to a nitrocellulose membrane. After blocking for 2 h in 5% skim milk, filters were incubated in a mouse mAb directed against murine and human p53 (1:10) (Ab-1; Oncogene Science, Cambridge, MA) overnight at 4°C. After five washes in TBST (.15 N NaCl, 20 mM Tris, pH 8.0, 0.1% Tween-20) (5 min each), filters were incubated for 1 h at 25°C in a goat anti-mouse secondary antibody conjugated to HRP. Filters were again washed five times in TBST for 5 min each wash. Blots were developed by the enhance chemiluminescence system

(ECL; Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Immunofluorescence

For immunofluorescence detection of human p53 delivered by adenovirus vectors, specimens were fixed for 5 min in methanol/acetone (1:1) and allowed to air dry for 5 min. After rehydration, cells were blocked in PBS containing 3% goat serum. A mouse mAb specific for an amino-terminal epitope of human p53 (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The primary antibody was diluted in this same blocking solution (1:50) and incubated on coverslips overnight at 4°C. After three washes in PBS, a goat anti-mouse secondary antibody conjugated with Cy3 (diluted at 1:2000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied to coverslips and incubated for 1 h at 25°C. After three washes in PBS, coverslips were mounted in glycerol and examined with a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NJ).

Isolation of Fragmented DNA

To examine DNA fragmentation, 10⁶ neurons were seeded on a 60-mm dish under the usual culture conditions. Cells were infected with recombinant adenovirus 3 d after plating and were harvested 48 h after infection. Cells were harvested, washed once with PBS, and used for DNA isolation as described previously (40). 1.2 ml lysis buffer was added to 100 μ l of cells suspended in TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Lysis was allowed to proceed at room temperature for 15 min after which the lysate was centrifuged for 15 min at 12,000 rpm. The gelatinous pellet was removed with a pipette, and the supernatant was digested with 100 μ g/ml RNase A at 37°C for 30 min. The DNA was then precipitated by adding an equal volume of 100% ethanol and NaCl, such that the final concentration was 0.5 M. After centrifugation, the pellet was washed with 70% ethanol and resuspended in 50 μ l of TE buffer. Fragmented DNA was end labeled with [³²P]dCTP using Klenow (Promega) for 15 min at room temperature. DNA ladders were resolved by running end-labeled DNA on a 2% agarose gel using a 100-bp ladder as a standard.

TUNEL Staining

To assay apoptosis immunohistochemically, terminal transferase was used to visualize fragmented DNA (TUNEL staining). Parallel cultures were infected with Adwtp53 or Ad5CA17lacZ at 50 MOI. After 72 h cells were fixed in acetone/methanol (1:1) for 10 min at -20°C. 50 μ l of a cocktail consisting of 1.0 μ l biotin dUTP (catalogue 109307; Boehringer Mannheim Biochemicals, Indianapolis, IN), 1.5 μ l terminal transferase (catalogue M187/1; Promega), 20 μ l of 5 \times terminal deoxy-transferase buffer (Promega), and 78 μ l distilled water was added to each coverslip. After a 1 h incubation at 37°C, coverslips were washed three times in PBS, pH 7.4, and once in TBS, pH 8.0, to stop the reaction. Coverslips were incubated with a streptavidin Cy3 (Jackson ImmunoResearch Laboratories, Inc.) diluted at 1:2,000 for 30 min. After three 5-min washes in PBS, samples were mounted in glycerol and examined with a Zeiss Axioplan microscope.

Results

Gene Transfer Efficiency of Recombinant Adenovirus vs Herpes Simplex Virus in Sympathetic Neurons

To determine the most effective and nontoxic gene transfer vector for postmitotic sympathetic neurons, we initially conducted parallel studies with the adenovirus vector, Ad5CA17LacZ, and the herpes simplex virus-1-based vector, RH105 (16), both of which express the *Escherichia coli* β -galactosidase (lacZ) reporter gene. Pure cultures of neonatal sympathetic neurons were infected with replication-defective viruses of both types at similar titers and, 48 h after infection, were stained with X-gal to visualize transgene expression. These experiments demonstrate that both herpes simplex virus (HSV)-and adenovirus-derived vectors are able to transduce sympathetic neurons in vitro (Fig. 1). Closer examination of lacZ staining and

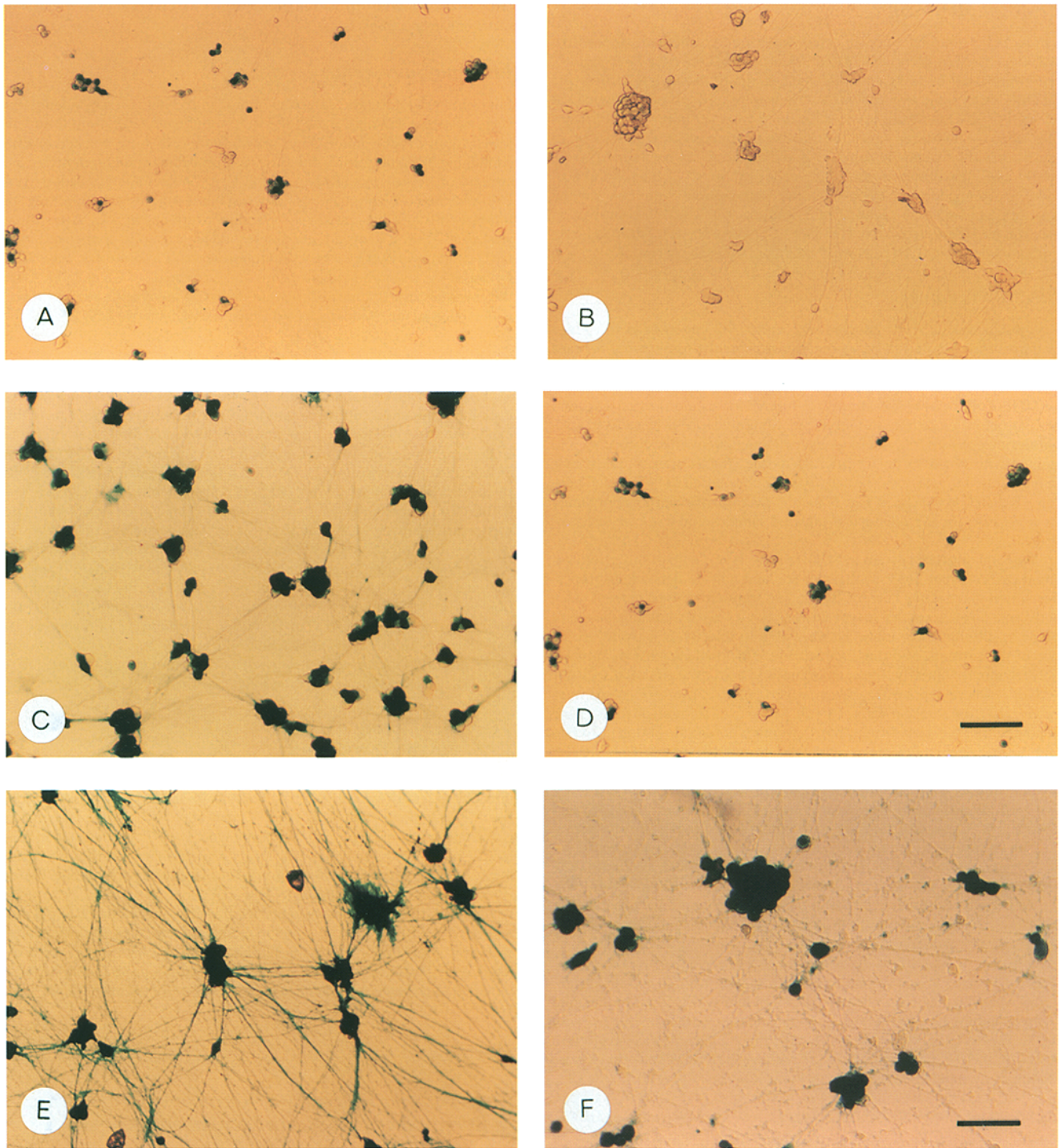
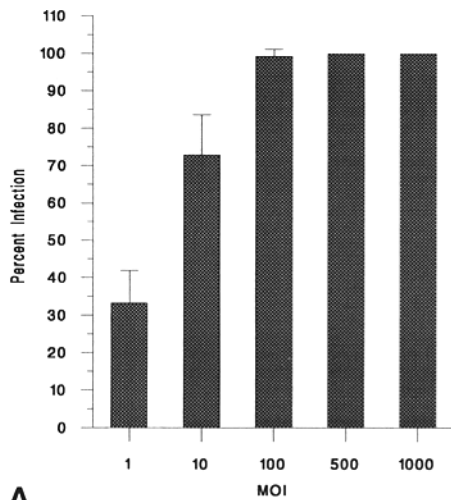


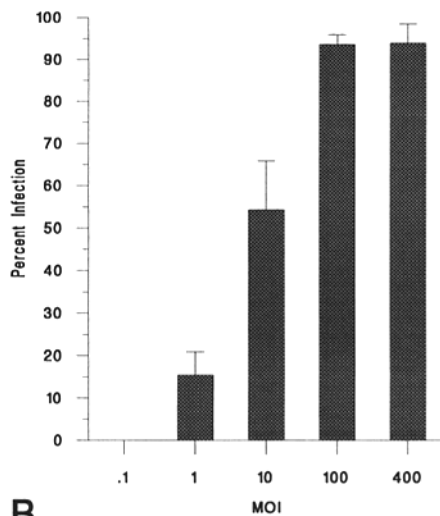
Figure 1. Morphology of cultured sympathetic neurons after viral infection. Sympathetic neurons were isolated from P0 rat pups and cultured in 6-well dishes in medium containing 50 ng/ml NGF. At 72 h medium was removed and replaced with 25% of the medium containing virus. After 1-h incubation, the remainder of the medium was added to each well, and morphology was examined after 48 h. (A, C, and E) Cells infected with adenovirus at the following MOI: A, 1; C, 10; E, 100. (B, D, and F) Cells infected with herpes virus at the following MOI: B, 1; D, 10; F, 100. Bars: (D) 200 μ M; (F) 80 μ M.

cell morphology, however, indicated clear differences in the efficacy of these two vectors. At titers of 1 MOI, adenovirus vector infection led to a higher proportion of lacZ-positive neurons (Fig. 1, A and B). At MOIs of 10, virtually all of the adenovirus vector-infected neurons expressed the transgene, whereas many neurons in the HSV-1-infected

cultures were negative (Fig. 1, C and D). At the highest titer of 100 MOI, neurons infected with adenovirus recombinants appeared normal morphologically, exhibiting no indication of cytotoxicity, and transgene expression was high enough that both cell bodies and processes were stained (Fig. 1 E). In contrast, neurons infected with the



A



B

Figure 2. Infectivity comparison between recombinant adenovirus and HSV-1. Sympathetic neurons were isolated from newborn rat pups and cultured in 6-well dishes in medium containing 50 ng/ml NGF. At 72 h cells were infected with virus and infectivity was assayed by X-gal staining after 48 h. (A) Neurons infected with recombinant adenovirus; (B) neurons infected with recombinant HSV-1. To estimate the percentage of cells that were infected, the total cell number and lacZ-positive cells were counted in five random fields. The data are expressed as the average of two separate experiments with error bars representing the range. The number of cells per field was 125 ± 50 for experiment 1, and 200 ± 59 for experiment 2.

HSV vector at 100 MOI displayed severe degeneration of neuritic processes within 48 h (Fig. 1 F).

Quantitation of the number of lacZ-positive cells in sister cultures of sympathetic neurons infected with these two viral vectors confirmed these qualitative conclusions (Fig. 2). While ~30% of sympathetic neurons were lacZ positive after recombinant adenovirus infection at titers of 1 MOI (Fig. 2 A), only 10% were transduced by the HSV vector at a similar titer (Fig. 2 B). At 10 MOI, transduction

efficiency was again higher with adenovirus than with the HSV-1 vector, with 75% and 50% lacZ-positive cells in the sister cultures, respectively (Fig. 2). Titers of ≥ 100 MOI with either vector resulted in transduction of $>95\%$, but remarkable differences in cytotoxicity became evident. We therefore assessed cell survival after infection with these two different vectors.

Neuronal Survival After Recombinant Virus Infection

To assess potential cytotoxicity in response to viral infection, sympathetic neurons were infected with varying titers of the adenovirus Ad5CA17LacZ or the HSV-1 RH105 vectors, and mitochondrial function was assayed 10 d later (Fig. 3). At 1 MOI, there was no significant difference in cytotoxicity between cells infected with recombinant adenovirus and those infected with recombinant HSV-1. However, as titers were increased, a sharp decrease in cell viability was evident; at 10 MOI, 90% of the neurons in the adenovirus vector-infected cultures remained alive, whereas only 45% of those in the HSV-infected cultures were alive. When these values are corrected for infectivity, it appears that almost all cells transduced with the HSV vector were lost after 10 d in culture (e.g., at 10 MOI HSV, 55% of cells were infected and 55% of cells were lost). In contrast, when adenovirus vector-infected neurons are corrected for infectivity data (i.e., 75% infected/10% lost), only 13% of infected cells were lost 10 d after infection at 10 MOI. A more striking difference appeared at 50 MOI; at this level of infection, the majority of the neurons in the HSV-infected cultures were lost (90%), while in adenovirus vector-infected cultures, only 15% of neurons were lost. It appears therefore that HSV-1 has a relatively narrow effective range, and titers necessary to transduce

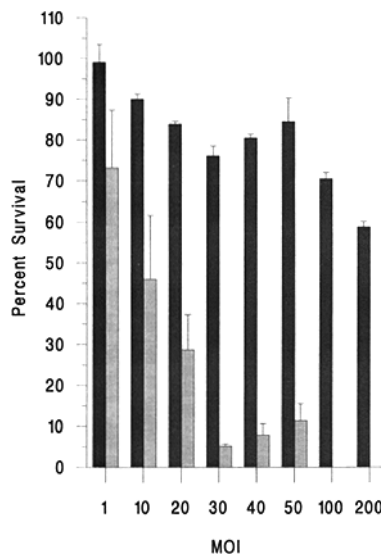


Figure 3. Comparison of neuronal viability after infection with recombinant adenovirus and HSV-1. Sympathetic neurons were isolated from newborn rat pups and infected with either recombinant adenovirus (black bars) or recombinant HSV-1 (hatched bars) after 72 h in culture. Cell survival was measured by the metabolic conversion of the tetrazolium salt into a formazan salt according to the Cell Titer 96 Assay Kit 10 d after infection. Bars represent the mean of three separate samples, with error bars representing the standard error.

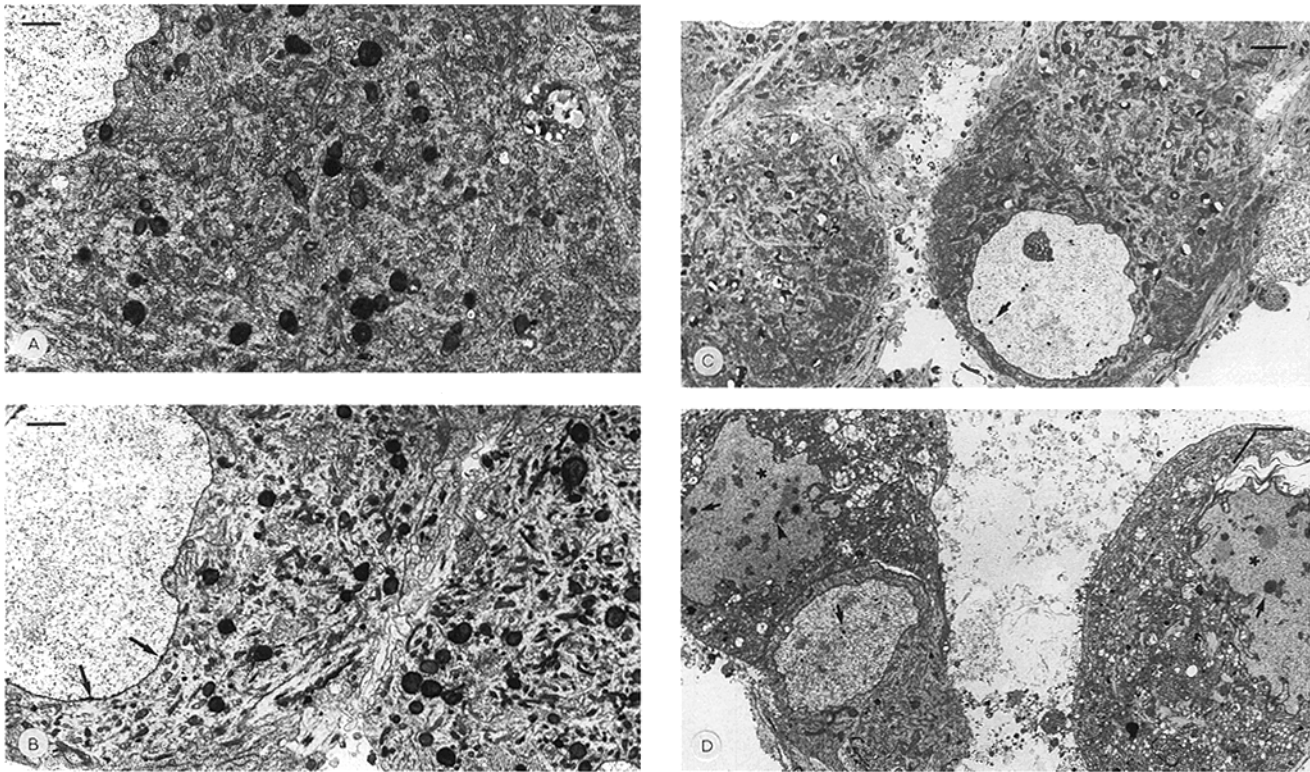


Figure 4. Changes in cytoarchitecture of sympathetic neurons after infection with recombinant adenovirus. Sympathetic neurons were infected with Ad5CA17LacZ after 72 h in culture and were fixed for EM 7 d later. Cells were harvested in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Specimens were processed for EM as described. (A) Uninfected sympathetic neuron showing normal cytoarchitecture. (B) Neurons after infection with 10 MOI adenovirus. Cells are indistinguishable from controls except for a number of small electron-dense inclusions found in the nucleus (arrows). (C) Neurons after infection at 50 MOI adenovirus exhibited normal cytoarchitecture with electron-dense nuclear inclusions. (D) Neurons infected at 100 MOI exhibited significant deterioration of cytoarchitecture. Cells exhibited chromatin disintegration (asterisks), very large electron-dense inclusions (arrows), and aggregates of filaments in nucleus (arrowhead). Bars: (A) 0.3 μm ; (B) 0.5 μm ; (C and D) 0.7 μm .

>75% of the cell population exhibit severe cytotoxic effects. The results indicate that recombinant adenovirus, at titers of 10 to 50 MOI, can transduce >70% of cells with minimal cytotoxicity up to 10 d. All further experimentation therefore was carried out with adenovirus-based vectors.

Cytoarchitecture of Transduced Neurons

While cells appeared normal and continued to survive after infection with adenoviral recombinants, EM was carried out at 7 d after infection to determine if the presence of a nonlytic virus carrying the reporter gene caused ultrastructural changes in surviving neurons. We first examined cells infected at the lower range of 10 MOI, which resulted in the transduction of $\sim 70\%$ of cells. The ultrastructure of these cells in general was indistinguishable from controls (Fig. 4 A); cells exhibited normal cytoplasm with healthy intact organelles, as well as nuclei containing normal chromatin (Fig. 4 B). In some cells very small electron-dense inclusions (Fig. 4 B) were found in nuclei of otherwise healthy cells. At 50 MOI, cell organelles remained intact and nuclear structure appeared normal, although slightly more nuclear inclusions were visible (Fig. 4 C).

At higher concentrations of adenovirus vector where significant cytotoxicity was evident, as assayed by mitochondrial function, nuclear abnormalities were readily ap-

parent (Fig. 4 D). These included large electron-dense inclusions as well as an accumulation of filamentous aggregates that are commonly found in neurons undergoing degeneration. At high titers some neurons exhibited disintegration of chromatin (Fig. 4 D). Ultrastructural examination revealed no features characteristic of apoptosis even at the highest titers examined (500 MOI) relative to uninfected controls. These results indicate that recombinant adenovirus can be used at titers that infect the majority of cells without provoking adverse cytological changes. Vector concentrations, however, should be carefully controlled, as excessive infection rates result in deterioration of nuclear structure.

Electrophysiological Function of Neurons Infected With Recombinant Adenovirus

Although cells infected with 10 to 50 MOI of recombinant adenovirus were normal with respect to mitochondrial function and cytoarchitecture, we determined whether the presence of vector modified neuronal function in any way by characterizing the electrophysiological properties. Specifically, we examined K currents on sympathetic neurons infected with Ad5CA17LacZ at 50 MOI for 7 d using whole-cell patch clamp. Previously, we have shown that the total outward current in uninfected sympathetic neu-

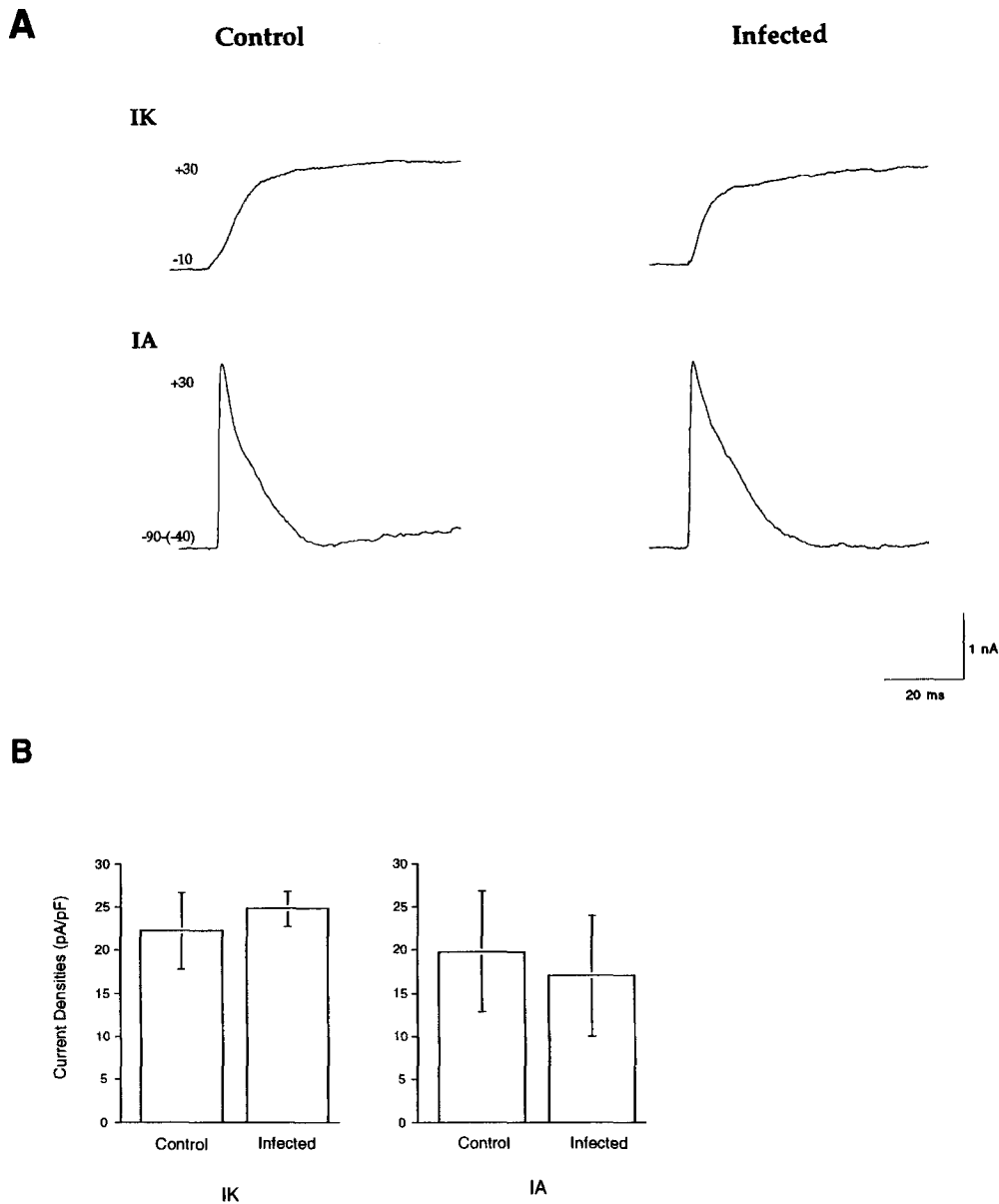


Figure 5. Voltage-gated K currents on adenovirus-infected, β -galactosidase-expressing sympathetic neurons. (A) Voltage-gated K currents on sympathetic neurons infected at 50 MOI Ad5CA17LacZ for 7 d relative to control, uninfected sympathetic neurons. (Upper panel; IK) Depolarizing voltage steps from a V_h of -10 mV activate only a slowly activating, non-inactivating current (IK). IA (lower panels) was isolated by subtracting currents from V_h of -40 mV (IK+IAs) from the corresponding currents evoked from a V_h of -90 mV (IK+IAs+IA). (B) K current densities are not altered by adenovirus vector infection: current density distribution of IK and IA for control and Ad5CA17LacZ-infected SCG neurons. Current densities (pA/pF) were measured as the peak isolated K current (pA) divided by the membrane capacitance (pF). Only SCG neurons from which electrophysiological recordings were obtained and which exhibited β -gal activity were considered to be infected neurons ($n = 6$ for β -galactosidase-positive neurons; $n = 5$ for control neurons).

rons is made up of three voltage-gated currents that differ in their kinetic and voltage-dependent properties: a non-inactivating current (IK); a fast transient A-type current (IA) that inactivates with a time course of 10–30 ms; and a slow transient A-type current (IAs) that inactivates with two components, one with a time constant of 100–300 ms, and the other with a time constant of 1–3 s (26). In sympathetic neurons expressing β -galactosidase 7 d after infection, the current densities (pA/pF) for IK and IA were found to be similar to those previously reported for cultured SCG neurons (27) and not significantly different from those of control, uninfected SCG neurons (Fig. 5). Neurons infected at 50 MOI with recombinant adenovirus therefore appear to function normally for up to 7 d.

Overexpression of p53 Induces Apoptotic Cell Death in Postmitotic Neurons

These results indicated that adenovirus vectors could be

used to genetically manipulate primary neurons within controlled parameters. We therefore used this approach to determine whether overexpression of p53 was sufficient to induce programmed cell death in postmitotic neurons. A recombinant adenovirus [Adwtp53] carrying a wild-type p53 expression cassette on the same pJM17 vector backbone as the lacZ reporter gene was used for these studies. Initially, to determine if adenovirus-mediated delivery of the human wild-type p53 could lead to stable overexpression in cultured sympathetic neurons, cells were infected at 50 MOI with recombinant adenoviruses carrying either human p53 [Adwtp53] or lacZ [Ad5CA17LacZ] and immunostained with an antibody specific for human p53. As predicted, neurons infected with Ad5CA17LacZ were not immunoreactive for human p53 (Fig. 6 A), while those infected with Adwtp53 exhibited strong nuclear staining in >80% of cells (Fig. 6 B).

To determine more precisely the extent of p53 overexpression relative to endogenous levels, transduced sympa-

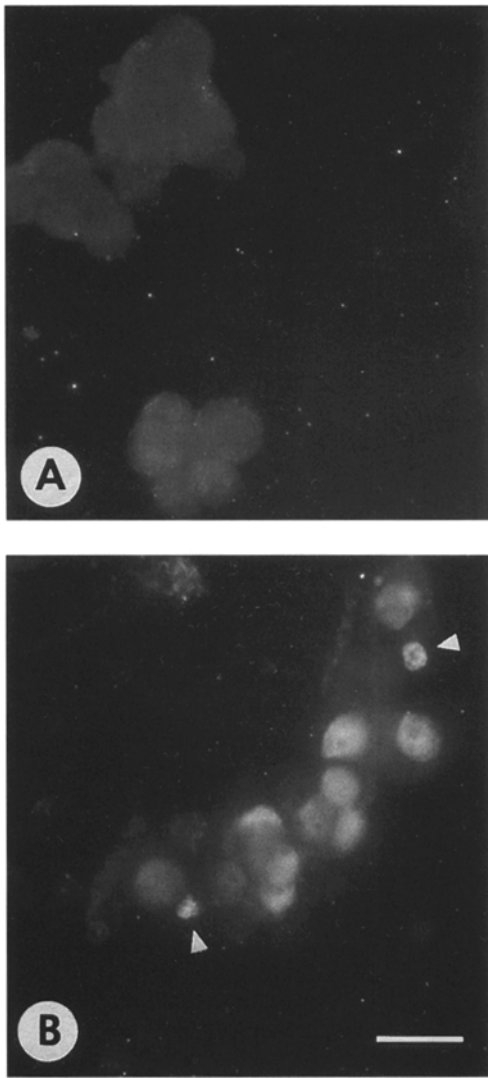


Figure 6. Human p53 overexpression is localized to the nucleus of sympathetic neurons. 3 d after plating on coverslips, sympathetic neurons were infected with recombinant adenovirus encoding β -galactosidase (AdCA17lacZ) (A), or human wild type p53 (Adwtp53) (B). 48 h after infection, cells were fixed in acetone/methanol (1:1) for 5 min and stained with an mAb (DO-1) that reacts specifically with human p53. Arrows point to pyknotic nuclei overexpressing human p53. Bar, 20 μ M.

thetic neurons were harvested at 30 and 48 h after infection with 50 MOI of Ad5CA17LacZ or Adwtp53, and the levels of p53 protein were examined by Western blot analysis with an antibody that recognized both rodent and human p53. These experiments demonstrated that endogenous p53 was stably expressed in sympathetic neurons, and that infection with Ad5CA17LacZ did not affect endogenous p53 expression (Fig. 7 B). In contrast, by 30 h after infection with Adwtp53, p53 protein was detectably overexpressed in sympathetic neurons (Fig. 7 A), while by 48 h, expression was much higher than endogenous levels (Fig. 7 B). Coincident with this increased expression of p53 at 48 h after infection, morphological changes characterized by cell shrinkage became evident in cells infected with Adwtp53, while those carrying Ad5CA17LacZ appeared normal. Moreover, a noticeable number of dead and pyk-

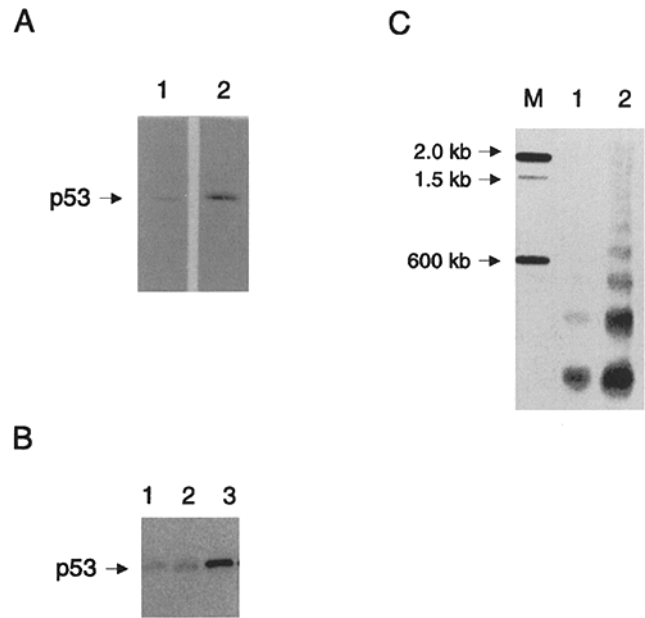


Figure 7. Overexpression of p53 as detected by Western blot analysis. (A) Neurons were infected with Adwtp53 at 50 MOI and harvested after 30 h. Lysates containing protein extracted from 100,000 cells were loaded on each lane, and Western blots were probed with a mouse mAb Ab-1 that reacts with both rodent and human p53. (Lane 1) Uninfected neurons; (lane 2) infected neurons showing expression of the p53 protein. (B) Neurons were uninfected (lane 1), infected with 50 MOI Ad5CA17lacZ (lane 2), or infected with 50 MOI Adwtp53 (lane 3) and harvested 48 h after infection. Western blots were performed as for A. (C) Overexpression of p53 leads to DNA fragmentation. Neurons were infected with 50 MOI of recombinant adenovirus Ad5CA17LacZ (lane 1) or Adwtp53 (lane 2) and harvested 48 h after infection. Nucleosomal DNA was extracted from 300,000 cells (each sample) and end labeled with [32 P]dCTP. Samples were run on a 2% agarose gel, dried, and visualized with a phosphorimager. M, molecular weight markers.

notic, p53-positive neurons were observed (Fig. 6 B) relative to controls, suggesting that overexpression of p53 leads to neuronal death.

To quantitate the extent of neuronal death after p53 overexpression, cell survival was measured with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) metabolic assay. 3 d after plating sympathetic neurons were infected in parallel with Ad5CA17LacZ and Adwtp53 at titers ranging from 5–500 MOI. The MTT survival assay of lacZ-infected neurons revealed no changes in cell viability 72 h after infection even at the highest MOI of 500 (Fig. 8 A). In contrast, cells infected with Adwtp53 under identical conditions exhibited a 40% decrease in cell survival at 5 MOI and a 65% decrease at 10 MOI (Fig. 8 A). Higher levels of virus resulted in a dramatic 75–85% loss of cell viability by 72 h. As an additional control, sister cultures were infected with wild-type adenovirus at similar MOIs. Surprisingly, even wild-type adenovirus did not affect the survival of sympathetic neurons for up to 72 h (Fig. 8 A).

These data indicated that overexpression of human p53 leads to the death of sympathetic neurons. To ascertain the time course of neuronal cell death after p53 overex-

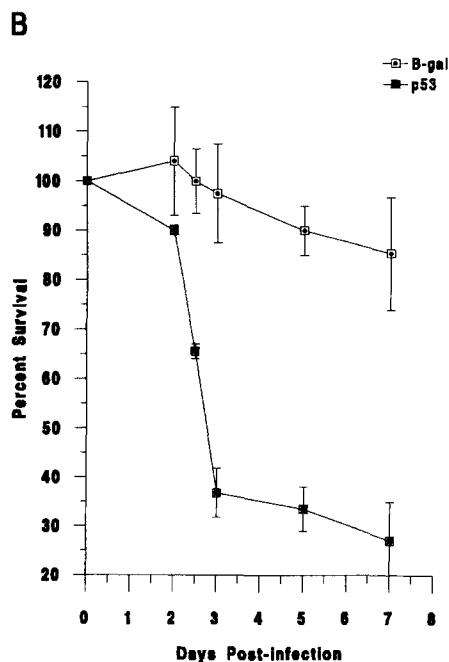
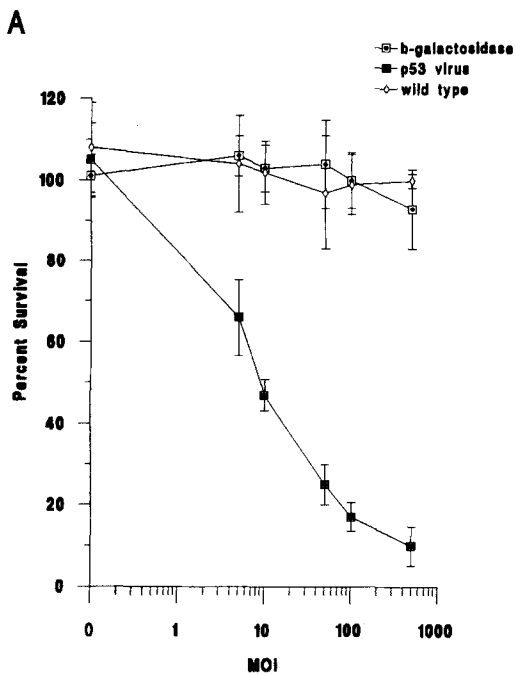


Figure 8. Recombinant adenovirus-mediated expression of p53 leads to death of postmitotic sympathetic neurons. (A) Neurons were infected in parallel with wild-type adenovirus, recombinant adenovirus (Ad5CA17LacZ), and a recombinant adenovirus carrying wild-type p53 (Adwtp53) at titers of 0, 5, 10, 50, 100, and 500 MOI. After 72 h cell survival was measured by metabolic conversion of tetrazolium salt into formazan salt using a Cell Titer 96 Assay Kit. Results are the average of three separate experiments, with error bars representing standard error. (B) Neurons were infected in parallel with recombinant adenoviral vectors carrying LacZ (Ad5CA17LacZ) and p53 (Adwtp53) at a titer of 50 MOI. After a time course of 2, 2.5, 3, 5, and 7 d, cell survival was measured as described for A. Results are the average of two separate experiments, with error bars representing range.

pression, parallel experiments were carried out with neurons infected at 50 MOI with Ad5CA17LacZ and Adwtp53. Survival was measured by MTT assay at 2, 2.5, 3, 5, and 7 d after infection. Neuronal cell death was first detectable by 48 h when a 10% decrease in cell survival was evident (Fig. 8 B). By 72 h a dramatic 60–70% loss in cell viability was detected. Cell death then continued at a very low level for the remainder of the assay. Thus, cell death begins 48 h after infection with Adwtp53, with the majority of neurons dying between 2 and 3 d.

To determine if p53-induced cell death was due to apoptosis we conducted three different assays: (a) the isolation of nucleosomal DNA to visualize DNA ladders; (b) TUNEL staining to visualize apoptosis immunohistochemically; and (c) EM. All three of these assays, which were conducted in parallel with cultures infected at 50 MOI with Ad5CA17LacZ or Adwtp53, indicated that overexpression of p53 leads to neuronal apoptosis. First, neurons infected for 48 h with Adwtp53 showed significantly more DNA fragmentation than those infected with Ad5CA17LacZ (Fig. 7 C), as demonstrated by DNA gel electrophoresis. Second, TUNEL staining revealed significantly higher levels of apoptosis in neurons overexpressing p53 relative to controls at 72 h after infection (Fig. 9, A and B). In Adwtp53-infected cultures there were many pyknotic, TUNEL-positive nuclei (Fig. 9 F), while only the occasional TUNEL-positive nucleus was observed in cultures infected with Ad5CA17LacZ (Fig. 9, A and D). Coincident with this increase in TUNEL labeling, p53-overexpressing neurons displayed dramatic neuritic degeneration (Fig. 9 E), while those expressing lacZ displayed normal morphology (Fig. 9 C). Finally, analysis of these cultures by EM demonstrated enhanced apoptosis of sympathetic neurons infected with 50 MOI Adwtp53, as indicated by chromatin collapse and condensation (data not shown). In contrast, sympathetic neurons infected with Ad5CA17LacZ did not display enhanced apoptosis relative to uninfected controls, even at 500 MOI, at least as measured ultrastructurally. Thus, adenovirus-mediated overexpression of p53 is sufficient to cause the apoptotic death of postmitotic sympathetic neurons.

Discussion

The data presented in this paper support two important conclusions. First, we demonstrate that adenovirus-derived vectors can be effectively used as gene transfer agents for postmitotic neurons. When used between 10 and 50 MOI, recombinant adenovirus can infect >75% of sympathetic neurons with little perturbation in cell survival, cytoarchitecture, and physiological function for at least 7 d after infection. Secondly, working within the parameters we have defined with the Ad5CA17LacZ recombinant adenovirus, we have demonstrated that overexpression of p53 is sufficient to induce programmed cell death in postmitotic neurons.

The observation that overexpression of the tumor suppressor, p53, is sufficient to induce apoptosis in postmitotic neurons has major implications for the mechanisms of cell death in the traumatized nervous system. Recent studies have demonstrated p53 upregulation during acute neurological disease such as transient middle cerebral ar-

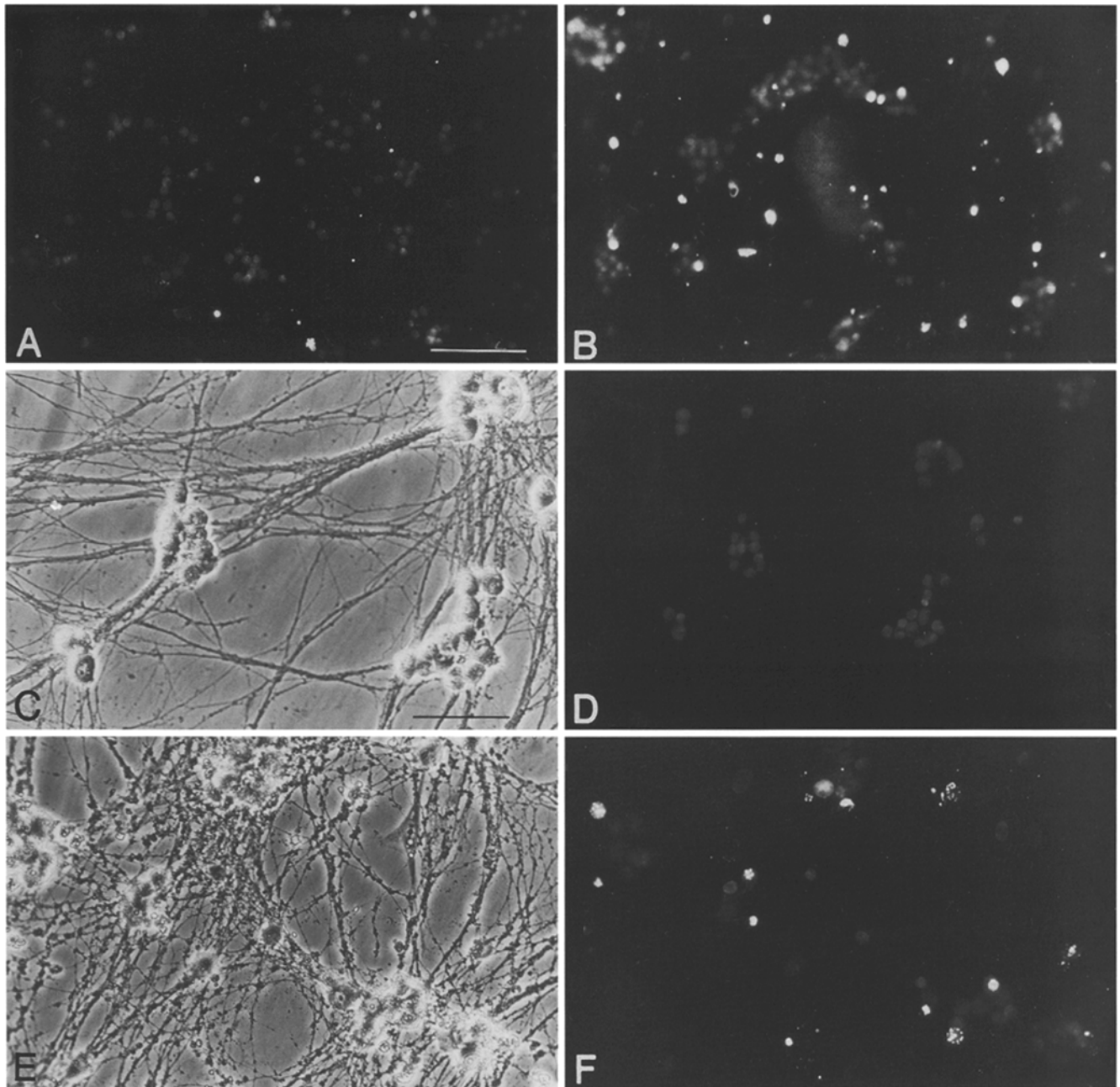


Figure 9. TUNEL staining reveals enhanced apoptosis in neurons overexpressing p53. Neurons were infected at 50 MOI with either Adwtp53 or Ad5CA17lacZ. After 72 h, cells were fixed with methanol/acetone (1:1) for 10 min and treated with a cocktail containing the terminal transferase enzyme and biotin-dUTP. Incorporation of biotin-dUTP was detected by Cy3-conjugated streptavidin. *A* and *B* were photographed at low magnification to show a representative sampling of the cell population. A markedly enhanced level of TUNEL staining is observed in cells overexpressing p53 (*B*) relative to neurons expressing lacZ (*A*). At higher magnification, phase contrast of the same fields reveals degeneration of neuritic processes in cell infected with Adwtp53 (*E*) relative to controls (*C*). The corresponding TUNEL staining (*D*, cells infected with Ad5CA17lacZ; and *F*, cells infected with Adwtp53) shows pyknotic nuclei indicative of apoptosis in cells infected with Adwtp53 (*F*). Bars: (*A*) 400 μ M; (*C*) 160 μ M.

tery occlusion (22) and kainic acid-induced excitotoxicity (36). Furthermore, p53 has been implicated in apoptosis after ionizing radiation in cerebellar granule cells (42). Since our data indicate that endogenous p53 is stably expressed in SCG neurons, it appears that this protein must be expressed at a given threshold level before the apoptotic pathway is triggered. Once this threshold is reached,

our data indicate that p53 alone is sufficient to induce neuronal apoptotic cell death, suggesting that the upregulation of p53 after neuronal injury may well be the key signal leading to the demise of injured neurons in at least some situations.

In contrast to the proposed role for p53 in stress-induced neuronal death, this protein does not apparently play a

major role in naturally occurring cell death. Mice carrying null mutations for p53 develop normally (7), and neurons derived from such mice exhibit normal apoptosis after neurotrophin withdrawal (5). Moreover, no enhancement of p53 expression was observed after NGF withdrawal in sympathetic neurons (13). Together, these studies indicate that if p53 does play a role during naturally occurring neuronal death, it is of relatively minor developmental importance.

It is somewhat surprising that p53 mediates apoptosis in neurons, since these cells are postmitotic, and thus pose no apparent threat of tumorigenesis. In cycling cells, the DNA-binding function of p53 is essential to mediate cell cycle arrest (34). However, the functional domains of p53 required to induce apoptosis are less well defined. A number of studies suggest that p53-mediated transactivation may induce apoptosis in some cell types (28, 29, 32, 35). In particular, recent studies indicate that cross talk may exist between p53 and the bcl-bax signaling cascade (17, 30). The gene for bax (30), one member of the bcl2 family that induces apoptosis, contains p53 consensus sites in its promoter (29). Wild-type but not mutant p53 was shown to transactivate the bax promoter and gel retardation assays demonstrated that p53 could directly bind the p53 consensus sequence of this promoter (28). The fact that p53-mediated apoptosis can be inhibited by overexpression of bcl-2 is consistent with the interpretation that the bcl-bax pathway is downstream of p53 (for review see 41).

Although p53 may mediate neuronal apoptosis via transcriptional activation, alternative mechanisms cannot yet be ruled out (4). For example, in other systems, p53 can inhibit transcription of genes required for cell survival by physically interacting with and inhibiting the single-stranded DNA-binding protein complex replication protein A (9, 21). Moreover, other transcription-independent mechanisms have recently been implicated in p53-mediated apoptosis of cell types other than neurons (for review see 41). Thus, the mechanism by which p53-induced cell death occurs is a function of the cell type and situation. Future studies using adenovirus-derived vectors to introduce p53 deletion mutants will help to define the mechanism of p53 action in postmitotic neurons.

The studies described here also demonstrate the use of recombinant adenoviral vectors for carrying out mechanistic studies in postmitotic neurons. We have found that titers sufficiently high to transduce the majority of neurons can be used without perturbing cell survival, neuronal function, or cytoarchitecture. It should be noted, however, that there are definite limitations with respect to transduction levels that sympathetic neurons are able to tolerate. At titers in excess of 100 MOI, assays of mitochondrial function indicate a decrease in cell survival. Cells surviving excessive infection rates exhibited remarkable changes in the nuclear ultrastructure manifested by electron-dense inclusions, filamentous aggregates, and, in severe cases, disintegration of chromatin. The nature of these inclusions is unknown and clearly distinct from either the cytopathic effect induced by wild-type adenovirus in respiratory epithelial cells, or apoptosis. Pathological changes resulting from recombinant viral transduction is an area that requires further investigation, particularly with regards to viral-mediated gene therapy.

In conclusion, adenovirus vectors provide valuable tools to examine molecular pathways in postmitotic neurons. By using these vectors, we have shown that overexpression of p53 is sufficient to induce apoptosis in sympathetic neurons. These results suggest that the signaling cascade responsive to p53 is functional in postmitotic neurons and may well be a key regulator of cell death in the traumatized nervous system.

The adenovirus vectors Ad5CA17LacZ and Adwtp53 were constructed by Christina Addison and Sylvia Bacchetti, respectively. We thank Dr. Rob Dunn for providing the herpes simplex virus, RH105, and Drs. Gyula Acsadi and Agnes Jani for many helpful discussions. The technical assistance of Audrey Speelman, Peter Ripstein, and Steve Prescott is gratefully acknowledged. We also thank Dr. Hewei Li for plaque purification of the adenovirus recombinants.

This work was supported by grants from the Canadian Neuroscience Network and the Medical Research Council of Canada (MRCC) to F.D. Miller. F.D. Miller is a Killam Scholar; R.S. Slack and R. Aloyz were supported by a Neuroscience Network fellowship; D.J. Belliveau was supported by an MRCC Genentech Inc. fellowship; and H. Lochmüller was supported by a grant from the Savoy Foundation of Canada.

Received for publication 28 March 1996 and in revised form 11 July 1996.

References

1. Bacchetti, S., and F.L. Graham. 1993. Inhibition of cell proliferation by an adenovirus vector expressing the human wild-type p53 protein. *Int. J. Oncol.* 3:781-788.
2. Bett, A.J., W. Haddara, L. Prevec, and F.L. Graham. 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA.* 91:8802-8806.
3. Boviatis, E.J., M. Chase, M.X. Wei, T. Tamiya, R.K. Hurford, N.W. Kowall, R.I. Tepper, X.O. Breakefield, and E.A. Chiocca. 1994. Gene transfer into experimental brain tumors mediated by adenovirus, herpes simplex virus, retrovirus vectors. *Hum. Gene Ther.* 5:183-191.
4. Caelles, C., A. Helmborg, and M. Karin. 1994. p53-dependent apoptosis in the absence of transcriptional activation of p53 target genes. *Nature (Lond.)* 370:220-223.
5. Davies, A.M. and A. Rosenthal. 1994. Neurons from mouse embryos with a null mutation in the tumour suppressor gene p53 undergo normal cell death in the absence of neurotrophins. *Neurosci. Lett.* 182:112-114.
6. Deckwerth, T.L., and E.M. Johnson. 1993. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* 123:1207-1222.
7. Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, and C.A. Montgomery. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature (Lond.)* 356:215-221.
8. Dou, Q.-P., J.L. Fridovich-Keil, and A.B. Pardee. 1991. Inducible proteins binding to the murine thymidine kinase promoter in late G1/S phase. *Proc. Natl. Acad. Sci. USA.* 88:1157-1161.
9. Dutta, A., J.M. Ruppert, J.C. Aster, and E. Winchester. 1993. Inhibition of DNA replication factor RPA by p53. *Nature (Lond.)* 365:79-82.
10. El-Deiry, W.S., S.E. Kern, J.A. Pietenpol, K.W. Kinzler, and B. Vogelstein. 1992. Definition of a consensus binding site for p53. *Nat. Genet.* 1: 45-49.
11. El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 75:817-825.
12. Elledge, R.M., and W.-H. Lee. 1995. Life and death by p53. *Bioessays.* 17: 923-930.
13. Freeman, R.S., S. Estus, and E.M. Johnson. 1994. Analysis of cell cycle related gene expression in postmitotic neurons: selective induction of cyclin D1 during programmed cell death. *Neuron.* 12:343-355.
14. Graham, F.L., and L. Prevec. 1991. Manipulation of adenovirus vectors. In *Methods in Molecular Biology*. E.J. Murray, editor. The Humana Press, Totowa, NJ, 109-128.
15. Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.I. Elledge. 1993. The p21 cdk-interacting protein cip1 is potent inhibitor of G1 cyclin-dependent kinases. *Cell.* 75:805-816.
16. Ho, D.Y., and E.S. Mocarski. 1988. Beta-galactosidase as a marker in the peripheral and nervous tissues of the herpes simplex virus-infected mouse. *Virology.* 174:279-283.
17. Hockenbery, D., G. Nunes, C. Millman, R.D. Shreiker, and S.J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (Lond.)* 348:334-336.

18. Katayose, D., R. Wersto, K.H. Cowan, and P. Seth. 1995. Effects of a recombinant adenovirus expressing WAF1/Cip1 on cell growth, cell cycle and apoptosis. *Cell Growth Differ.* 6:1207-1212.
19. Lane, D.P. 1993. A death in the life of p53. *Nature (Lond.)* 362:786-787.
20. Le Gal La Salle, G., J.J. Robert, S. Berrard, V. Ridoux, L.D. Stratford-Perricaudet, M. Perricaudet, and J. Mallet. 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. *Science (Wash. DC)* 259: 988-990.
21. Li, R., and M.R. Botchan. 1993. The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. *Cell* 73:1207-1221.
22. Li, Y., M. Chopp, Z.G. Zhang, C. Zaloga, L. Niewenhuis, and S. Gautam. 1994. p53-immunoreactive protein and p53 mRNA expression after transient middle cerebral artery occlusion in rats. *Stroke* 25:849-856.
23. Lipson, K.E., S.T. Chen, J. Koniecki, D.H. Ku, and R. Baserga. 1989. S-phase specific regulation by deletion mutants of the human thymidine kinase promoter. *Proc. Natl. Acad. Sci. USA* 86:6848-6852.
24. Lochmüller, H., A. Jani, J. Huard, S. Prescott, M. Simoneau, B. Massie, G. Karpati, and G. Acsadi. 1994. Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants during multiple passages in 293 cells. *Hum. Gene Ther.* 5:1485-1491.
25. Ma, Y., R.B. Campenot, and F.D. Miller. 1992. Concentration-dependent regulation of neuronal gene expression by nerve growth factor. *J. Cell Biol.* 117:135-141.
26. McFarlane, S., and E. Cooper. 1992. Postnatal development of voltage-gated K currents in rat sympathetic neurons. *J. Neurophys.* 67:1291-1300.
27. McFarlane, S., and E. Cooper. 1993. Extrinsic factors influence the expression of voltage-gated K currents in neonatal rat sympathetic neurons. *J. Neurosci.* 13:2591-2600.
28. Miyashita, T., and J.C. Reed. 1995. p53 is a direct transcriptional activator of the human bax gene. *Cell* 80:293-299.
29. Miyashita, T., S. Krajewski, M. Krajewska, H.G. Wang, H.K. Lin, D.A. Liebermann, B. Hoffman, and J.C. Reed. 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 9:1799-1805.
30. Oltvai, Z.N., C.L. Millman, and S.J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell* 74:609-619.
31. Oppenheim, R.W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453-501.
32. Owen-Schaub, L., W. Zhang, J.C. Cusack, L.S. Angelo, S.M. Santee, T. Fujiwara, J.A. Roth, A.B. Deisseroth, W-W. Zhang, E. Kruzel, and R. Radinsky. 1995. Wild-type human p53 and a temperature-sensitive mutant induce Fas/Apo-1 expression. *Mol. Cell. Biol.* 15:3032-3040.
33. Picksley, S.M., and D.P. Lane. 1994. p53 and Rb: their cellular roles. *Curr. Opin. Cell Biol.* 6:853-858.
34. Pietsenpol, J.A., T. Tokino, S. Thiagalingam, W.S. El-Deiry, K.W. Kinzler, and B. Vogelstein. 1994. Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl. Acad. Sci. USA* 91: 1998-2002.
35. Sabbatini, P., J. Lin, A.J. Levine, and E. White. 1995. Essential role for p53-mediated transcription in apoptosis but not growth suppression. *Genes & Dev.* 9:2184-2192.
36. Sakhi, S., A. Bruce, N. Sun, G. Tocco, and M. Baudry. 1994. p53 induction is associated with neuronal damage in the central nervous system. *Proc. Natl. Acad. Sci. USA* 91:7525-7529.
37. Sherley, J.L., and T.J. Kelly. 1988. Regulation of human thymidine kinase during the cell cycle. *J. Biol. Chem.* 263:8350-8358.
38. Silos-Santiago, I., L.J.S. Greenlund, E.M. Johnson, and W.D. Snider. 1995. Molecular genetics of neuronal survival. *Curr. Opin. Neurobiol.* 5:42-49.
39. Slack, R.S., and F.D. Miller. 1996. Viral vectors for use in modulating gene expression in neurons. *Curr. Opin. Neurobiol.* 6:576-583.
40. Slack, R.S., I.S. Skerjanc, B. Lach, J. Craig, K. Jardine, and M. W. McBurney. 1995. Cells differentiating into neuroectoderm undergo apoptosis in the absence of functional retinoblastoma family proteins. *J. Cell Biol.* 129:779-788.
41. White, E. 1996. Life, death, and the pursuit of apoptosis. *Genes & Dev.* 10: 1-15.
42. Wood, K.A., and R.J. Youle. 1995. The role of free radicals and p53 in neuron apoptosis in vivo. *J. Neurosci.* 15:5851-5857.
43. Xiong, Y., G. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature (Lond.)* 366: 701-704.