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# Hydrogen peroxide induces nuclear translocation of p53 and apoptosis in cells of oligodendroglia origin

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**Abstract**

The observation that apoptosis is an inherent pathway in oligodendrocytes development coupled with the notion that wild-type p53 is expressed in these cells, prompted us to investigate the interrelationship between the two phenomena. Using a permanent oligodendroglia-like cell line (OLN 93), we examined the role of p53 protein in apoptosis following a DNA insult induced by a brief exposure to $H_2O_2$. A marked translocation of p53 from the cytosolic to the nuclear compartment was notable by 20 min, following a 5 min treatment with 1 mM $H_2O_2$ as identified by cell immunostaining. By 48 h following $H_2O_2$ addition, nearly 60% of the cells exhibited p53 in the nuclei. At this time, a large proportion of the cells underwent apoptosis as identified by DAPI nuclear staining. The genotoxic-induced p53 relocalization appeared to be cell cycle phase specific; thus OLN 93 cultures enriched for cells in the $G_0/G_1$ stage by serum starvation, and abundant in nuclear-associated p53, were more susceptible to $H_2O_2$-induced apoptosis than their untreated counterparts and than double thymidine block, $G_1/S$ enriched, cultures. Analysis of the expression of p53 downstream genes indicated that p21 and mdm2 were upregulated following p53 nuclear translocation. From the kinetics of protein accumulation, it appears that mdm2 enhancement accelerated the exit of p53 from the nucleus to the cytosol. Our results suggest that following stress, oligodendroglia-like cells are induced to undergo p53-dependent apoptosis, an event that coincides with p53 nuclear translocation and is cell-cycle related. 

*Keywords:* Oligodendrocyte; Oxidative stress; p53; Subcellular localization; Suppressor gene; Oligodendroglia; Stress; $H_2O_2$

## 1. Introduction

The tumor suppressor gene p53 plays a central role in the maintenance of genomic stability [17,22,24,26,28,29]. Genotoxic stress was shown to induce p53 protein stabilization, leading to either cell growth arrest [6], DNA repair [27,44], onset of apoptosis [54] or induction of cell differentiation [2,18,46,48,49]. Versatility in p53 activity, as a response to various external or internal signals. may serve as a control mechanism which underlies central decisions in developmental pathways in vivo [1].

The development and physiological performance of the neural system involves ongoing processes of cell differentiation and apoptosis [8,31,34]. Furthermore, a number of studies indicate the involvement of p53 in developmental pathways of neural cells. Differentiation of rat primary cultures of oligodendrocytes and neurons in culture was shown to be accompanied by the migration of p53 protein into the nuclear compartment. Inhibition of the endogenous wild-type p53, by introduction of the C-terminal mini protein, blocked both apoptosis and differentiation of these cells [14]. Enhancement of p53 expression was also demonstrated during cerebellar granule neurons maturation in vitro [52]. The conclusion that p53 is involved in normal brain development is substantiated by the observation that p53 deficient embryos exhibit neural developmental abnormalities. Defects in neural tube development, such as exencephaly, were observed in about 8–16% of p53 null female embryos, thus suggesting that p53 plays a role in neural tube closure [3,43].

The subcellular localization of p53 seems to play an important role in the activity of the p53 molecule. Both import into the nucleus and export to the cytoplasm appear

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to be mediated by specific pathways that are associated with either the stabilization of the active protein or its inactivation by degradation, respectively. Nuclear localization of p53 was shown to be mediated by nuclear localization signals (NLS) inherent in the C-terminus of the protein [45]. Interruption of nuclear localization was shown to interfere with the p53 activity [47]. Following stress, p53 was shown to accumulate within the nucleus where it appears to be protected from degradation by the proteosome machinery [25]. It was recently shown that the export of p53 from the nucleus to the cytoplasm is controlled by direct interactions with the mdm2 protein. It was suggested that following this complex formation, the two proteins are exported to the cytoplasm through the Nuclear Export Signal (NES) contained within the mdm2 molecule. Within the cytoplasm, p53 is degraded [42].

To further investigate the mechanism that underlies the role of p53 in the apoptosis of oligodendrocytes we have utilized an oligodendrocyte permanent cell line (OLN 93), derived from spontaneously transformed cells in primary rat brain glial cells [41] which is exposed to a brief genotoxic stress using $H_2O_2$. It is well established that free hydroxyl radicals generated from excess $H_2O_2$ have a deleterious effect not only on proteins and lipid membranes, but also on DNA, causing base modifications, single–double-strand breaks, strand excision, and the cross linking of bases [19]. The nervous tissue and oligodendrocytes in particular, show a high sensitivity to oxidative stress [23].

In this report we demonstrate that treatment of OLN 93 cells with $H_2O_2$ enhance translocation of the p53 protein from the cytoplasm to the nucleus leading to apoptosis. The kinetic patterns of protein accumulation, and the subcellular distribution of mdm2 support the recent notion that export of p53 to the cytosol may be shuttled by mdm2 [42].

## 2. Materials and methods

### 2.1. Cell growth and $H_2O_2$ application

A permanent cell line, OLN 93, derived from a primary culture of rat oligodendroglia cells [41], was used. Cells were plated onto 50 μg/ml poly-L-lysine precoated dishes and routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 units/mg penicillin/streptomycin (GIBCO), and 10% fetal calf serum at 37°C and an atmosphere of air enriched with 7.5% $CO_2$. For $H_2O_2$, addition, cells were plated on poly-L-lysine and 12 h after attachment, 1 mM $H_2O_2$ was added in DMEM containing 0.1% serum. After 5 min, the medium was discarded, replaced with complete DMEM containing 10% serum and grown at 37°C for specified time periods. Enrichment of cells in the $G_0/G_1$ cell cycle phase was attained by growth in DMEM containing 0.1% serum for 48 h. Enrichment of cells in the $G_1/S$ cell cycle phase done by a double thymidine block, briefly consisted of treating cells for 16 h with 2 mM thymidine, followed by an 8 h incubation with 24 mM deoxycytidine and 16 h with a second 2 mM tymidine pulse [50].

### 2.2. Immunocytochemical staining

For immunocytochemistry, OLN 93 cells were plated on poly-L-lysine coated coverslip. Cultures were fixed for 30 min in 50 mM PBS containing 4% paraformaldehyde. Following several rinses in PBS, cells were incubated for 20 min in Tris-buffer saline (pH 7.4) containing 3% $H_2O_2$ to block endogenous peroxidase staining. Cells were permeabilized by exposure to 0.02% Triton X-100 in PBS for 5 min followed by incubation for 24 h at 4°C with the primary antibodies. After several rinses, cells were processed using the avidin–biotin complex Kit (ABC Elite Kit, Vector Laboratories). In order to verify the specificity of the chromogen reaction, control cells were processed identically, except that the cells were incubated with the primary antibody solvent only. The cells were viewed and photographed using a Zeiss microscope. The percentage of p53 nuclear localization was established by counting at least five representative fields from each coverslip. The statistical significance of differences between the values was tested by analysis of variance. Data are presented as the mean ± S.E.M. of at least three experiments.

### 2.3. Antibodies

Monoclonal anti-p53 PAb-421 antibody [20] was purified from ascitic fluids. Commercial anti $p21^{waf-1}$ antibodies were used. Anti-mdm-2 monoclonal antibodies (2A10) were a generous gift from A. Levine (Princeton University, USA).

### 2.4. Cell fractionation

For nuclear and cytosol fractionation, cells were incubated with hypotonic buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 10 mM KCl) for 15 min, lysed by adding NP-40, and timely stirred by vortexing. The samples were centrifuged, and supernatants (cytosol fraction) were separated from the pellet (nuclear fraction). The cell pellet was resuspended for 15 min in buffer C (20 mM HEPES, 25% glycerol, 40 mM NaCl, 1.5 mM $MgCl_2$ 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF), and then centrifuged. The ensuing supernatant, corresponding to the nuclear fraction was collected. All samples were aliquoted and stored at −80°C.

### 2.5. Western blot analysis

Samples were subjected to 10% or 12% SDS-polyacrylamide gel electrophoresis. Gels were blotted onto nitro-

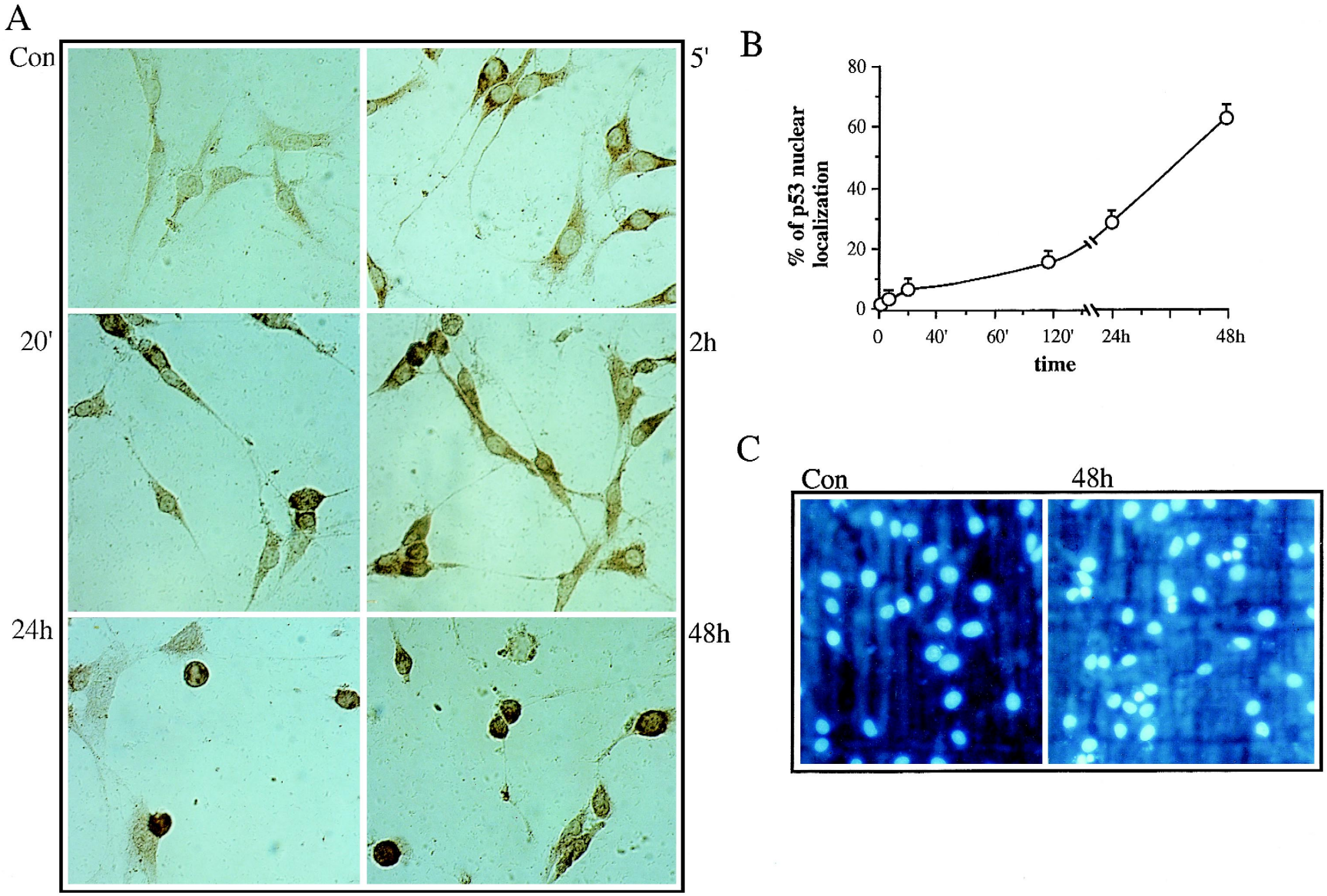


Fig. 1. Time course of p53 subcellular distribution and DAPI stain in OLN 93 cells following $H_2O_2$ treatment. OLN 93 cells were treated for 5 min with 1 mM $H_2O_2$. Medium was removed and cells were returned to 10% serum containing medium for the designated time periods as detailed under Section 2. Panel A depicts the time course of p53 translocation from the cytosolic to the nuclear compartment after exposure to $H_2O_2$. Panel B depicts the percentage ± S.E.M. of p53 positive nuclei counted from at least three different experiments. Panel C shows a DAPI staining of $H_2O_2$ treated cell (48 h) compared to control (Con). Notice the abundance of shrunken nuclei indicative of apoptosis.

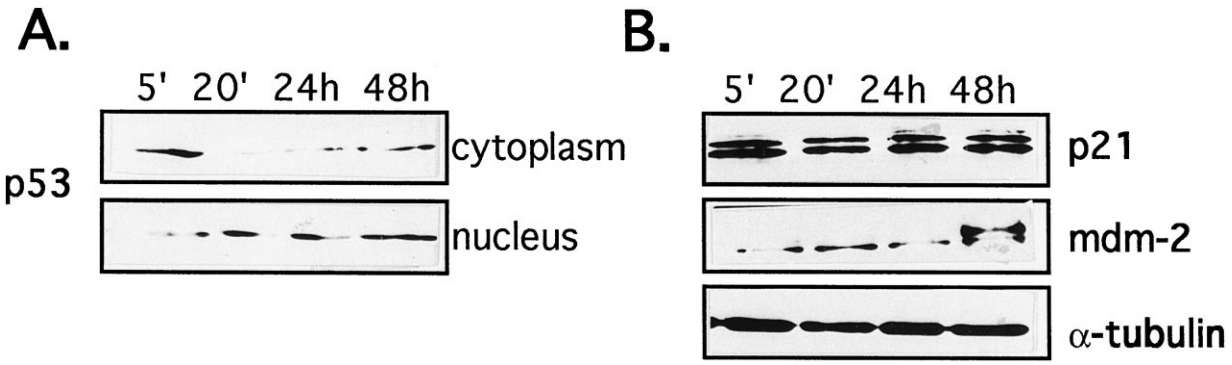


Fig. 2. Subcellular distribution of p53, p21 and mdm2. OLN 93 cells were exposed for 5 min to 1 mM $H_2O_2$ and returned to the growth medium. After designated time periods cells were harvested and subcellular fractionation was carried out as detailed under Section 2. p53, p21 and mdm2 levels in the cytoplasmic and nuclear protein fractions following $H_2O_2$ treatment was carried out with the anti-p53 monoclonal antibody PAb-421 (panel A), p21 polyclonal antibody, and anti-mdm2 monoclonal antibody 2A10 (panel B). Anti-a tubulin monoclonal antibody was used as controls.

cellulose (Amersham) and incubated for 30 min with PBS containing 3% (by vol.) skim milk. After washing with PBS containing 0.05% of Tween (PBST), blots were incubated with appropriated antibodies for 1 h. After rinsing,

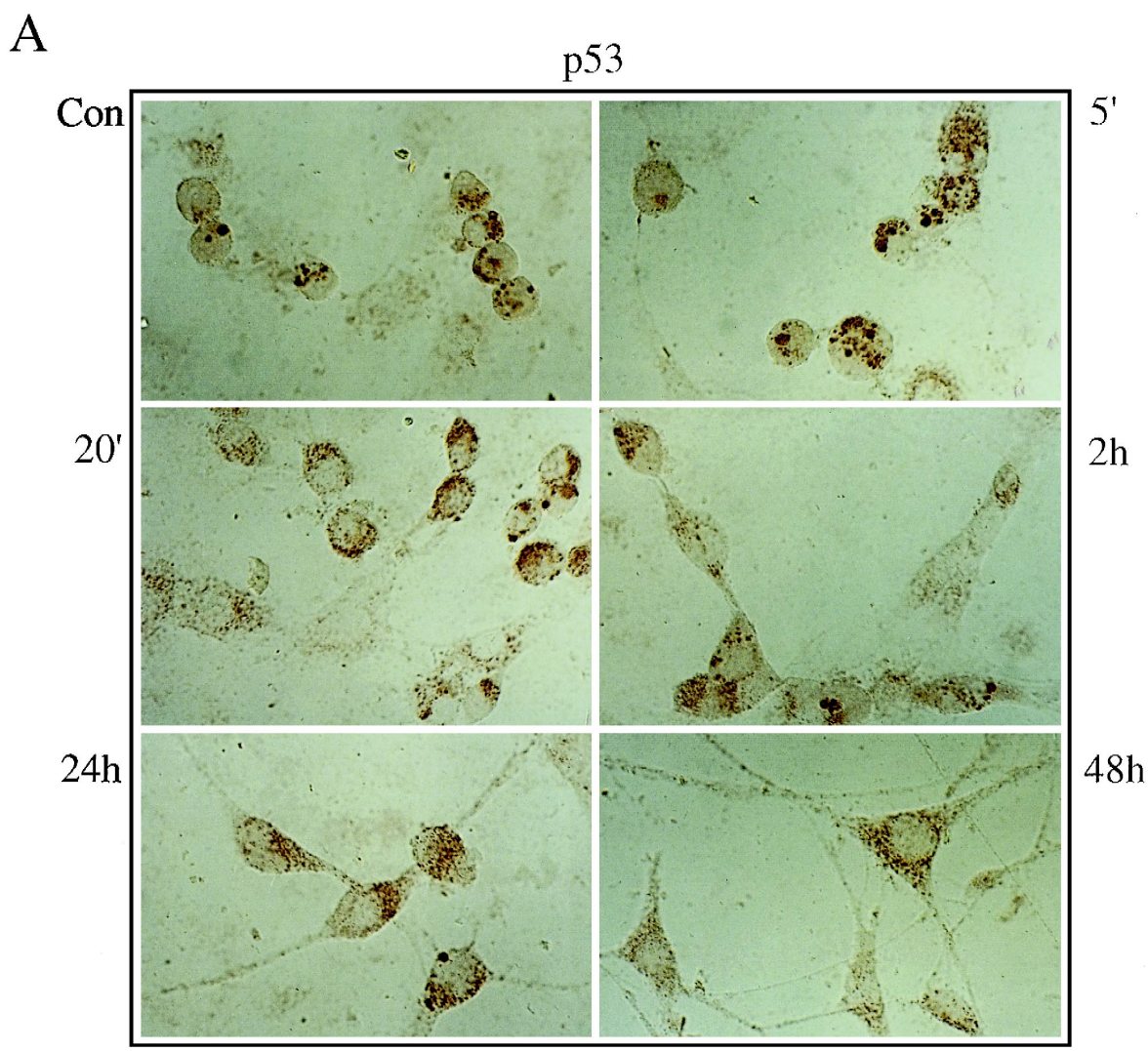


Fig. 3. Subcellular localization of p53 in $G_0/G_1$ enriched OLN 93 cells after $H_2O_2$ treatment. OLN 93 cells were grown for 48 h in 0.1% serum and subsequently treated for 5 min with 1 mM $H_2O_2$. Experimental details were similar to those described in Fig. 1. The composite depicts the time course of p53 immunostaining distribution between the cytosolic and the nuclear compartments.

the second antibody linked to horse radish 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 done with ECL Plus (Western blotting system) reagent from Amersham (UK).

### 2.6. Cell cycle analysis

Cells were cooled to 4°C, rinsed twice in PBS, scraped off the plate, collected, centrifuged and fixed in 70% ethanol at −20°C. After 2 h at −20°C, cells were centrifuged, rinsed once with PBS, and resuspended in 1 ml of PBS containing 0.5 mg/ml RNase A. After incubation at 37°C for 1 h, fixed cells were stained for 15 min with 50 μg/ml Propidium iodide (PI). Cell cycle reading (10–15.000 cells) was performed on 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 statistical significance of differences between the values was tested by analysis of variance. Data are presented as the mean ± S.E.M. of at least three experiments.

## 3. Results

### 3.1. $H_2O_2$-dependent p53 nuclear translocation

In order to evaluate the effects of $H_2O_2$ on p53 expression and cell survival, we used OLN 93, an oligodendrocyte permanent cell line. This line, derived from spontaneously transformed cells of primary rat brain glial cells,

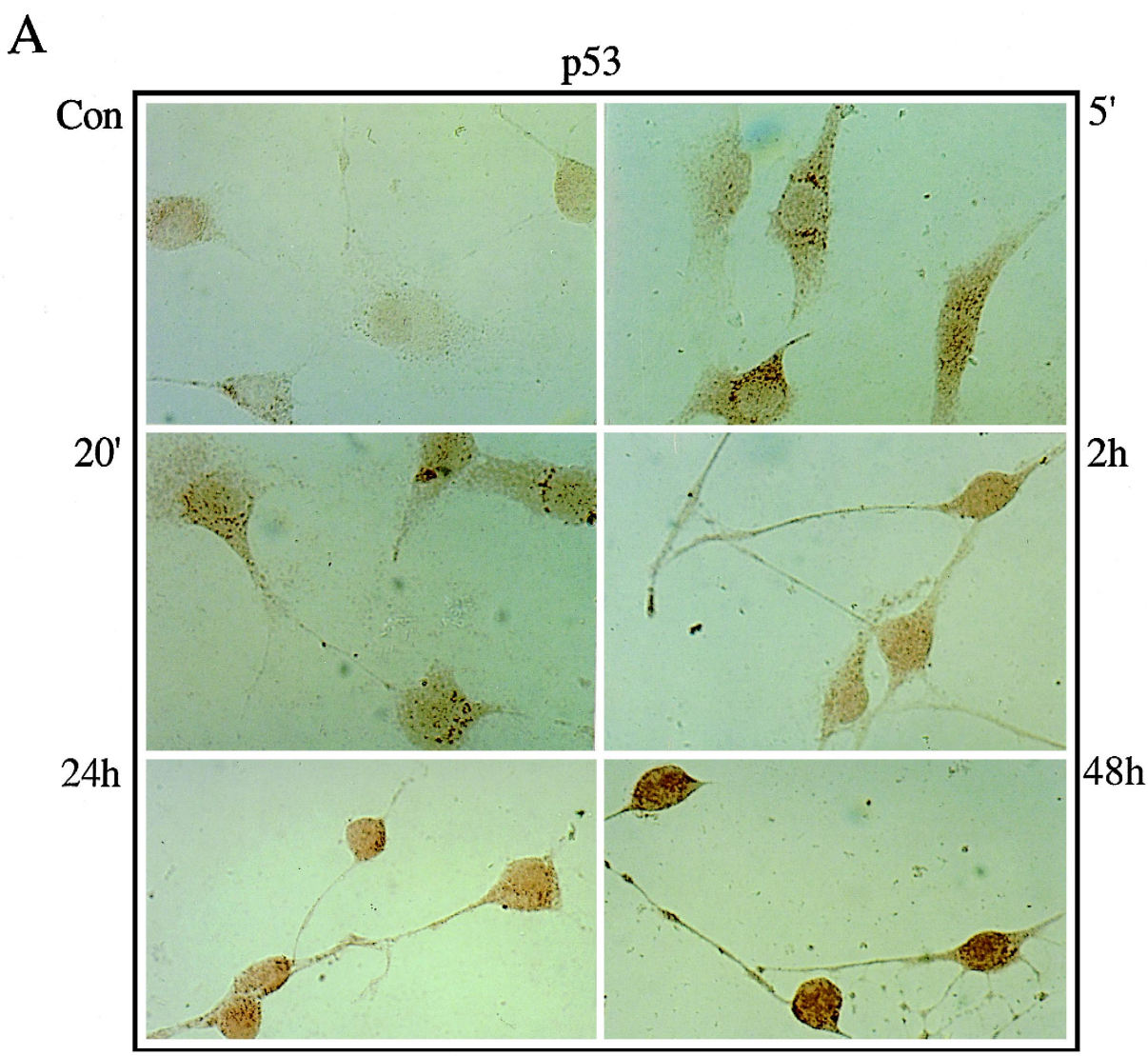


Fig. 4. Subcellular localization of p53 in $G_1$/S enriched OLN 93 cells after $H_2O_2$ treatment. OLN 93 cells were subjected to double thymidine block as detailed under Section 2 and treatment with $H_2O_2$ was done as detailed in Fig. 1. The composite depicts the time course of p53 immunostaining distribution between the cytosolic and the nuclear compartments.

exhibited morphological features and antigenic properties comparable to 5 to 10 day old (postnatal time) cultured rat brain oligodendrocytes [41]. Cells were briefly treated with 1 mM $H_2O_2$ for 5 min after which the genotoxic agent was replaced with fresh medium. At different time points, cells were fixed and stained with PAb-421, a p53 specific monoclonal antibody. To analyse apoptotic cell death, parallel plates were subjected to DAPI staining. Fig. 1 (panel A) depicts a representative panel of cells stained at various time periods following the oxidative stress. Notable, untreated cells exhibited a weak cytoplasmic p53 signal. Five min following the $H_2O_2$ insult, while still confined to the cytoplasmic compartment, the p53 signal was enhanced. Only scattered p53 positive nuclei were observed after 20 min. After 24 h, the number of cells exhibiting a p53 nuclear localization was significantly increased, and 2 days later, more than 60% of cells expressed p53 nuclear immunostaining (panel B). As indicated by the DAPI stain, pyknotic and shrinking nuclei, indicative of apoptosis [21] were observed 48 h following treatment (Fig. 1C). Under the same conditions, the cell nuclei of untreated cells exhibited a normal morphology.

To corroborate the immunochemical visualization of p53 translocation into the nucleus, the content of p53 in the nuclear and cytoplasmic fractions was evaluated by Western blot analysis. As noticed in Fig. 2 (panel A), 5 min following $H_2O_2$ treatment, p53 protein was mainly detected in the cytoplasmic fraction while fewer or no changes were seen in the nuclear fraction. However, 20 min later, the nuclear fraction showed high levels of p53 protein. At this time point p53 disappeared from the cytoplasm and seemed to accumulate in the nuclear compartment (Fig. 2 panel A). By 24 and 48 h following the $H_2O_2$ stress, p53 levels remained high in the nuclear fraction but low in the cytoplasmic fraction. These patterns agree with the immunostaining profiles observed in Fig. 1.

Since both p21 and mdm2 are rapidly activated by functional p53 [5,16,33], we have examined whether expression of these target genes was altered in cells exposed to $H_2O_2$. Western blot analysis of the total cell extracts using either a polyclonal antibody against p21, or a monoclonal antibody 2A10 against the core domain of the mdm2 protein [36] was performed. For comparison, the levels of α-tubulin, detected by a monoclonal antibody were analyzed under the same conditions. As shown in Fig. 2 (panel B), p21 expression was already high as early as 5 min after $H_2O_2$ addition and remained as such for at least 48 h. In contrast, mdm-2 was progressively elevated

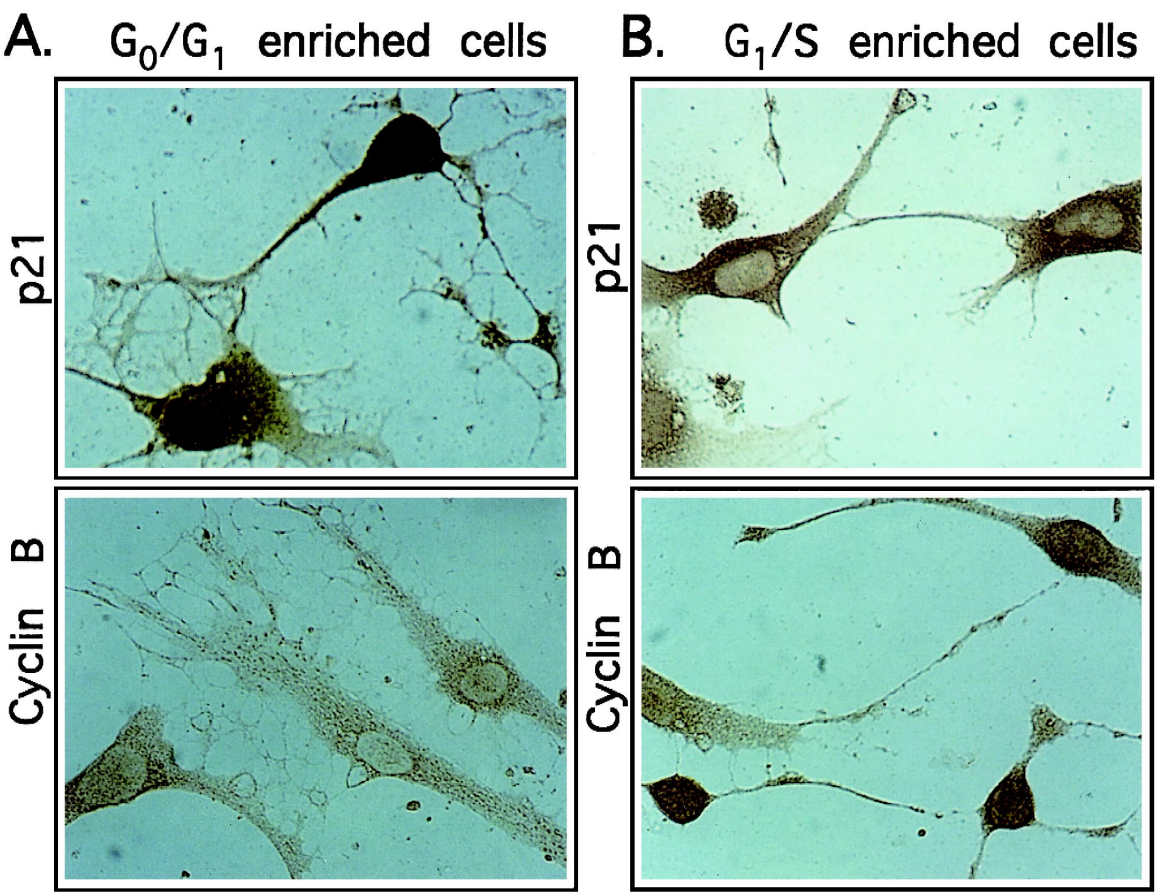


Fig. 5. Immunostaining characterization of cell cycle proteins in OLN 93 cells enriched for $G_0/G_1$ or $G_1/S$ cells. $G_0/G_1$ enriched cultures (panel A) obtained as detailed in Fig. 3 and $G_1/S$ enriched cultures (panel B) obtained as in Fig. 4, were stained with antibodies to p21 or cyclin B1 proteins expression in the control $G_O/G_1$ enriched cells respectively.

following the $H_2O_2$ pulse, reaching a peak 48 h after treatment. This suggests that OLN 93 cells constitutively express p21 in a p53-independent manner, while expression of mdm2 is induced in a p53-dependent manner.

### *3.2. Cell cycle-dependent p53 nuclear translocation and apoptosis*

Recent experiments pointing to a relationship between $G_1$-arrested OLN 93 cells and $H_2O_2$ induced cell death [21], prompted us to examine whether p53 nuclear translocation is also cell-cycle dependent. To that end, serum starvation and double thymidine block procedures [50] were used to enrich the proportion of OLN cells in the $G_0/G_1$ and $G_1/S$ phases, respectively. Morphological and DAPI examination of serum-deprived cultures after 2 days showed no pyknotic nuclei which are characteristic of cell death (data not shown). Nevertheless, as a result of serum starvation, many cells exhibited a remarkable p53 nuclear localization (Fig. 3). Treatment with $H_2O_2$, did not modify this pattern. In fact, as a result of 10% serum supplement after the starvation session, p53 shuttled back to the cytosol in a time-dependent fashion after 2 h; both $H_2O_2$-treated (Fig. 3) and untreated (micrographs not shown) cells showed p53 in the cytoplasm. Unlike serum-starved cells, which expressed p53 nuclear localization, cells enriched for $G_1/S$ by the double thymidine block showed little or no p53 staining in association with the nuclear compartment (Fig. 4). In that case, and similar to the data shown in Fig. 1, p53 protein was still largely evident in the cytoplasm up to 24 h following $H_2O_2$ treatment. Only after 48 h, was p53 protein detected in the nucleus.

Additional characterization of the $G_0/G_1$ or $G_1/S$ enriched cell populations, was done by immunostaining with anti-p21 and cyclin B1 antibodies [13,35] and $G_2/M$ [4,39]. The expression of p21 typical for the $G_0/G_1$ phase and expression of cyclin B1, typical for the $G_1/S_1$ phase were evaluated. As seen in Fig. 5 (panel A), after serum starvation, p21 was highly expressed in the nucleus while cyclin B1 was weakly detected in the cytoplasm, in accordance with a $G_0/G_1$ cell-cycle stage. In contrast, after the double thymidine block, p21 was mainly localized in the cytoplasm, while cyclin B1 was associated with the nucleus (Fig. 5 panel B), as expected for cells residing in the $G_1/S$ phase.

Since $G_0/G_1$ and G1/S enriched cell populations responded differently to nuclear p53 protein translocation after oxidative stress, we investigated whether these different patterns could also affect the induction of apoptosis in cells resting at different cell cycle phases. Cells at the $G_0/G_1$ and $G_1/S$ phases, each containing about 2.0% and 3.8% of cells in the sub-G1 phase respectively, were treated with $H_2O_2$ and 48 h later, fixed with 70% ethanol, stained with Propidium iodide and fluorescence analyzed by a cell sorter apparatus for apoptotic cells. Fig. 6 depicts the percent incidence of apoptotic cells contained in the sub-$G_1$ fraction, of either $G_0/G_1$ or $G_1/S$ enriched,

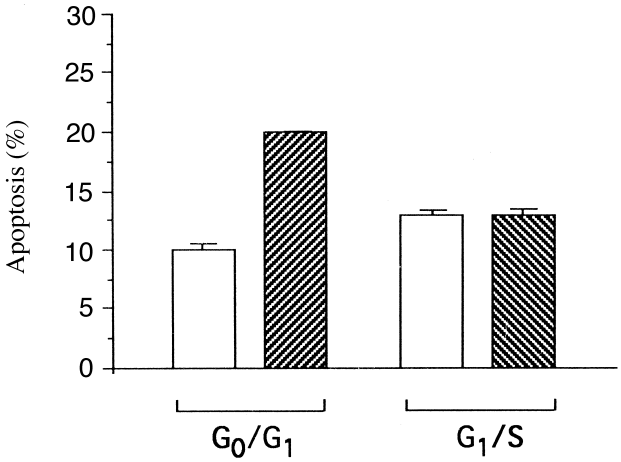


Fig. 6. Cell cycle analysis of $H_2O_2$ treated $G_0/G_1$ and $G_1/S$ enriched OLN 93 cells. OLN 93 cultures enriched with $G_0/G_1$cells (as in Fig. 3) and $G_1/S$ cells (as in Fig. 4) were subjected to 1 mM $H_2O_2$ for 5 min. Medium was discarded and fresh medium containing 10% serum was added. After 48 h non-treated cells (open bars) and $H_2O_2$ treated cells (striated bars) were fixed, stained with PI and analyzed by FACS for the sub-G1 fraction (as detailed under Section 2). Apoptosis is expressed as percentage of sub-G1 cells are $\pm$S.E.M. from triplicate analyses.

$H_2O_2$-treated or untreated cultures. A ten fold increase in the sub-$G_1$ cell fraction representative of apoptotic cells was observed in $G_0/G_1$ enriched cultures treated with $H_2O_2$ while only a five fold increase was observed in untreated, sister cultures. In contrast, $H_2O_2$ did not exhibit an additive increase of sub-$G_1$ cells beyond the basal value of the untreated, $G_1/S$ enriched sister cultures (Fig. 6). This is in line with our previous observations that, under these conditions $H_2O_2$ induced apoptosis, seems to be predominately expressed in cells at $G_0/G_1$ phase [21].

## 4. Discussion

Apoptosis is an inherent event in the life of the central nervous system, which permits a continuous remodeling of nerve cell networks, especially during development [37]. Previously, Raff et al. put forward the hypothesis that oligodendrocytes may undergo a salient commitment to apoptosis that will antagonize survival signals [40]. Furthermore, it was shown that free radicals of oxygen, generated from glucose and hypoxanthine oxidation, by glucose and xanthine oxidase, respectively, induced cell death of bovine oligodendrocytes in cultures [23].

Recent work has shown that p53 is directly involved in the IL-2 induced apoptosis of primary cultures of rat oligodendrocytes [14]. The relatively high constitutive levels of p53 expressed in normal differentiated oligodendrocytes suggests that p53 is involved in the control of the physiological growth arrest of these differentiated oligodendrocytes [15]. Thus, p53 may be involved in both differentiation and apoptosis of oligodendrocytes, possibly operating through separate pathways. In the present study, we report that a brief treatment of OLN 93 cells with

$H_2O_2$ induces the stabilized p53 tumor suppressor protein to migrate into the cell nucleus. Ultimately, this process was followed by cell apoptosis, thus suggesting that p53, may play a pivotal role in apoptosis of oligodendrocytes.

To evaluate the p53 down stream genes expressed following the $H_2O_2$ insult, we focused on the analysis of the expression of cyclin-dependent kinase inhibitor p21 [16] and mdm2 [5,33], both typical p53 target genes. The observation that the increase in p21 occurred concomitantly with stabilization and nuclear translocation of p53 protein hints that the latter may be dependent on a $G_0/G_1$ growth arrest controlled by p21. As expression of p21 precedes p53 translocation, we assumed that in this case the $H_2O_2$ insult induces p21 in a p53-independent manner. It is well accepted that p21 may be induced by either a p53-dependent or p53-independent mechanism [10,12, 30,32].

The finding that mdm2 expression seem to linger behind p53 overexpression confirms the existence of a loop control mechanism between these two proteins. Indeed, it was suggested that p53 induces mdm2 expression, which in turn causes p53 degradation [53]. It was recently shown that hdm2, the human homologue of the mouse mdm2, forms a complex with p53 and shuttles from the nucleus to the cytoplasm [42]. This nuclear export is controlled by a typical nuclear export signal contained within the hdm-2 protein. Cytoplasmic localization subjects p53 to proteosome degradation.

Various p53 activities were shown to be associated with the different cell cycle phases. p53-dependent $G_0/G_1$ arrest was associated with apoptosis [9]. Likewise, it was shown that cells at S-phase can be induced to undergo apoptosis in a p53-dependent manner. In addition it was suggested that p53 also plays a role in the $G_2/M$ checkpoint [51] and spindle formation [11]. Komarova et al. [25] suggested that cell-cycle selectivity of p53 nuclear accumulation was controlled by nuclear functions rather than by cytoplasm modification of p53. They demonstrated that nuclear accumulation of p53 following DNA damage will thereafter induce the activation of its downstream target gene that occurs during a specific window of the cell-cycle. Breder et al. [7] have shown that cell death of monocytes can be induced in the same cell-cycle phase in which cytokines are applied. It was suggested that the entire sequence of signal transduction and the effector mechanism of cell death may take place in the same phase without further cell cycle progression.

The present data suggest that $H_2O_2$ rapidly enhances p53 nuclear migration which in turn may activate specific target genes, such as p21 and mdm2. Presentation of $H_2O_2$ to cultures deprived of serum ($G_0/G_1$ enrichment) leads to a remarkable increase (from 2% to 20%) in apoptotic cell death (Fig. 6). Nevertheless, as evident in the p53 stain data (Fig. 3), a relocation of p53 from the nucleus to the cytosol takes place. The survival of these cells is most likely attributed to the addition of serum which restores the proliferative properties of OLN 93 cells. The differential sensitivity leading to either cell death or survival thereof is not yet understood. Based on our observations and recent emerging data from other experimental models [42], it is tempting to speculate that cells which escaped apoptosis, signal p53 to exit the nucleus, probably via interaction with mdm2; a process which subsequently ends in the degradation of the former.

In summary, our data support the conclusion that p53 plays a central role in controlling apoptosis of oligodendrocytes, an important pathway in the normal life of brain cells. Clearly under stressful conditions the mechanism by which p53 functions requires the subcellular translocation of the protein to the nucleus. In that context, it is worth mentioning that inactivation of p53 in neuroblastoma was found to be associated with aberrations in nuclear translocation of the p53 protein [38].

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