Oligodendrocytes and their precursors require phosphatidylinositol 3-kinase signaling for survival

Geeta S. Vemuri and F. Arthur McMorris*

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

*Author for correspondence (e-mail: mcmorris@wista.wistar.upenn.edu.)

INTRODUCTION

Insulin-like growth factors I and II (IGF-I and II) regulate the development of oligodendrocytes and the synthesis of myelin by promoting proliferation of oligodendrocytes and their precursors (McMorris et al., 1986, 1990; McMorris and Dubois-Dalcq, 1988), inducing immature oligodendrocyte precursors to develop into oligodendrocytes (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990), regulating myelin gene expression and the amount of myelin produced per oligodendrocyte (McMorris et al., 1990; Carson et al., 1993), and supporting the survival of oligodendrocytes (Barres et al., 1992, 1993). The biological effects of IGF-I and IGF-II on oligodendrocyte development and myelination are mediated by the type I IGF receptor, which is expressed both by oligodendrocyte precursors and by galactocerebroside (GC)-positive oligodendrocytes (McMorris et al., 1986, 1990). Binding of IGF-I or IGF-II to the type I IGF receptor activates the intrinsic tyrosine kinase of the receptor β-subunit and induces receptor autophosphorylation, leading to receptor-mediated phosphorylation of tyrosines within insulin receptor substrate-1 (IRS-1), IRS-2, and Shc, which then serve as sites for docking and activation of signal transduction molecules including phosphatidylinositol 3-kinase (PI3-K), the adapter molecule Grb2, the protein tyrosine phosphatase Syp (also known as SHPTP-2), and possibly others (reviewed by White and Kahn, 1994). Each of these molecules activates additional downstream signal transduction molecules leading ultimately to biological bo}

SUMMARY

Signal transduction in response to several growth factors that regulate oligodendrocyte development and survival involves the activation of phosphatidylinositol 3-kinase, which we detect in oligodendrocytes and their precursors. To investigate the role of this enzyme activity, we analyzed cell survival in cultures of oligodendrocytes treated with wortmannin or LY294002, two potent inhibitors of phosphatidylinositol 3-kinase. Cell survival was inhibited by 60-70% in these cultures within 24 hours, as quantitated by a tetrazolium staining assay for viable cells and by measurement of DNA content. Similar results were obtained with oligodendrocyte precursor cells. Nuclear of the dying cells contained fragmented DNA, as revealed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling assays, indicating that the cells were dying by apoptosis. Moreover, a significant increase in the number of cells with fragmented nuclear DNA was detected as early as 4 hours, well before any significant differences could be detected in glucose transport or cell viability. Exogenous addition of insulin-like growth factor-I, neurotrophin-3, platelet-derived growth factor, basic fibroblast growth factor, ciliary neurotrophic factor, N-acetyl cysteine, vitamin C, vitamin E, progesterone or serum did not prevent cell death in the presence of wortmannin or LY294002. These findings indicate that survival of oligodendrocytes and their precursors depends on a phosphatidylinositol 3-kinase mediated signaling pathway. Inhibition of this critical enzyme activity induces apoptotic cell death, even in the presence of exogenous growth factors or serum.

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; CNP, 2’,3’-cyclic nucleotide 3’-phosphohydrolase; CNTF, ciliary neurotrophic factor; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; GC, galactocerebroside; HEPES, N-2-Hydroxyethylpiperazine-N’-2-ethanesulphonic acid; IGF-I and II, insulin-like growth factor-I and II; IRS-1 and 2, insulin receptor substrate 1 and 2; MAP kinase, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl cysteine; NT-3, neurotrophin-3; OM-5 and -6, oligodendroglial cell medium-5 and -6; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PI(3)P, phosphatidylinositol(3)phosphate; PI(3,4)P2 , phosphatidylinositol(3,4)bisphosphate; PI(3,4,5)P3 , phosphatidylinositol(3,4,5)trisphosphate; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; TCA, trichloroacetic acid; TLC, thin layer chromatography; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling assay.

Key words: phosphatidylinositol 3-kinase, apoptosis, oligodendrocytes, cell survival, cell death, growth factors, antioxidants
responses such as alterations in gene activity, cell proliferation, and changes in energy metabolism (White and Kahn, 1994). Many of these same signal transduction pathways, including the PI3-K pathway, are activated by multiple receptor types, including the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and neurotrophin receptors (reviewed by Barbacid, 1994; Malarkey et al., 1995). Many of the same growth factors also play a major role in the regulation of oligodendrocyte development (McMorris et al., 1986; Richardson et al., 1988; Barres et al., 1993). How activation of similar signal transduction cascades by different growth factors or in different cell types leads to very different biological responses remains a major unsolved puzzle.

PI3-K phosphorylates phosphatidylinositol (PI), PI(4)-phosphate [Pi(4)P], and PI(4,5)P2 at the 3 position to produce PI(3)P, PI(3,4)P2 and PI(3,4,5)P3, respectively (reviewed by Kapeller and Cantley, 1994). PI(3,4)P2 and PI(3,4,5)P3 accumulate rapidly in agonist-stimulated cells and activate the δ, ε, ζ, and η isoforms of protein kinase C (Nakanishi et al., 1993; Toker et al., 1994) and the serine/threonine protein kinase encoded by the akt proto-oncogene (Franke et al., 1995). There is considerable evidence indicating that agonist-elevated 3-phosphorylated phosphoinositides act as second messengers in a wide range of cellular responses. Inhibition of PI3-K activity by the fungal metabolite wortmannin, a potent inhibitor of PI3-K expression is widespread, its presence in many of these same signal transduction pathways, including the PI3-K pathway, are activated by multiple receptor types, including the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and neurotrophin receptors (reviewed by Barbacid, 1994; Malarkey et al., 1995). Many of the same growth factors also play a major role in the regulation of oligodendrocyte development (McMorris et al., 1986; Richardson et al., 1988; Barres et al., 1993). How activation of similar signal transduction cascades by different growth factors or in different cell types leads to very different biological responses remains a major unsolved puzzle.

PI3-K activity assay

Oligodendroglial precursor cells and mature oligodendroglial cells were obtained by immunopanning as described by Barres et al. (1992) using monoclonal antibodies against the cell surface antigens A2B5 (expressed by oligodendroglial cell precursors), O4 (expressed by oligodendrocytes and intermediate precursors), O1 (GC, expressed by oligodendrocytes) and Run-2 (expressed by astrocytes) (Eisenbarth et al., 1979; Bartlett et al., 1981; Sommer and Schachner, 1981). Briefly, mixed glial cell cultures were established from 1-day postnatal rat cerebra as described by McMorris et al. (1986), and an enriched population of oligodendrocytes and precursors was isolated by differential shakeoff at days 5 or 7 as described by McCarthy and de Vellis (1980). The cell suspension was passed sequentially over a series of bacteriological Petri dishes coated with monoclonal antibodies Run-2, O1, O4, and A2B5, and O1+/Run-2- cells (oligodendrocytes) or A2B5-/Run-2-/O1+/O4- cells (oligodendrocyte precursors) were collected and inoculated into 16-well culture slides (Lab Disposable Products). In some experiments, shakeoff-enriched cells were used without further purification.

MATERIALS AND METHODS

Antibodies, culture media and reagents

Monoclonal antibodies used for immunopanning were prepared as tissue culture supernatants from hybridoma cells. Antibodies used were, Run-2 (Bartlett et al., 1981), O1, O4 (Sommer and Schachner, 1981) and A2B5 (Eisenbarth et al., 1979). Rabbit antisera against 2,3'-cyclic nucleotide 3'-phosphohydrolase (CNP; Raible and McMorris, 1989) or O1 antibody were used for immunostaining. Anti-PI3-K p85 antibody was purchased from Upstate Biotechnology Incorporated. OM-5 and OM-6 culture media (oligodendroglial cell medium-5 and -6) were prepared as described by Raible and McMorris (1990). OM-5 contains 10% fetal bovine serum, whereas OM-6 medium is serum-free unless stated otherwise. Wortmannin, NAC, vitamin C, the sodium salt of vitamin E, PI(4,5)P2 and phosphatidylinerine were obtained from Sigma Chemical Company. LY294002 was purchased from BioMol. Wortmannin or LY294002 were dissolved in dimethylsulfoxide (DMSO) and then added to cells (final DMSO concentration = 0.2%); controls received 0.2% DMSO alone. DMSO by itself had no detectable effect on the cells. Silica gel H thin-layer chromatography (TLC) plates were from Whatman. [γ-32P]ATP and [3H]thymidine were obtained from ICN Biomedical. Terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP were purchased from Boehringer Mannheim.

Preparation of purified precursor cells and oligodendrocytes

Oligodendroglial cells and their precursors were identified and purified by immunopanning as described by Barres et al. (1992) using monoclonal antibodies against the cell surface antigens A2B5 (expressed by oligodendroglial cell precursors), O4 (expressed by oligodendrocytes and intermediate precursors), O1 (GC, expressed by oligodendrocytes) and Run-2 (expressed by astrocytes) (Eisenbarth et al., 1979; Bartlett et al., 1981; Sommer and Schachner, 1981). Briefly, mixed glial cell cultures were established from 1-day postnatal rat cerebra as described by McMorris et al. (1986), and an enriched population of oligodendrocytes and precursors was isolated by differential shakeoff at days 5 or 7 as described by McCarthy and de Vellis (1980). The cell suspension was passed sequentially over a series of bacteriological Petri dishes coated with monoclonal antibodies Run-2, O1, O4, and A2B5, and O1+/Run-2- cells (oligodendrocytes) or A2B5-/Run-2-/O1+/O4- cells (oligodendrocyte precursors) were collected and inoculated into 16-well culture slides (Lab Disposable Products). In some experiments, shakeoff-enriched cells were used without further purification.

MTT cell survival assay

Cell survival was measured by a modification of the MTT tetrazolium assay (Mosmann et al., 1983) wherein viable cells with active mitochondria reduce MTT to a dark blue formazan product. Briefly, cells were incubated with 100 ng of MTT in 200 μl culture medium for 1 hour. The medium was then removed, the cells were rinsed once with phosphate-buffered saline (PBS) and the blue formazan product was solubilized in acidic isopropanol (0.04 M HCl in isopropanol) and quantitated by absorbance at 630 nm.
Quantitation of DNA

DNA content of the culture was measured by fluorimetry using the DNA-binding dye bis-benzimidazole (Hoechst 33258) as described by Labarca and Paigen (1980). Cells in culture plates were rinsed with cold 0.85% NaCl and sonicated in the wells in 400 μl of PBS per well, and the homogenate was transferred to a polypropylene tube. Hoechst 33258 was added (1 μg/ml in 1 ml PBS), the sample was vortexed, and fluorescence was measured at 365 nm excitation and 460 nm emission. Calf thymus DNA was used as the standard.

[^3H]thymidine incorporation

DNA synthesis was determined as [^3H]thymidine incorporation into trichloroacetic acid (TCA)-insoluble material. Cell cultures were incubated with 1 μCi/ml of [^3H]thymidine for 24 hours, cells were washed to remove unincorporated radioactivity, and radioactivity insoluble in 10% TCA was measured by liquid scintillation counting.

Immunostaining

Cells were fixed for 20 minutes using 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and then equilibrated in 60% glycerol and stored at −20°C (McMorris et al., 1986). After warming to room temperature and re-equilibration in 50 mM Tris-0.4 M NaCl buffer (pH 7.5), slides were stained by the avidin-biotin complex immunoperoxidase method using rabbit antiserum against CNP (McMorris et al., 1986; Raible and McMorris, 1989). Alternatively, cells were stained by the indirect immunofluorescence method with O1 antibody (culture supernatant) at 1:10 dilution.

[^3H]-2-deoxyglucose uptake assay

Glucose uptake was measured by using [^3H]-2-deoxyglucose. For the last hour in culture, medium was removed and replaced with glucose-free medium containing 1 μCi/ml [^3H]-2-deoxyglucose. Cells were then washed with PBS to remove excess radioactivity, and the incorporated radioactivity was determined by liquid scintillation counting.

TUNEL assay

Degradation of nuclear DNA in cultured cells was detected by the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method as described by Gavrieli et al. (1992); Yasuda et al. (1995). Briefly, cells grown on 16-well slides were fixed as described above and rinsed in phosphate-buffered 140 mM NaCl, pH 7.4. Slides were then pre-equilibrated with TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM CoCl2) for 2 minutes at room temperature. TdT (18 units/well) and biotinylated dUTP (300 pmole/well) were added in TdT buffer, and slides were incubated for 60 minutes at 37°C. They were then rinsed in the phosphate buffer described above, the reaction was terminated using TB buffer (300 mM NaCl, 30 mM sodium citrate) for 15 minutes at room temperature, and slides were rinsed in PBS containing 2% bovine serum albumin (w/v) for 10 minutes. For fluorescence assay, slides were incubated for 20 minutes at room temperature with 50 μg/ml streptavidin-rhodamine (Vector Laboratories) at 1:100 (v:v) dilution in PBS, rinsed and mounted in Vecta Shield mounting medium (Vector Laboratories) for fluorescence microscopy. For peroxidase detection, slides were incubated for 30 minutes at 37°C with streptavidin-peroxidase (Vector Laboratories) diluted 1:20 in 100 mM Tris, pH 7.2, stained for 20 minutes at room temperature using 3- amino-9-ethyl carbazole as substrate, and mounted in Gurr’s neutral mounting medium for bright-field microscopy. Tracts of biotinylated deoxyuridine incorporated at DNA nicked ends were then detected by fluorescence microscopy or bright-field microscopy, respectively.

RESULTS

PI3-K activity in oligodendroglial cells

To assess PI3-K activity free from interference from other cellular kinases or phosphatases, we immunoprecipitated PI3-K with specific antibody and measured enzyme activity in the immunoprecipitates. Enzyme assay was performed using PI(4,5)P2 and [γ-[^32]P]ATP as substrate, and the PI(3,4,5)P3 product was isolated by TLC and quantitated by liquid scintillation counting. PI3-K activity was readily detected in immunopanned oligodendrogial precursor cells and oligodendrocytes, whereas, activity was reduced by approximately 90% by the addition of wortmannin at 50 nM (Fig. 1), a concentration that inhibits PI3-K but not other kinases such as protein kinases A, C or G, myosin light chain kinase, mitogen-activated protein kinases, receptor protein tyrosine kinases, Ca2+/calmodulin-dependent protein kinase II, or phosphatidylinositol 4-kinase (Arcaro and Wymann, 1993; Yano et al., 1993; Ui et al., 1995).

Inhibition of PI3-K activity by wortmannin or LY294002 results in cell death

A2B5-positive oligodendrocyte precursors were purified by immunopanning, plated in medium containing 10% or 1% serum and allowed to attach to the culture surface for 16-24 hours. Immunostaining and counting of control cultures showed that the cells were >99.8% pure (data not shown), in agreement with previous reports (Barres et al., 1992). The medium was then replaced with OM-6 medium containing 1% serum, with or without test substances. The cells survived well for at least 2 days under these conditions. There was no significant change in cell number, as measured by DNA content of control cultures during the 24-hour period after the change to OM-6 medium containing 1% serum (Table 1). Addition of 100 ng/ml IGF-I, which acts as both a mitogen and a survival factor for oligodendroglial precursor cells (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990; Barres et al.,...
Fig. 2.  Cell survival in oligodendroglial precursor cell cultures treated with wortmannin. Immunopanned oligodendroglial precursors (>99.8% pure) were plated in OM-6 medium containing 1% serum for 24 hours before addition of 50 nM wortmannin ( ), 100 ng/ml IGF-I ( ) or both ( ). Controls received no additions ( ). Cells were incubated for an additional 24 hours prior to the assay. To determine the DNA content, cells were sonicated in the culture well and DNA was quantitated in the whole lysate by Hoechst 33258 fluorescence. Data represent the mean as a percentage of control (± s.e.m.) (control = 619±38 ng) for two experiments done in duplicate. To measure DNA synthesis, cells were incubated with 1 μCi/ml of [3H]thymidine for 24 hours, washed, and the radioactivity incorporated into TCA-insoluble material was determined. Data represent the mean as a percentage of control (± range) of one experiment done in duplicate (control = 6161±332 c.p.m.).

Results obtained using the MTT assay for cell survival were in very close agreement with those of the DNA assay, i.e., treatment of oligodendroglial precursor cells with 50 nM wortmannin reduced cell survival by 60%, and 100 ng/ml IGF-I, which by itself caused an increase in viable cells, did not protect cells from the effects of wortmannin (Fig. 2). Similarly, addition of 100 ng/ml IGF-I doubled [3H]thymidine uptake in the cultures, whereas addition of 50 nM wortmannin, in the absence or presence of IGF-I, resulted in a 70% decrease in [3H]thymidine uptake (Fig. 2), probably as a consequence of the reduction in viable cells.

To determine whether wortmannin induces the death of mature oligodendrocytes, O1-positive oligodendrocytes were purified by immunopanning, exposed to 50 nM wortmannin for 36 hours, and then observed by immunofluorescence microscopy. Fig. 3 shows that wortmannin greatly reduced the number of oligodendrocytes that survived. When oligodendrocytes in similar cultures were counted after immunostaining with anti-CNP antiserum (which stains the same population of cells as O1 antibody), the number of oligodendrocytes in wortmannin-treated cultures was reduced by 78% as compared with control (Table 2). These results indicate that PI3-K-mediated signal transduction is necessary for the survival of oligodendrocytes as well as their precursors.

We also tested LY294002, another inhibitor, which is structurally unrelated to wortmannin, is highly specific for PI3-K and inhibits PI3-K by a different mechanism (Vlahos et al., 1994; Ui et al., 1995). Fig. 4 shows that LY294002, like wortmannin, inhibited the survival of oligodendrocyte precursors, and that both inhibitors are effective at concentrations similar to those used for wortmannin.

Table 1. Cell survival in A2B5-positive oligodendroglial precursor cells

<table>
<thead>
<tr>
<th>Time (hours) after drug addition</th>
<th>DNA content, fluorescence units ± s.e.m.</th>
<th>IGF-I</th>
<th>Wortmannin</th>
<th>IGF-I + Wortmannin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>481±22</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>509±14</td>
<td>200±11</td>
<td>946±26</td>
<td>229±35</td>
</tr>
<tr>
<td>IGF-I + Wortmannin</td>
<td>[2500±35]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Immunopanned A2B5-positive oligodendroglial precursor cells (>99.8% pure) were allowed to attach to the culture surface for 16 hours in OM-5 medium containing 10% serum. The medium was then replaced with OM-6 medium containing 1% serum with or without 50 nM wortmannin or 100 ng/ml IGF-I as indicated. Cells were incubated for 24 hours and DNA content per culture was determined. Data are from one representative experiment done in triplicate.

Table 2. Wortmannin-induced cell death in mature oligodendroglial cells

<table>
<thead>
<tr>
<th>CNP-positive oligodendrocytes/mm²</th>
<th>Total ± s.e.m.</th>
<th>% of Control ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>491±53</td>
<td>100±12</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>90±8</td>
<td>22±9</td>
</tr>
</tbody>
</table>

Shakeoff-enriched oligodendrocyte cultures were incubated with or without 50 nM wortmannin for 36 hours in OM-6 medium containing 1% serum, fixed, and immunostained with anti-CNP antibody to identify oligodendrocytes. CNP-positive cells were counted at 500x magnification under bright-field microscopy. Data shown are from one representative experiment done in triplicate.
Cell death caused by inhibition of PI3-K is apoptotic

To examine the nature of the cell death in wortmannin-treated cultures, immunopanned oligodendroglial precursor cells were incubated with wortmannin for 4 or 24 hours, fixed, and stained by the TUNEL method for visualization of nuclei containing extensively nicked DNA, a diagnostic feature of apoptosis. After 24 hours of exposure to wortmannin, 62% of the cells were TUNEL positive, as opposed to 3.3% in control cultures (Fig. 5, Table 3), indicating that inhibition of PI3-K by wortmannin induces apoptosis. Moreover, 20% of the cells were TUNEL positive as early as 4 hours after the addition of wortmannin, before cell death could be detected by MTT assay, indicating that apoptosis is induced rapidly when PI3-K activity is inhibited (Fig. 5, Table 3).

Studies with 3T3-L1 cells have shown that PI3-K regulates the translocation of GLUT-1 and GLUT-4 glucose transporters to the cell surface where they become actively engaged in glucose transport (Clarke et al., 1994). To determine whether apoptosis in oligodendrocytes and precursors might be the consequence of glucose starvation, we used [3H]2-deoxyglucose to measure glucose uptake in wortmannin-treated cultures. After 4 hours of treatment, when apoptosis was well underway in the wortmannin-treated cultures as determined by TUNEL assay (Table 3, Fig. 5), there was no detectable difference in glucose transport between wortmannin-treated and control cultures (Table 3). After 24 hours of treatment, however, when the number of viable cells per culture was reduced 60-70%, total glucose uptake per culture was reduced by 80% (Table 3). Thus, onset of apoptosis precedes any detectable change in glucose transport, and cell death was not simply the consequence of glucose starvation.

Polypeptide survival factors, serum, antioxidants, and steroids do not protect oligodendroglial cells from wortmannin- or LY294002-induced cell death

We then tested five different polypeptide factors that signal via the activation of protein tyrosine kinases and that are known to act as survival factors for oligodendrocytes and oligodendrocyte precursors for their ability to prevent oligodendroglial cells from undergoing apoptosis in response to wortmannin or LY294002. We tested IGF-I, NT-3 and......

Table 3. Glucose transport, viable cell number and TUNEL-positive cells in wortmannin-treated cultures

<table>
<thead>
<tr>
<th></th>
<th>[¹⁴C]2-deoxyglucose uptake [cpm/well]</th>
<th>MTT assay for viable cells (A₆₃₀)</th>
<th>TUNEL-positive cells (% of total cells*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hours</td>
<td>24 hours</td>
<td>4 hours</td>
</tr>
<tr>
<td>Control</td>
<td>17.4±1.8</td>
<td>30.5±1.7</td>
<td>0.71±0.14</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>18.7±1.6</td>
<td>6.1±0.5</td>
<td>0.74±0.08</td>
</tr>
</tbody>
</table>

Immunopanned A2B5-positive cells were plated in OM-6 medium containing 1% serum for 24 hours, and 50 nM wortmannin or control medium was then added. Cells were incubated for an additional 4 or 24 hours.

* A minimum of 500 cells were counted per well in duplicates for each condition.

Data are mean values ± s.e.m. from one representative experiment for each assay procedure.
PDGFAA (Barres et al., 1992, 1993), which are known to activate signal transduction via the PI3-K pathway as well as via other signal transduction pathways (Barbacid, 1994; Malarkey et al., 1995), and bFGF (Yasuda et al., 1995) and CNTF (Louis et al., 1993), which are not known to signal through PI3-K (Vainikka et al., 1994; Stahl and Yancopoulos, 1994). Immunopanned A2B5-positive cells were incubated with each of these factors, either individually or in combination, at concentrations reported to be optimal for induction of oligodendroglial cell survival, with or without the addition of 50 nM wortmannin. After 24 hours in the presence of wortmannin, 60-65% of the cells were TUNEL-positive and therefore undergoing apoptosis (Fig. 6). None of the oligodendroglial survival factors, either singly or as a combination of all five, significantly reduced the number of cells undergoing apoptosis in response to wortmannin (Fig. 6).

Serum significantly promotes the survival of a wide variety of cell types under many different physiological conditions in vitro. Some of the survival-promoting activity of serum is due to the presence of IGF-I, PDGF, and other well-defined survival factors, whereas some of this activity is probably due to other, as yet unknown factors or combinations thereof. In immunopanned oligodendroglial precursor cells cultured in medium containing 1% serum, the number of TUNEL-positive cells remained below 3% for at least 24 hours in control cultures, whereas in wortmannin-treated cultures, the number increased to 20% within 4 hours of the addition of wortmannin and reached 62% of the total by 24 hours (Table 3). Likewise, in the presence of 10% serum, 65% of the cells were TUNEL-positive after a 24-hour exposure to wortmannin (Fig. 6). Therefore, serum is not able to support the survival of oligodendroglial cells in the absence of active PI3-K.

The antioxidants NAC, vitamin C and vitamin E, and the steroid hormone progesterone, promote cell survival in neurons and oligodendrocytes and can block apoptosis in response to growth factor deprivation or in the presence of toxic levels of glutamate or tumor necrosis factor-α (TNF-α) (Mayer and Noble, 1994). These survival agents appear to function through pathways at least partially distinct from tyrosine kinase-dependent signaling pathways and can substitute for or augment the activity of survival factors such as CNTF (Mayer and Noble, 1994). Because PI3-K is involved in signaling from many different tyrosine kinase-coupled receptors, we tested whether wortmannin-triggered apoptosis of oligodendroglial cells could be rescued by NAC, vitamin C, vitamin E or progesterone, individually or in combination.

Oligodendroglial cells were enriched by differential shakeoff, cultured for 24 hours with test substances in the presence or absence of wortmannin, and assayed for viable cells by the MTT method. Wortmannin reduced the number of viable cells by approximately 50%, and none of the antioxidants or progesterone, alone or in combination, reduced the loss of viable cells in these cultures (Fig. 7).
PI3-K and oligodendroglial cell survival 2535

Table 4. LY294002-induced cell death in the presence of polypeptide survival factors, antioxidants, and steroids

<table>
<thead>
<tr>
<th>Cell viability (% of control ± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>LY294002</td>
</tr>
<tr>
<td>LY294002 + growth factors</td>
</tr>
<tr>
<td>LY294002 + antioxidants + progesterone</td>
</tr>
</tbody>
</table>

Immunopanned oligodendroglial precursor cells were plated in OM-6 medium containing 1% serum for 24 hours, and then fresh medium was added with or without 30 μM LY294002, either by itself or together with a mixture of growth factors (IGF-I, NT-3, PDGF AA, CNTF and bFGF) or antioxidants and progesterone (N-acetyl cysteine, vitamin C, vitamin E and progesterone) at the concentrations shown in Figs 6 and 7. Cell viability was determined by the MTT assay method as described in Fig. 7.

Because NAC, vitamin C and vitamin E can significantly augment the efficacy of survival factors that signal through tyrosine kinase-coupled receptors (Mayer and Noble, 1994), we tested whether these agents in combination with the oligodendroglial survival factors IGF-I or CNTF could rescue oligodendroglial cells from wortmannin-induced cell death. The IGF-I receptor has intrinsic tyrosine kinase activity and signals through the PI3-K pathway as well as other pathways, whereas the CNTF receptor signals through janus kinase (JAK) non-receptor tyrosine kinases and is not known to directly activate the PI3-K pathway. However, combinations of either IGF-I or CNTF with all three antioxidants had no significant effect in rescuing wortmannin-treated cells (Fig. 7).

In the absence of wortmannin, all of the agents tested resulted in an increase in cell number and/or survival as compared with controls (Fig. 7), possibly due to a reduction in the basal level of cell death that occurs in the absence of wortmannin, an increase in cell proliferation, or both. The effect was greatest with IGF-I (Fig. 7), which is known to be a mitogen as well as survival factor for oligodendroglial cells (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1990; Barres et al., 1992, 1993).

Finally, we tested the same survival factors, antioxidants and steroids for their ability to prevent cell death in the presence of the PI3-K inhibitor LY294002. As shown in Table 4, the results of these experiments were virtually identical to those using wortmannin: exposure of oligodendrocyte precursors to LY294002 for 24 hours reduced the number of viable cells by 50-60%, and none of the survival factors, antioxidants or steroids significantly reduced the loss of viable cells.

DISCUSSION

In the present study we have examined the role of PI3-K signal transduction in the development and survival of oligodendrocytes and oligodendroglial precursor cells, using two inhibitors of PI3-K, wortmannin and LY294002. Wortmannin has been tested in a wide variety of systems and, at the concentration used here (50 nM), has been shown to inhibit PI3-K without inhibiting other kinases, lipases, or other enzymes or receptor functions (e.g., ligand binding), and therefore, it has been considered to be a specific inhibitor of PI3-K (reviewed by Ui et al., 1995). Secondy, we used another inhibitor of PI3-K, LY294002 (Vlahos et al., 1994), which is also highly specific to PI3-K. LY294002 is structurally different from wortmannin, and inhibits PI3-K by a different mechanism (Vlahos et al., 1994; Ui et al., 1995). We obtained essentially identical results with wortmannin and LY294002. Unexpectedly, it was recently reported that 50 nM wortmannin inhibits bombesin-stimulated phospholipase A2 (PLA2) activity in Swiss 3T3 cells (Cross et al., 1995). In contrast, LY294002 does not appreciably inhibit PLA2 (C. Vlahos, personal communication). Therefore, our results indicate that the death of the oligodendrocytes and oligodendroglial precursors in the presence of wortmannin or LY294002 is due to the inhibition of PI3-K.

Finally, neither wortmannin nor LY294002 is normally toxic to cells, even after prolonged exposure (Arcaro and Wymann, 1993; Yano et al., 1993; Ui et al., 1995; Vlahos et al., 1994).

Four isoforms of mammalian PI3-K have been well characterized (Kapeller and Cantley, 1994). Three of them have the same 110×10^3 M_{r} (p110) catalytic subunit but different 85×10^3 M_{r} (p85) regulatory subunits. The two subunits associate tightly with each other by binding of the SH3 domain of p85 with a proline-rich SH3-binding motif of p110. The fourth isoform of PI3-K consists of a single p110 subunit which is activated by the βγ subunit of activated heterotrimeric G-proteins (Stephens et al., 1994). Mammalian p110 is closely related to yeast VPS34, a PI3-K that transduces signals regulating membrane trafficking and secretion (Kapeller and Cantley, 1994). Wortmannin strongly inhibits all four mammalian PI3-Ks but not yeast VPS34 (Ui et al., 1995).

Many different agents have been shown to promote survival of oligodendroglial cells, but no known mechanism is common to them all. Several agents, such as IGF-I, PDGFAA, and NT-3, transduce signals through the PI3-K signal transduction pathway by activating the intrinsic tyrosine kinase activity of their respective receptors, leading to docking of PI3-K at phosphotyrosines within YXXM consensus motifs (Kapeller and Cantley, 1994). Phosphorylation of tyrosines within other motifs results in the docking and activation of other signal transduction molecules and activation of their downstream pathways, such as the mitogen-activated protein kinase (MAP kinase) pathway (Malarkey et al., 1995). All three of the above factors activate PI3-K and MAP kinase and some (but not all) of the three also activate PLCγ, Src-family kinases and protein tyrosine phosphatases (Barbicad, 1994; White and Kahn, 1994; Malarkey et al., 1995). In contrast, EGF is not known to regulate oligodendroglial cell survival (Barres et al., 1992), even though its receptor activates the PI3-K and MAP kinase pathways, as do the IGF-I receptor, PDGF receptor, and the NT-3 receptor trk C. bFGF is an oligodendroglial survival factor (Yasuda et al., 1995) that activates the MAP kinase pathway but is not known to activate PI3-K (Vainikka et al., 1994). CNTF is a potent oligodendroglial survival factor (Louis et al., 1993; Barres et al., 1993) that acts via a different tyrosine kinase-dependent signaling system: the CNTF receptor activates JAK non-receptor tyrosine kinases that phosphorylate STAT proteins which, in turn, assemble into active transcription factor complexes that migrate into the nucleus and regulate gene activity (Stahl and Yancopoulos, 1994). Other oligodendroglial survival factors, such as NAC, vitamin C, vitamin E and progesterone are believed to act via mechanisms partially or completely distinct from tyrosine kinases, and their action can augment the survival activity of factors that act at tyrosine kinase receptors (Mayer and Noble, 1994).
Although activation of PI3-K is not known to be a common feature of all these oligodendroglial survival factors, our results show that none of them can support oligodendroglial survival if PI3-K activity is blocked by wortmannin. Therefore, PI3-K is essential for the survival of oligodendroglial cells and their precursors. We speculate that signal transduction mechanisms involved in oligodendroglial cell survival require direct or indirect activation of PI3-K, since in the absence of PI3-K, the cells die by apoptosis and cannot be rescued by external addition of survival factors. Our findings are consistent with a recent study in which wortmannin treatment of PC-12 pheochromocytoma cells induced apoptosis which could not be prevented by the neuronal cell survival factor, nerve growth factor (Yao and Cooper, 1995).

Cell death plays a very important role in development, especially in the immune and nervous systems (reviewed by Raff, 1992) by effecting the removal of cells that are defective, improperly located, in excess, or inappropriately connected with other cells. Within the nervous system, both neurons and glial cells are generated in excess during development, and compete for limited amounts of survival factors; only those cells that are able to obtain adequate amounts of survival factors from cells with which they are properly connected and from other sources within their environments are able to survive (Raff, 1992). Oligodendroglial cells and their precursors require survival factors both in vitro and in vivo. Withdrawal of survival factors in vitro reduces oligodendroglial cell survival and, under some conditions, can result in massive oligodendroglial cell death; supplementation with IGF-1, PDGF, CNTF, or neurotrophins greatly increases oligodendroglial cell number (Barres et al., 1992, 1993; Louis et al., 1993). In vivo, approximately 50% of the oligodendroglial cells in the optic nerve normally die during development. Delivery of exogenous survival factors to the nerve prevents cell death and results in the doubling of oligodendroglial cell number (Barres et al., 1992).

Various pathological processes involve inappropriate triggering of apoptosis. Cytotoxic stimuli such as glutamate in periventricular white matter injury frequently seen in premature infants and TNF-periventricular white matter injury. Cytotoxic stimuli such as glutamate in cell death and results in the doubling of oligodendroglial cell number (Barres et al., 1993). Delivery of exogenous survival factors to the nerve prevents cell death and results in the doubling of oligodendroglial cell number (Barres et al., 1992).

We thank Dr Reema Mewar, Georgia Kraiber and Mark J. Engleka for valuable advice and many helpful discussions. Dr J. S. Grinspan for teaching us the TUNEL assay and for her generous gift of growth factors, Dr C. J. Vilahos for communicating his results prior to publication. The Wistar Institute Histology Core Facility for assistance with histological procedures, and Marina Hoffman for editorial assistance. This work was supported by NS 32394, NS 32122, NS 26119, and the H. H. Smith Foundation (F.A.M.).

REFERENCES


(Accepted 5 May 1996)