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H_2O_2-Induced Apoptotic Death in Serum-Deprived Cultures of Oligodendroglia Origin Is Linked to Cell Differentiation

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When deprived of serum, oligodendroglialike (OLN 93) cells grown on poly-L-lysine-coated culture dishes cease to proliferate after 3 days and morphologically extend many fibers resembling morphologically differentiated, immature oligodendrocytes. At this time no cell death is apparent unless serum deprivation is extended for a period longer than 1 week. After 3 days in serum-deprived medium, treatment of cells with 1 mM H_2O_2 for 30 min facilitates apoptotic cell death, even when serum is added during the recovery period. Both serum-deprived, differentiated cells, and proliferating cells, respond to H_2O_2 by an initial growth arrest followed by growth resumption after 48 hr. However proliferating cells show resistance to the apoptotic effect of H_2O_2. This is correlated with growth arrest in the S phase at different stages of DNA replication, as well as with different timing of induced $p21^{Waf1}$ expression. Thus, cells grown in serum, express elevated $p21^{Waf1}$ protein levels after 4 hr, whereas serum-deprived, differentiated cells, only after 24 hr. The mRNA levels of $p21^{Waf1}$ follow a similar timed pattern. Hence $p21^{Waf1}$ may protect OLN 93 cells against the genotoxic effect of H_2O_2. The data suggest an intimate relationship between G1-arrest, morphological differentiation, and H_2O_2-mediated apoptosis. J. Neurosci. Res. 56:447–456, 1999.

Key words: oligodendroglial cells; oxidative stress; hydrogen peroxide; apoptosis; differentiation

INTRODUCTION

Cellular proliferation and differentiation in the nervous system are closely linked processes, controlled by a number of growth factors and depend on environmental conditions. In tissue culture, serum addition provides neural cells with necessary extrinsic growth factor supplements that promote cell growth and enable survival. Serum deprivation, on the other hand, causes an arrest in cell division and may induce cellular differentiation (Chakravarthy et al., 1995; Eves et al., 1996). Extended periods of serum deprivation may eventually lead to cell death due to the lack of growth factors (Batistatou and Greene, 1993; Behl et al., 1994) or increased oxidative stress (Atabay et al., 1996). The latter has been implicated in a variety of human neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, ischemic stroke, and multiple sclerosis (Olanow, 1993; Cuzner and Norton, 1996; Lyras et al., 1997) and is a common cause for cell death. Cells in the nervous system are highly sensitive to oxidative stress because they utilize high levels of oxygen for normal function, and the brain has relatively poorly developed antioxidant mechanisms (Halliwell, 1992; Wood and Youle, 1994; Dawson and Dawson, 1996).

Oligodendrocytes and their precursor cells exhibit a high sensitivity to oxidative stress. Oligodendrocyte precursors, for example, are easily damaged by hypoxic events because of low antioxidant levels and high iron content (Thorburne and Juurlink, 1996). The cytotoxic potential of oxygen radical-generating systems on bovine oligodendrocytes prepared from adult brains was described (Kim and Kim, 1991). Also, oxidative stress in mature rat brain oligodendrocytes, exposed to hydrogen peroxide, leads to the onset of programmed cell death involving the activation of the immediate-early genes c-fos and c-jun (Richter-Landsberg and Vollgraf, 1998). Maturation-dependent differences in the vulnerability of oligodendrocytes to oxidative stress are observable, specifically oligodendrocyte precursors show a higher sensitivity to oxidative stress-induced death caused by glutathione depletion (Back et al., 1998). There is little information at the present time on the effects of toxic radicals on the molecular mechanisms regulating cell survival and death

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in proliferating compared to differentiating oligodendroglia cells.

We hypothesized that the molecular events that lead to cell death resulting from serum deprivation or from diverse oxidative stresses may be closely linked and distinctly regulated in proliferating compared to growth-arrested cells. To address this issue we used a permanent cell line derived from spontaneously transformed primary cultures of rat brain glial cell, namely, OLN 93 (Richter-Landsberg and Heinrich 1996). As recently demonstrated, in response to H_2O_2, p53 undergoes rapid translocation to the nucleus in these cells (Uberti et al., 1999).

In the present study we set to investigate in more details, the consequences of a double insult generated by growth arrest and hydrogen peroxide on cell cycle events, $p21^{Waf1}$ expression and cell death. We demonstrate that actively dividing cells, which rapidly respond to H_2O_2 stress by elevating $p21^{Waf1}$ expression show less cell death than the serum-deprived cells. The latter show a delayed appearance of $p21^{Waf1}$. We also conclude that H_2O_2 treatment facilitates apoptotic death most prominently in a serum-deprived cell population that undergoes morphological differentiation and consists predominantly of cells arrested in the G1 phase.

MATERIALS AND METHODS

Cell Culture and H_2O_2 Addition

The procedure for generation and propagation of the OLN 93 clonal cell line used throughout these experiments was recently published (Richter-Landsberg and Heinrich, 1996). Experiments were carried out with cells not passaged more than 40 times. For propagation, cells were grown on plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and without pyruvate (GIBCO, Grand Island, NY), 100 U/µg penicillin/streptomycin (GIBCO), and 10% fetal calf serum at 37°C and an atmosphere of air enriched with 7.5% CO_2. For H_2O_2-induced stress, cells were seeded on culture plates precoated with 50µg/ml poly-L-lysine (PLL, MW 78,000, Sigma, St. Louis, MO) and grown for specified time periods in the absence or presence of serum containing DMEM. H_2O_2 at various concentrations was added in DMEM containing 0.1% serum for a period of 30 min. After incubation, medium was discarded and cells were returned to the incubator in DMEM containing 10% serum, until the time of the assay. For the present experiments, two states of cell populations were elected, based on the percentage of cells present in the G1 cell-cycle; one population with a low (around 40%) G1-enriched, and a second, with a high G1-enriched (>80% and mostly above 90%) cell population. To obtain the former, cells were seeded in 10% serum containing DMEM on culture plates precoated with PLL and allowed to grow logarithmically usually between 2 and 3 days. To obtain the high G1-enriched population, cells were switched to serum-free medium and grown under these conditions for 3–5 days. H_2O_2 was added in 0.1% serum containing DMEM using conditions detailed above.

Fluorescence and Phase Contrast Light Microscopy Visualization

For fluorescence studies, cells seeded on PLL-coated coverslips were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were fixed for 5 min in methanol at −20°C followed by three times rinsing before exposure to 0.5 mg/ml DAPI at room temperature. After 10 min, the coverslips were rinsed three times with PBS and mounted with Moviol 4–88. Cells were visualized by an Axioplan Zeiss microscope equipped with appropriate filter settings for fluorescence. Photography was assisted with a CCD camera (COHU, San Diego, CA) and frames were collected by a Quadra 950 station using an 1.55 NIH Image program software.

Cell Viability Assay

Cells grown in 0.1 ml medium on 96-well poly-L-lysine precoated plates were incubated at 37°C for 2 hr with 10 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml PBS from Sigma). Medium was discarded and 0.1 ml of 0.04 *N* HCl in isopropanol was added to each well. After shaking, color reading was done in the ELISA reader at 540 nm and 630 nm.

Cell-Cycle Analysis

Cells were scraped off the PLL-coated plates in DMEM and after a brief centrifugation (2 min at 1,000*g*) the supernatant was discarded. Cell pellets were washed with 0.1 ml phosphate buffer saline (PBS, pH 7.4), again centrifuged and the washed cells pellet fixed in 70% ethanol at −20°C. After 2 hr at −20°C, cells were centrifuged at 1,000*g* for 2 min, rinsed once with PBS, and resuspended in 1 ml PBS containing 0.5 mg/ml RNase A (Sigma). After incubation at 37°C for 1 hr fixed cells were stained for 15 min with 50 µg/ml propidium iodide (Sigma). Cell-cycle reading of 10,000–5,000 cells was done using a Becton-Dickinson FACSort (Becton-Dickinson, Mountain View, CA). PI fluorescence (''FL2''), forward scatter (FCS), and side scatter (SSC) were all measured simultaneously and recorded in a Quadra 950 station using a Cell Quest FACSort software program. The FCS threshold value was set experimentally and remained constant for all measurements.

For staining with the BrdU antibody, cells were incubated for 30 min with 0.01 mM BrdU (Sigma). Medium was discarded and cells were collected in 0.1 ml PBS followed by 5 ml cold 70% ethanol (−20°C). After 1 hr or more, cells were centrifuged for 10 min and the solvent discarded. Pellet was resuspended in 1 ml 2 *N* HCl with 0.5% Triton X-100 (v/v) and incubated at room temperature for 30 min. Cells were precipitated by centrifugation and resuspended in 1 ml of 0.1 M $Na_2B_4O_7$, pH 8.5. After 15 min cells were precipitated by centrifugation and resuspended in 35 µl 0.5% Tween 20 (v/v) plus 1.0% BSA (w/v) in PBS followed by addition of 15 µl anti-BrdU FITC (Bactolab). After 1 hr incubation at room temperature, 1 ml PBS was added and cells were precipitated by centrifugation and resuspended in 1 ml PBS containing 5µg/ml propidium iodide. FACSort analysis was done and recorded as detailed above.

DNA Fragmentation Analysis

After treatment, OLN cells were harvested and collected in microfuge tubes and centrifuged at 1,500 rpm for 7 min at 4°C. The pellet was resuspended in PBS centrifuged as above, and to the resulting pellet 0.1 ml lysis buffer (containing 1% NP-40 in 20 mM EDTA and 50 mM Tris-HCl pH 8.0) was added. After gentle tapping, tubes were centrifuged for 5 min at 4,500 rpm and supernatants transferred to separate tubes. The pellet was once again resuspended in 0.1 ml lysis buffer and extraction repeated. The combined supernatants were adjusted to 1% SDS and RNAse (final concentration 2.5 mg/ml) was added for 2 hr at 55°C followed by an additional 2 hr with proteinase K (final concentration 2.5 mg/ml) treatment for 2 hr at 37°C. The fragmented DNA was then precipitated by addition of 0.l ml of 10 M ammonium acetate and three volumes of −20°C absolute ethanol. The tubes were left at −20°C overnight and subsequently centrifuged at 10,000 rpm for 10 min to obtain the fragmented DNA pellet. The pellet was once rinsed in 70% ethanol and dissolved in 10 mM Tris buffer containing 1 mM EDTA (pH 8.0). The fragmented DNA was fractionated on 1.5 % agarose gel, prestained with ethidium bromide and visualized with a UV lamp.

$p21^{Waf1}$ mRNA and Protein Analysis

Cells were collected, lysed in TRI reagent, and RNA extracted according to manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Total RNA was fractionated on 1% agarose formaldehyde gels and transferred overnight onto Hybond-N+nitrocellulose membranes (Amersham, Buckinghamshire, UK). For the purpose of quantitation, GAPDH expression was used to normalize for RNA content. DNA probes were labeled using a Prime-a Gene® labeling system, from Promega (Madison, WI), Klenow fragment, nucleotide triphosphate and [α-^{32}P]dCTP. The probes were purified by passing the reaction mixture through a Sepadex G-25 column. Prehybridization and hybridization were carried out with 50% formamide at 45°C for 2–3 hr and overnight, respectively. Posthybridization washes were performed at 50°C for 3–4 hr in 2× of 3M Citrate/NaCl, pH 7.0 buffer (SSC) and later for 90 min in 1× SSC buffer. Membranes were wrapped in saran plastic wrap and after overnight exposure to an X-ray film (Fuji, Tokyo) were developed and quantified using a scanner (Umax, Power Look-II, Hsinchu, Taiwan).

For protein analysis, cells were collected in PBS and centrifuged at 1,500 rpm for 10 min at 4°C. The cell pellet was then resuspended in PBS containing protease inhibitors (2 mM PMSF, 25 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A) and immediately frozen in liquid nitrogen. To the cell suspension, 1 mM dithiothreitol (DTT), 0.1 mM sodium vanadate,1% Triton-X-100, 0.5% NP-40 in 150 mM NaCl were added and the slurry passed through a syringe needle (0.50 mm × 16 mm) two to three times to make a uniform suspension. Protein content was measured according to Bradford (1976) and samples were resolved on a 12% SDS-PAGE gel. The fractionated proteins on SDS-PAGE, were then transferred onto a Protran BA83 cellulose nitrate membrane (Schleicher & Schuell, Germany) and the membrane blocked with 10% skim milk powder and 0.05% Tween 20 in PBS. The membrane was then incubated with antibodies against $p21^{Waf1}$ (Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing, the second antibody linked to horseradish peroxidase (HRP conjugated anti rabbit IgG; Sigma Nes Ziona, IL) in PBS containing 0.5% Tween 20 and 0.5% skim milk powder was added. After 90 min, the second antibody was removed and following several rinses, immunoidentification was carried out with ECL Plus (Western blotting system) reagent from Amersham (UK).

RESULTS

H_2O_2 Effects on Cell Survival and Growth

OLN 93 cells grown on poly-L-lysine-coated dishes for 3 days in the absence of serum reach a state of quiescence, and characteristically show a flat appearance with numerous cellular processes (Fig. 1B). MTT uptake asssay indicates that after 3 days in the absence of serum cells remained growth arrested and viable even in the presence of serum for the next 4 days at least (Fig. 2B), while cells pregrown in 10% serum continued to grow exponentially in serum (Fig. 2A). To exert oxidative stress, hydrogen peroxide in DMEM containing 0.1% serum was added to the cultures for 30 min, and thereafter

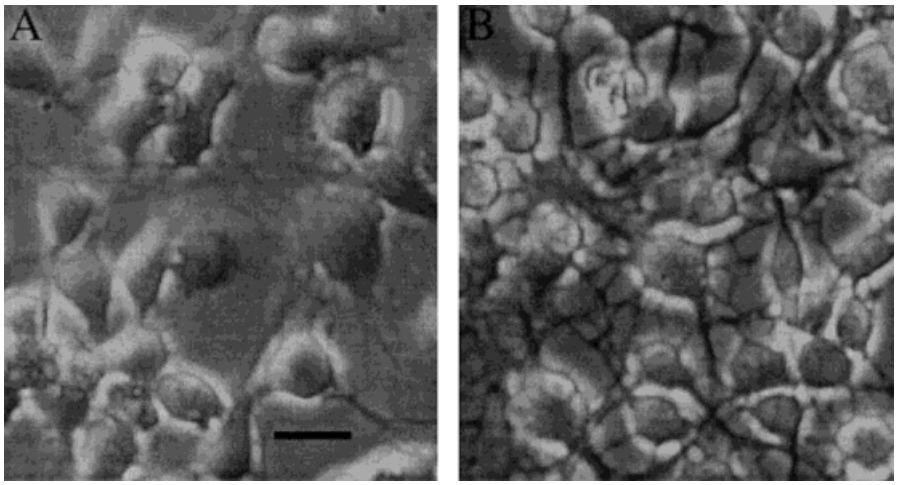

Fig. 1. Effect of serum deprivation on OLN 93 cell differentiation. OLN 93 cells were seeded on glass coverslips precoated with 0.1 mg/ml PLL. **A:** Grown at 37°C in the presence of 10% serum in DMEM for 2 days. **B:** Grown at 37°C in serum-free medium for 4 days. Bar = 10 μm.

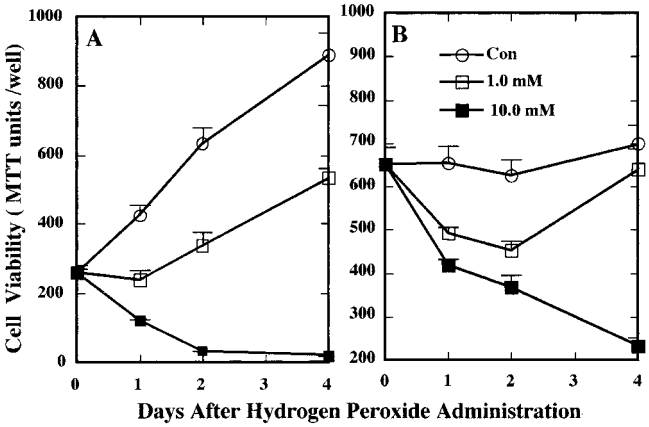

Fig. 2. Effect of H_2O_2 on cell viability. OLN 93 cells were seeded onto 96-well plates precoated with 50 μg/ml PLL. **A:** Grown for 2 days in the presence of 10% serum in DMEM. **B:** Grown for 3 days in the absence of serum. H_2O_2 was addded at 0.1–10 mM concentration for a period of 30 min. After incubation medium was discarded, fresh DMEM containing 10% serum was added and cells returned to 37°C incubation for 1–4 days. MTT assay was performed as detailed in the Materials and Methods section. Each value is a mean ± SE of 6–8 wells, and the experiment was repeaated at least three times.

medium was replaced by DMEM supplemented with 10% serum in all cultures.

Short-term exposure to 1 mM H_2O_2, caused a significant reduction in cell viability as notable after 1 and 2 days. A partial recovery in the metabolic activity was noticed after 4 days in culture. By contrast, cells grown in 10% serum for 48 hr were only slightly affected by 1 mM after day 1, and growth was resumed thereafter (Fig. 2A). At 10 mM H_2O_2 prominent cell death was noticed irrespective whether cells were precultured in the presence or absence of serum. These results suggest that H_2O_2 exerts a complex effect on OLN 93 cell viability depending on the state of cell growth and the amount of H_2O_2 added. It should be noted that serum was always added to cells after the H_2O_2 stress.

Further evidence for the effect of H_2O_2 on cell viability was observed using DAPI nuclear staining. As illustrated in Figure 3, cells precultured in 10% serum treated with 1 mM H_2O_2, exhibited a uniform nuclear stain as evident by the fluorescence image (Fig. 3A′). By contrast, in cultures pregrown in serum-free medium for 3 days, the same treatment led to the appearance of condensed and fragmented nuclei, indicating apoptotic cell death (Fig. 3B′). Additional support for the differential effect of H_2O_2 on cell death depending on the growth state was evident from cell sorting after staining with propidium iodide. Cells were treated as above with 1 mM H_2O_2, and analyzed after 16 hr of recovery in DMEM supplemented with 10% serum. Figure 4 shows the distribution of cells in the G1, S, and sub-G1 stages after analysis on the cell sorter. Notably, approximately 80% of cells resided in the G1 phase under serum-deprived conditions and only approximately 4% were present in the S phase. By contrast, in the presence of serum, 42% and 14% of the cells were in the G1 and S phases, respectively. Treatment with H_2O_2 increased the percentage of sub-G1 cells from a value of 3% to a value of 23% in the serum-deprived cultures. This fractional population is reminiscent of cells undergoing apoptosis (Rodriguez-Tarduchi et al., 1990). H_2O_2 did not increase significantly the amount of sub-G1 cells in actively proliferating, serum-supplemented cells. A twofold and threefold in-

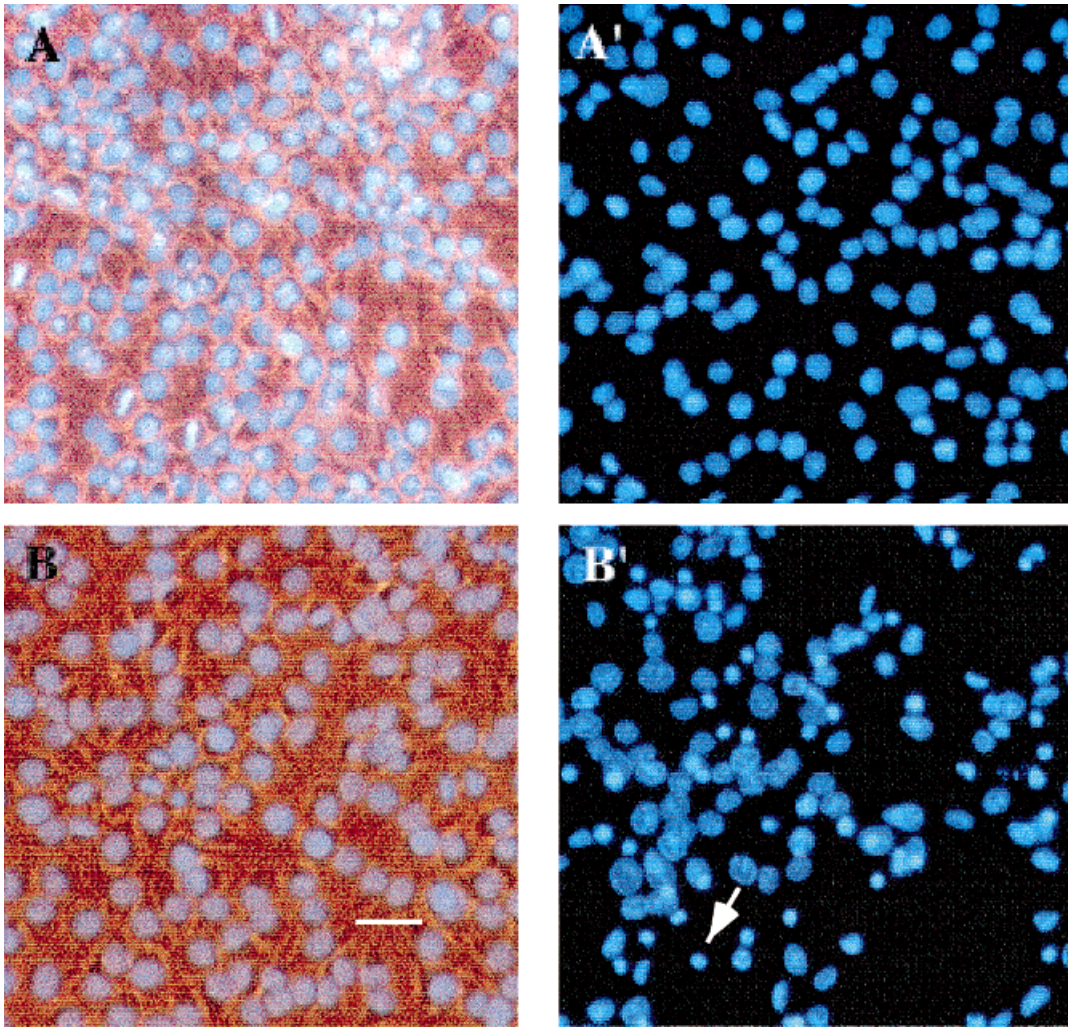

Fig. 3. Fluorescence microscopy images of DAPI-stained cells after H_2O_2 treatment. OLN 93 cells were seeded on glass coverslips precoated with 0.1 mg/ml PLL. **A:** Grown at 37°C in the presence of 10% serum in DMEM for 2 days. **B:** Grown at 37°C in serum-free medium for 4 days. Coverslips were incubated then with 1 mM H_2O_2 for 30 min at 37°C in DMEM and medium discarded. Fresh medium supplemented with 10% serum was added, and cells incubated for 24 hr at 37°C. Cells were fixed for 5 min with methanol (−20°C) and subsequently rinsed in PBS prior to staining with 0.5 mg/ml DAPI at 22°C for 10 min. Coverslips were rinsed three times with PBS and mounted with Moviol 4–88 before observation with a Zeiss microscope. Phase-contrast micrographs of serum-supplemented (A) and serum-free cells (B) are shown in the background of the DAPI fluorescence. H_2O_2 treated cultures (**A′** and **B′**) are presented only by DAPI fluorescence to illustrate the appearance of condensed apoptotic nuclei (arrow) particularly in panel B′. Bar = 25 μm.

crease in the amount of cells arrested in the S phase was noticed in serum-supplemented and serum-deprived cultures, respectively.

Further evidence for the effect of H_2O_2 on cell-cycle is provided by the BrdU distribution in the cell sorter. As shown in Figure 5A, actively dividing cells showed a high proportion (24.8%) of BrdU-labeled nuclei in the S phase in contrast to a relatively low proportion (4.5%) of BrdU-stained nuclei in serum-deprived cultures (Fig. 5B). After 30 min exposure to 1 mM H_2O_2, followed by 16 hr recovery, OLN cells under both conditions were arrested in the S phase (24.4% and 14.3% in Fig. 5A′ and B′ respectively). At this time the apoptotic cell population identified as sub-G1, increased from a near basal value to 1.5% and 15.1% for serum-supplemented and serum-deprived cultures (Fig. 5A′,B′), respectively. This is consistent with lack of cell growth demonstrated by MTT uptake after 24 hr following 1 mM H_2O_2 administration in both types of cultures (Fig. 1).

The high percentage of cells residing in the sub G1 phase after H_2O_2 treatment of serum-deprived cultures, raised the question as to the extent of DNA fragmentation

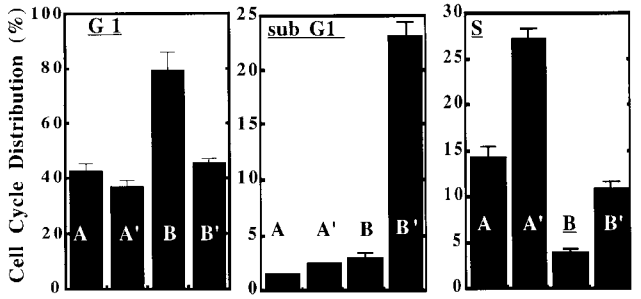

Fig. 4. Cell-cycle analysis of OLN 93 cells after H_2O_2 treatment. Cells were seeded on 6-cm-diameter culture plates precoated with 50 µg/ml PLL, and grown for 2 days at 37°C in the presence of 10% serum in DMEM (**A bars**) or 3 days in the absence of serum (**B bars**). Medium was replaced with fresh DMEM containing 0.1% serum and to designated cultures 1 mM H_2O_2 was added for 30 min at 37°C. The H_2O_2 solution was then substituted with fresh DMEM containing 10% serum and after 16 hr incubation, cell-cycle analysis was done in the presence of propidium iodide as detailed in the Materials and Methods section. The panels from left to right depict G1, sub-G1 and S phase cells distribution, respectively, from untreated (bars A and B) and H_2O_2-treated (**bars A**′and **B**′) cultures. The bars represent % cells ±SEM of triplicate cultures. The experiment was repeated on several occasions with practically similar results.

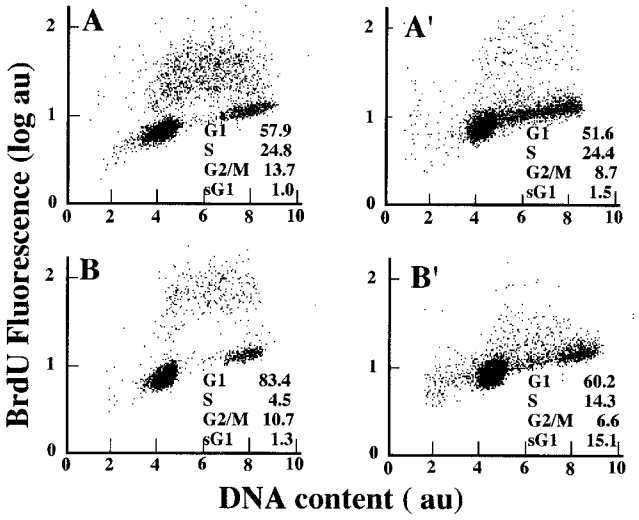

Fig. 5. Cell-cycle analysis and BrdU distribution of OLN 93 cells after H_2O_2 treatment. Experimental conditions were similar to those described for Figure 4. One mM H_2O_2 was added for 30 min to either cells maintained for 2 days in 10% serum (**A**′) or 3 days in serum-free medium (**B**′). Fresh DMEM containing 10% serum was added and after 24 hr incorporation of BrdU and propidium iodide staining were done as detailed in the Materials and Methods section. **A** shows a large population of actively dividing cells (S phase) stained with BrdU in contrast to H_2O_2-treated cells, which show S-arrested BrdU nonstained cells (A′). **B** shows a relatively low population of BrdU-stained cells, which, after H_2O_2 treatment, is arrested in the S phase (B′). The experiment was repeated twice on different occasions with similar results.

under these conditions. Agarose gel electrophoresis of DNA extracts (Fig. 6) showed that after 6 hr no ladderlike DNA fragmentation was noticed. After 24 hr, however, a substantial increase in DNA fragmentation was seen at 1 mM H_2O_2 and above. This result corroborates previous data, suggesting that H_2O_2-induced cell death in the serum-deprived culture was apoptotic.

H_2O_2 Enhances $p21^{Waf1}$ mRNA and Protein Expression

The selective effect of H_2O_2 on stimulating apoptotic death in serum deprived cultures and the high content of arrested G1 cells prompted us to examine downstream cell-cycle-related signals following these changes. $p21^{Waf1}$ seemed as an appropriate candidate for induction as already shown previously in other cells of non neural origin (Qiu et al., 1996) and based on our own recent data (Uberti et al., 1999). OLN cells grown in 10% serum or in the absence of serum were subjected to various concentrations of H_2O_2 and $p21^{Waf1}$ mRNA measured after 4 hr and 24 hr following the stress. As illustrated in Figure 7, 4 hr after exposure to H_2O_2, no changes in $p21^{Waf1}$ mRNA levels were observed in serum-deprived cultures (Fig. 7, lane a). After 24 hr, however, the levels of $p21^{Waf1}$ mRNA increased (Fig. 7, lane b), particularly at the higher H_2O_2 concentrations. In contrast to serum-deprived cultures, actively dividing, serum-supplemented cultures showed a high level of $p21^{Waf1}$ mRNA already after 4 hr and even at the lowest H_2O_2 concentration (Fig. 7, lane c). After 24 hr there were no differences between H_2O_2-treated and -untreated cultures (Fig. 7, lane d) with respect to mRNA levels. The relative changes in $p21^{Waf1}$ normalized to GAPDH mRNA suggest a rapid response of the proliferating cells to the H_2O_2 stress. The changes in the mRNA levels were also accompanied by changes in the amount of $p21^{Waf1}$ protein. As shown in Figure 8, serum-deprived cultures showed an increase in $p21^{Waf1}$ levels after 24 hr following the H_2O_2 stress. By contrast, a maximum increase was noticed by 4 hr after H_2O_2 treatment in serum-supplemented cultures. Collectively, these results indicate that growth in serum protects the OLN cells against H_2O_2 induced apoptosis. This is correlated with elevation of $p21^{Waf1}$, which was shown previously to protect cells from apoptosis.

DISCUSSION

Oligodendrocytes, the myelin-forming cells of the central nervous system (CNS), are derived from bipoten-

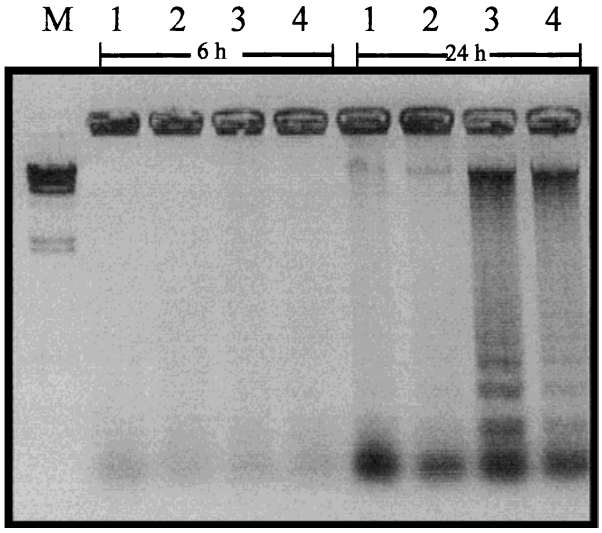

Fig. 6. DNA fragmentation after H_2O_2 treatment. OLN 93 cells were seeded on 10 cm diameter PLL precoated culture plates, and grown for 4 days in serum-free medium. H_2O_2 at 0.1 mM (**lane 2**), 1 mM (**lane 3**) and 5 mM (**lane 4**) was added for 30 min at 37°C. Medium was discarded and fresh DMEM containing 10% serum was added for 6 and 24 hr. DNA fragments were isolated and chromatographed as detailed in the Materials and Methods section. A fragmented DNA fraction was used as marker (**M**) to identify the size of the base pairs.

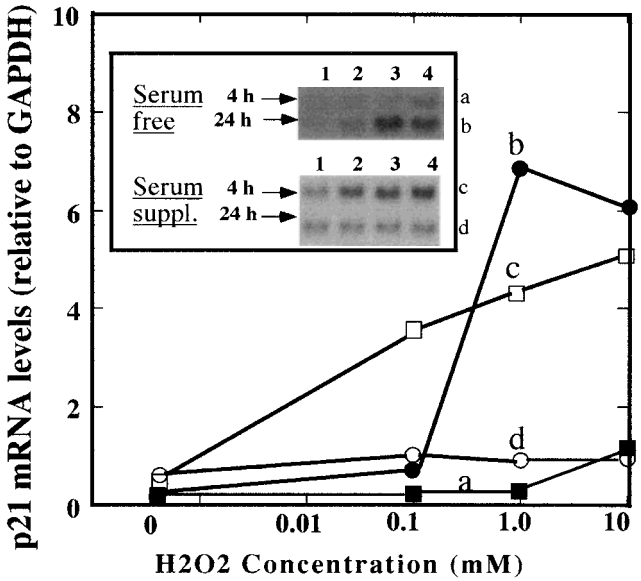

Fig. 7. $p21^{Waf1}$ mRNA expression levels after H_2O_2 treatment. Experimental conditions were similar to those described for Figure 6. H_2O_2 at 0.1 mM (**lane 2**), 1 mM (**lane 3**), 5 mM (**lane 4**) or none (**lane 1**) was added for 30 min at 37°C to either cells maintained for 4 days in serum-free medium (serum-free) or 2 days in 10% serum (serum-suppl). After the genotoxic stress cells were incubated for either 4 hr (**lanes a** and **c**) or 24 hr (**lanes b** and **d**) with fresh DMEM containing 10% serum total RNA was extracted using a commercial isolation kit and Northern blot analysis was carried out using a c-DNA radiolabeled probe followed by autoradiography. Radioactive bands corresponding to $p21^{Waf1}$ mRNA were compared to GAPDH mRNA. The experiment was repeated at least twice and similar results were obtained.

tial precursor cells. In culture, these cells differentiate to astrocytes type II in the presence of serum, die in the absence of serum, and proliferate or differentiate to mature oligodendrocytes when certain growth factors are added (Barres et al., 1993). Depending on their developmental stage, oligodendrocyte precursors differentially respond to mitogenic signals, phorbol esters, and environmental stimuli (Pfeiffer et al., 1993; reviewed in McMorris and McKinnon, 1996). In this article, OLN 93 cells, a cell line with oligodendroglial properties (Richter-Landsberg and Heinrich, 1996), were used to search for a link between cell-cycle stages and the consequences of oxidative stress on cellular death. These cells respond to serum deprivation by a cessation of cell proliferation and by an increase in morphological differentiation. Cell-cycle analysis revealed that after 3 days of serum removal, 80% of the cells resided in the G1 phase and only 4% were present in the S phase, with almost no concommittant signs of cell death (Figs. 4 and 5). By contrast, cells grown in medium containing 10% serum revealed a mixed population of 42% and 14% of the cells in the G1 and S phase, respectively. The application of a second stress, that is, H_2O_2, revealed that cells in these different stages of the cell cycle exhibited a differential response. In both populations a shift of cells from G1 to S phase and an arrest in the S phase were observed, but only serum-deprived cells underwent the onset of an apoptotic death program. Thus, serum removal not only caused growth arrest and morphological differentiation, as has been described in other systems (Howard et al., 1993; Eves et al., 1996; Qi et al., 1997; Ma et al., 1998; Yoshida et al., 1998), but also rendered the cells more susceptible to other stresses. This indicates a complex relation of events, which may ultimately lead to either survival, differentiation, or cell death.

At this time, there are many gaps in our understanding of the mechanism of the genotoxic action exerted by H_2O_2 in cultured mammalian cells and, in particular, the window at which this agent may exert a regulatory or a cytotoxic action. Treatment of OLN 93 with H_2O_2 caused cell arrest in the S phase both in logarithmically growing and in quiescent cells, although only in the latter, cells died by apoptosis (Fig. 6). That points out to a specific vulnerable population of cells presumably existing in the serum-deprived cultures. On the other hand, in spite of relatively high concentrations of H_2O_2, the genotoxic stress did not result in complete cell death even after prolonged starvation. In fact, mitochondrial activity losses

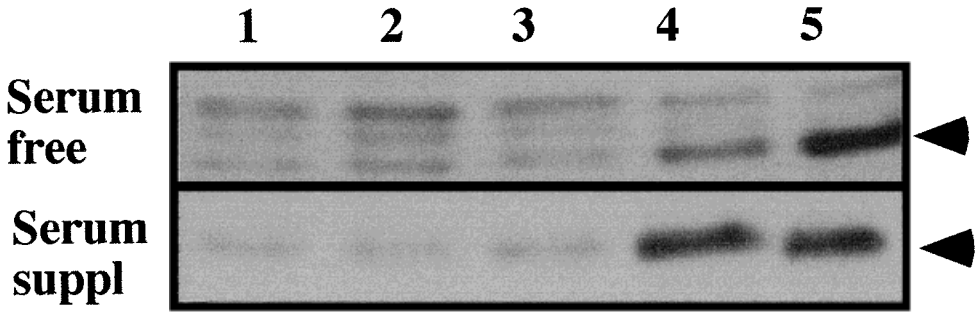

Fig. 8. $p21^{Waf1}$ protein levels after treatment with H_2O_2. Experimental conditions were similar to those described for Figure 7. One mM H_2O_2 was added for 30 min to either cells maintained for 2 days in 10% serum or 3 days in serum-free medium. The H_2O_2 containing medium was discarded and cells incubated in 10% serum containing DMEM for 1 hr (**lane 2**), 2 hr (**lane 3**), 4 hr (**lane 4**), and 24 hr (**lane 5**). Total cellular proteins were prepared and 0.04-mg aliquots were resolved on 12%–15% SDS-PAGE as detailed in the Materials and Methods section. After transblotting bands were subjected to $p21^{Waf1}$ antibody and visualization was done after using a second HRP-linked antibody stained with ECL. Control cells were run in **lane 1**.

after the first day following stress were restored to the original levels after 4 days (Fig. 2). Although not a reliable criteria for cell death (Hansen et al., 1989), the MTT measurement indicates a return to the normal metabolic status of the cells.

To account for the high proportion of apoptotically dying cells, a selective targeting of the genotoxic stress toward a discrete cell population, namely, that associated with the more differentiated state is suggested. This assumption is based on the finding that serum-deprived, in contrast to proliferating cells, acquire a certain degree of morphological differentiation and thus become more susceptible. This possibility is in accord with recent finding examining the relationships and transitions between cell cycle, differentiation, and apoptosis. For example, NGF deprivation enhanced apoptosis of terminally differentiated PC12 cells (Mills et al., 1997), whereas apoptotic death in retinoic acid-treated P19 carcinoma cells occurred simultaneously with cell differentiation (Ninomiya et al., 1997). Similarly, the hippocampal neuronal cells H19-7 differentiated with basic FGF at the nonpermissive temperature, died by apoptosis (Eves et al., 1996). The link between cell differentiation and cell death is an attractive possibility that may be resolved experimentally, through the use of specific oligodendrocyte cell differentiation markers along with apoptotic cell death markers.

To this stage, the linkage of H_2O_2-induced apoptotic cell death and cell differentiation is also inferred from our most recent studies using the ubiquitous p53 tumor suppressor protein and by the current studies using the $p21^{Waf1}$ protein, a universal inhibitor of cyclin-dependent kinases. We have shown that within 20 min after treatment with H_2O_2 the immunofluorescently identified p53 tumor suppressor protein was induced to migrate into the OLN 93 cell nucleus (Uberti et al., 1999). At a later time, this translocation was followed by a massive apoptotic cell death, notable particularly in serum-deprived cultures. The latter already exhibit high levels of p53 in the nucleus (Uberti et al., 1999). Given the fact that relatively high constitutive levels of p53 tumor suppressor are expressed in normal differentiated oligodendrocytes (Eizenberg et al., 1996) it is possible that p53 is involved in both controlling physiological growth arrest in the OLN 93 cells and also in enhancing cell death.

One of the more important genes activated by 53 is that encoding for $p21^{Waf1}$, an inhibitor of cyclin-dependent kinases and an important regulator of crucial cellular processes, including cell-cycle control, cellular differentiation, and the response to genotoxic stress. In this study we demonstrate that G1-arrested cells attained by serum deprivation after 3–4 days show low levels of $p21^{Waf1}$ and express little mRNA levels. These low levels are in accord with similar observations made in oligodendroglia cell lineages (Durand et al., 1997) and in mouse brain embryos (Parker et al., 1995). Following H_2O_2 addition, the rapid increase in $p21^{Waf1}$ expression in the proliferating OLN 93 cells may indicate a mobilization of this cell-cycle inhibitor protein to enable DNA repair and cell rescue. The slowing down of cell division accompanied by a transient arrest of cells in the S phase (Fig. 5) and the high levels of $p21^{Waf1}$ are indicative of downregulation of cyclin-dependent kinase activities (La Baer et al., 1997) and the cell rescue that follows are in accord with the resumption of cell growth (Fig. 2). Interestingly, $p21^{Waf1}$ expression is delayed in serum-deprived cultures after exposure to H_2O_2. Thus, it is tempting to speculate that a certain population of OLN cells although arrested in the G1 stage, respond to H_2O_2 by elevating their $p21^{Waf1}$ levels and eventually resume growth. This is in

accordance with the possible inhibitory role of $p21^{Waf1}$ in apoptosis shown in other stress conditions (Wang et al., 1997; Yu et al., 1998). Nevertheless, the mechanism by which $p21^{Waf1}$ confers protection and prevents death in G1 arrested cells while it fails to do so in differentiated cells, remains still a puzzle. It is possible that $_{p21^{Waf1}}$ can rescue cells only during a certain window of the cell-cycle and as previously hypothesized (Gorospe et al., 1996), $p21^{Waf1}$ is further emerging as an important cellular growth regulator. Thus elevation of $p21^{Waf1}$ in dividing cells after 4 hr is perhaps a crucial regulatory element for acquiring protection from cell death.

In conclusion, the principal observation stemming from this study is that the genotoxic effect of H_2O_2 on OLN 93 cells depends on previous growth conditions; OLN 93 cells arrested at G1 phase by serum deprivation exhibit a greater sensitivity to H_2O_2-mediated apoptosis than actively proliferating cells that show complete resistance. This effect is associated with the timing of $p21^{Waf1}$ expression; it is faster in proliferating cells than in, nondividing, differentiated cells, raising the possibility that $p21^{Waf1}$ expression protects from apoptosis.

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REFERENCES

Atabay C, Cagnoli CM, Kharlamov E, Ikonomovic MD, Manev H. 1996. Removal of serum from primary cultures of cerebellar granule neurons induces oxidative stress and DNA fragmentation: protection with antioxidants and glutamate receptor antagonists. J Neurosci Res 43:465–475.

Back SA, Gan X, Li Y, Rosenberg PA, Volpe JJ. 1998. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. J Neurosci 18:6241–6253.

Barres BA, Hart IK, Coles HSR, Burne JF, Voyvodic JT, Richardson WD, Raff MC.1992. Cell death and control of cell survival in the oligodendrocyte lineage. Cell 70:31–46.

Batistatou A, Greene LA. 1993. Internucleosomal DNA cleavage and neuronal cell survival/death. J Cell Biol 122 :523–532

Behl C, Davis JB, Klier FG, Schubert D. 1994. Amyloid β peptide induces necrosis rather than apoptosis. Brain Res 645:253–264.

Bradford MM. 1986. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:238–259.

Chakravarthy BR, Wong J, Durkin JP. 1995. Evidence that the modulation of membrane-associated protein kinase C activity by an endogenous inhibitor plays a role in N1E-115 murine neuroblastoma cell differentiation. J Neurochem 65:1569–1579.

Cuzner ML, Norton WT. 1996. Biochemistry of demyelination. Brain Pathol 6:231–242.

Dawson VL, Dawson TM. 1996. Free radicals and neuronal cell death. Cell Death Differentiation 3:71–78.

Durand B, Gao FB, Raff M. 1997. Accumulation of the cyclin-dependent kinase inhibitor p27/kip1 and the timing of oligodendrocyte differentiation. EMBO J 16:306–317.

Eizenberg O, Faber-Elman A, Gottlieb E, Oren M, Rotter V, Schwartz, M. 1996. p53 plays a regulatory role in differentiation and apoptosis of central nervous system-associated cells. Mol Cell Biol 16:5178–5185.

Eves EM, Boise LH, Thompson CB, Wagner AJ, Hay N, Rosner MR. 1996. Apoptosis induced by differentiation or serum deprivation in an immortalized central nervous system neuronal cell line. J Neurochem 67:1908–1920.

Gorospe M, Martindale JL, Sheikh MS, Fornace AJ Jr, Holbrook NJ. 1996. Regulation of $p21^{Cip1/Waf1}$ expression by cellular stress: p53-dependent and p53-independent mechanisms. Mol Cell Diff 4:47–65.

Halliwell B. 1992. Reactive oxygen species and the central nervous system. J Neurochem 59:1609–1623.

Hansen MB, Nielsen SE, Berg K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203–210.

Howard MK, Burke LC, Mailhos C, Pizzey A, Gilbert CS, Lawson WD, Collins MK, Thomas NS, Latchman DS. 1993. Cell cycle arrest of proliferating neuronal cells by serum deprivation can result in either apoptosis or differentiation. J Neurochem 60:1783–1791.

Kim YS, Kim SU. 1991. Oligodendroglial cell death induced by oxygen radicals and its protection by catalase. J Neurosci Res 29:100–106.

La Baer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. 1997. New functional activities for p21 family of CDK inhibitors. Genes Dev 11:847–862.

Lyras L, Cairns NJ, Jenner A, Jenner P, Halliwell B. 1997. An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. J Neurochem 68:2061–2069.

Ma X, Wang Y, Stephens NL. 1998. Serum deprivation induces a unique hypercontractile phenotype of cultured smooth muscle cells. Am J Physiol 274:1206–1214.

McMorris FA,McKinnon RD. 1996. Regulation of oligodendrocyte development and CNS myelination by growth factors: prospects for therapy of demyelinating disease. Brain Pathol 6:313–329.

Mills JC, Kim LH, Pittman RN. 1997. Differentiation to an NGF-dependent state and apoptosis following NGF removal both occur asynchronously in cultures of PC12 cells. Exp Cell Res 231:337–345

Ninomiya Y, Adams R, Morriss-Kay GM. 1997. Eto-K Apoptotic cell death in neuronal differentiation of P19 EC cells: cell death follows reentry into S phase. J Cell Physiol 172:25–35.

Olanow CW. 1993. A radical hypothesis for neurodegeneration. Trends Neurosci 16:439–444.

Parker S, Eichele G, Zhang P, Rawls A, Sands A, Bradley A, Olson A, Harper J, Elledge S. 1995. p53 independent expression of p21Cip1 in muscle and other terminally differentiating cells. Science 267:1024–1027.

Pfeiffer SE, Warrington AE, Bansal R. 1993. The oligodendrocyte and its many cellular processes. Trends Cell Biol 3:191–197.

Qi Y, Wang KT, McMillian M, Chikaraishi DM. 1997. Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. J Neurosci 17:1217–1225.

Qiu X, Forman HJ, Schonthal AH, Cadenas E. 1996. Induction of p21 mediated by reactive oxygen species formed during the metabolism of aziridinylbenzoquinones by HCT116 cells. J Biol Chem 271:31915–31921.

Richter-Landsberg C, Heinrich M. 1996. OLN 93: a new permanent oligodendroglia cell line derived from primary rat brain glial cultures. J Neurosci Res 45:161–171.

Richter-Landsberg C, Vollgraf U. 1998. Mode of cell injury and death after hydrogen peroxide exposure in cultured oligodendroglia cells. Exp Cell Res 244:218–229.

Rodriguez-Tarduchi G, Collins M, Lopes-Rivas A. 1990. Regulation of apoptosis in interleukin-3 dependent hemopoietic cells by interleukin-3 and calcium ionophores EMBO J 9:2997–3002.

Thorburne SK, Juurlink BH. 1996. Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. J Neurochem 67:1014–1022.

Tournier C, Thomas G, Pierre J, Jacquemin C, Pierre M, Saunier B. 1997. Mediation by arachidonic acid metabolites of the H_2O_2-induced stimulation of mitogen-activated protein kinases (extracellular-signal-regulated kinase and c-Jun NH2-terminal kinase). Eur J Biochem 244:587–595.

Uberti D, Yavin E, Gil S, Goldfinger N, Rotter V. 1999. Hydrogen peroxide induces nuclear translocation of p53 and apoptosis in cells of oligodendroglia origin. Mol Brain Res 65:167–175.

Wang YA, Elson A, Leder P. 1997. Loss of p21 increases sensitivity to ionizing radiation and delays the onset of lymphoma in atm-deficient mice. Proc Natl Acad Sci USA 94:14590–14595.

Wood KA, Youle RJ. 1994. Apoptosis and free radicals. Ann NY Acad Sci 738:400–407.

Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y. 1998. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates ''reserve cells.'' J Cell Sci 111:769–779.

Yu D, Jing T, Liu B, Yao J, Tan M, McDonnell TJ, Hung M-C. 1998. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of $p21^{Cip1}$, which inhibits $p34^{Cdc2}$ kinase. Mol Cell 2:581–591.