

C5b-9 Terminal Complement Complex Protects Oligodendrocytes from Death by Regulating Bad Through Phosphatidylinositol 3-Kinase/Akt Pathway¹

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Apoptosis of oligodendrocytes is induced by serum growth factor deprivation. We showed that oligodendrocytes and progenitor cells respond to serum withdrawal by a rapid decline of Bcl-2 mRNA expression and caspase-3-dependent apoptotic death. Sublytic assembly of membrane-inserted terminal complement complexes consisting of C5b, C6, C7, C8, and C9 proteins (C5b-9) inhibits caspase-3 activation and apoptotic death of oligodendrocytes. In this study, we examined an involvement of the mitochondria in oligodendrocyte apoptosis and the role of C5b-9 on this process. Decreased phosphatidylinositol 3-kinase and Akt activities occurred in association with cytochrome *c* release and caspase-9 activation when cells were placed in defined medium. C5b-9 inhibited the mitochondrial pathway of apoptosis in oligodendrocytes, as shown by decreased cytochrome *c* release and inhibition of caspase-9 activation. Phosphatidylinositol 3-phosphate kinase and Akt activities were also induced by C5b-9, and the phosphatidylinositol 3-phosphate kinase inhibitor LY294002 reversed the protective effect of C5b-9. Phosphatidylinositol 3-phosphate kinase activity was also responsible for the phosphorylation of Bad at Ser¹¹² and Ser¹³⁶. This phosphorylation resulted in dissociation of Bad from the Bad/Bcl-x_L complex in a Gi α -dependent manner. The mitochondrial pathway of oligodendrocyte apoptosis is, therefore, inhibited by C5b-9 through post-translational regulation of Bad. This mechanism may be involved in the promotion of oligodendrocyte survival in inflammatory demyelinating disorders affecting the CNS. *The Journal of Immunology*, 2001, 167: 2305–2311.

Oligodendrocytes (OLG)³ myelinate the axons of the CNS and undergo apoptosis during development (1–3). In developing CNS, OLG are selectively rescued from apoptosis by survival signals provided by axonal contact and growth factors (3–5). Apoptotic death of OLG, in contrast, plays a pathogenic role in myelin loss and failure of axonal conduction in inflammatory and degenerative disorders affecting CNS (6–8). Apoptotic OLG death is induced by a variety of factors, including TNF- α , nerve growth factor, and the interaction of CD95 and CD95 ligand *in vivo* and *in vitro* (8–11). Some OLG in multiple sclerosis (MS) lesions express Bcl-2, and the level of expression correlates with the extent of remyelination (12, 13). This finding suggests that OLG are capable of antiapoptotic response *in vivo*. Therefore, factors that rescue OLG from apoptosis may increase the survival and remyelinating potential of OLG.

Complement activation with assembly of the terminal complement complex consisting of C5b, C6, C7, C8, and C9 proteins

(C5b-9) plays a significant role in the pathogenesis of a variety of CNS diseases, including MS, Guillain-Barré syndrome, Alzheimer's disease, Parkinson's disease, and stroke (14–20). By forming pores in the cell membrane, C5b-9 causes cell death in part through unregulated Ca²⁺ influx (21, 22). However, OLG, like other nucleated cells, survive limited complement attack through protection by complement-inhibitory proteins and by elimination of membranes carrying C5b-9 complexes (23–28). We recently demonstrated that sublytic doses of C5b-9 inhibit the caspase-3-dependent OLG apoptosis induced *in vitro* by serum deprivation or TNF- α (29). The mechanisms and signaling pathways involved in this protective activity of C5b-9 are currently unknown. Sublytic C5b-9 induces Gi-dependent and G $\beta\gamma$ -mediated activation of Ras, proto oncogene serine/threonine-protein kinase (Raf)-1, mitogen-activated protein kinase-1/extracellular signal-regulated kinase-1 (MEK1), and extracellular signal-regulated kinase-1 (ERK1) in a JY B cell line, primary human aortic smooth muscle cells, and primary rat OLG (30–33). Phosphatidylinositol 3-phosphate kinase (PI-3K) signaling appears to be required for cell survival (34) including OLG and OLG progenitor cells (OPC) (35). Thus, the recent finding that PI-3K is activated by C5b-9 in aortic smooth muscle cells (33) may be significant in C5b-9-mediated protection from apoptosis.

Both mitogen-activated protein kinase and PI-3K pathways provide survival signaling that may neutralize proapoptotic Bcl-2 proteins (36). ERK induces Bax/Bcl-2 heterodimerization by directly phosphorylating Bcl-2 and regulates Bad/Bcl-x_L assembly by phosphorylating Bad through ribosomal protein 5b kinase 1 (RSK)1 (37, 38). Regulation of apoptosis by PI-3K is primarily mediated through Akt, a serine-threonine kinase, which in turn regulates Bad and caspase-9 (39, 40). BCL2-antagonist of cell death (Bad) is a cytoplasmic protein that promotes apoptosis by dimerization with Bcl-2 or Bcl-x_L (39, 41). Heterodimerization of Bad with Bcl-2 or Bcl-x_L reduces the number of Bcl-2 and Bcl-x_L

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³ Abbreviations used in this paper: OLG, oligodendrocytes; C5b-9, terminal complement complexes consisting of C5b, C6, C7, C8, and C9 proteins; cyto-c, cytochrome *c*; MS, multiple sclerosis; OPC, OLG progenitor cells identified by mAb A2B5; ERK1, extracellular signal-regulated kinase; PI-3K, phosphatidylinositol 3-phosphate kinase; MEK1, mitogen-activated protein kinase-1/ERK1; cox IV, cyto-c oxidase subunit IV; PTX, pertussis toxin; Bad, BCL2-antagonist of cell death; RSK1, ribosomal protein 5b kinase 1.

that bind to Bax or Bak, thus inhibiting the antiapoptotic activity of Bcl-2 and Bcl-x_L (42, 43). Phosphorylation of Bad enhances cell survival by inducing cytoplasmic sequestration of Bad through formation of the Bad/14-3-3 protein complex (42, 43).

In this report, we demonstrate that the decrease in PI-3K and Akt activities of OLG and OPC in response to serum growth factor withdrawal is associated with an induction of apoptosis through the mitochondrial pathway. Sublytic C5b-9 assembly protected OLG from apoptosis, and this rescue was mediated by Gi-dependent activation of PI-3K/Akt. This signaling pathway appears responsible for phosphorylation of Bad at Ser¹¹² and Ser¹³⁶ and the dissociation of Bad from Bcl-x_L. These findings suggest that sublytic C5b-9, a ubiquitously activated inflammatory mediator, may play a significant role in OLG survival in inflammatory CNS disorders.

Materials and Methods

Primary cultures of OLG progenitor cells and OLG

Primary OPC were prepared according to the method of Saneto and DeVellis (44). Briefly, glial cells isolated from neonatal Sprague Dawley rat brains were grown for 10 days as stratified mixed cultures. OPC, obtained by shaking, were differentiated in defined medium containing serum-free DMEM-Ham's F-12, transferrin (500 ng/ml; Sigma, St. Louis, MO), insulin (75 ng/ml; Sigma), basic fibroblast growth factor (75 μg/ml; Collaborative Research, Lexington, MA), and 1 mM sodium pyruvate. We have previously shown that after 56 h in defined medium, >85% of cells are mature OLG expressing myelin basic protein, proteolipid protein, and galactocerebroside (31). Less than 3% of cells negative for myelin basic protein are astrocytes, whereas the remaining cells stain with A2B5 Ab and are therefore progenitors in varying stages of differentiation (31). Thus, OPC cultured in defined medium for 56–72 h are designated as OLG.

Membrane assembly of sublytic C5b-9 using terminal complement proteins

In this study, sublytic C5b-9 was assembled using purified human complement proteins C5–C9 obtained from Quidel (San Diego, CA). The C5b6 complex was prepared from C5 and C6 as described (45) or purchased (Advanced Research Technologies, San Diego, CA). Cell monolayers at 10⁶/24-cm² flask were treated with 30 μg C5b-6 or 18 U C5b-6 (Advanced Research Technologies) for 15 min, then with C7 (10 μg) for 5 min at room temperature. C8 (10 μg) and C9 (15 μg) were added in a final volume of 1 ml, as described (21, 29, 32). Cells were incubated at 37°C for the indicated periods. The concentrations of complement proteins used in this study are sublytic for OLG, as determined by staining cells with vital dye trypan blue.

Cell death by MTS assay

Live cells were quantitated using CellTiter96Aqueous cell proliferation assay (Promega, Madison, WI), as previously described (29). In brief, OPC plated in 96-well plates at 10⁵ cells/well in 200 μl defined medium were cultured for 56 h. After exposure of cells to C5b-9, methyltetrazolium salt (40 μl) was added at the indicated time points. The plates were incubated 2 h at 37°C, and OD was measured at 540 nm. Results are expressed as mean percent surviving cells ± SE using the initial cell number at the beginning of the experiment as 100%. Each sample was assayed in triplicate. The Student *t* test was used to determine the statistical significance of the results.

PI-3K and Akt kinase activity assays

PI-3K assay was performed as previously described (33). In brief, cells were lysed in 30 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM DTT, 2 mM MgCl₂, 1 mM NaVO₄, 0.5 mM PMSF, 100 μg/ml aprotinin, and leupeptin (RIPA buffer). Equal amounts of cell lysates (150 μg protein) were immunoprecipitated overnight with anti-p85 PI-3K polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of protein A/G agarose (Calbiochem, La Jolla, CA). The beads were washed in RIPA buffer and then with kinase buffer (20 mM Tris-HCl (pH 7.5), 100 mM EDTA, 0.5 mM EGTA, 1 μM ATP, 10 mM MgCl₂). The immunoprecipitates were incubated with phosphatidylinositol and 10 μCi [³²P]ATP (NEN, Boston, MA) for 30 min at 37°C in kinase buffer. The reaction was terminated by adding 4 M HCl and chloroform-methanol (1:1), and samples were

analyzed on TLC by allowing migration of the phosphorylated substrates. The spots were excised, and the radioactivity was counted. For Akt assay, equal amounts of lysates (150 μg) were subjected to immunoprecipitation with rabbit IgG to human Akt1 (Santa Cruz Biotechnology) and protein A- and G-agarose, as described for PI-3K. Agarose beads were washed with RIPA buffer as above, and beads carrying the immunoprecipitates were incubated for 10 min at 37°C in kinase buffer containing 40 μM protein kinase A inhibitor peptide (Upstate Biotechnology, Lake Placid, NY) and 10 μM Akt substrate peptide RPRAATF (Upstate Biotechnology; Ref. 46). Peptide phosphorylation was assessed by loading samples on P81 phosphocellulose paper and counting the radioactivity.

Determination of cytochrome c (cyto-c) release

Cytoplasmic translocation of cyto-c from the mitochondrial intermembrane space was determined by digitonin permeabilization (47–49). Briefly, cells were lifted from plates by trypsinization and washed with ice-cold PBS and then with 0.25 mM EGTA. Cells were resuspended in a 20 mM HEPES buffer, pH 7.0, containing 125 mM KCl, 2 mM K₂HPO₄, 5 mM malate, 5 mM glutamate, and protease inhibitors. Cells were treated with 0.01% digitonin for 10 min on ice, a condition that permeabilizes >95% of cells. Permeabilized cells were centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant containing cytoplasmic proteins was recovered and stored at –70°C. The pellets containing mitochondria were lysed in RIPA buffer. The protein concentration was measured by the bicinchoninic acid method (Pierce, Rockford, IL). The presence of cyto-c in the supernatant is not due to mitochondrial damage by digitonin, as shown by the release of cytoplasmic lactate dehydrogenase but not mitochondrial succinyl dehydrogenase (47). The supernatant and pellet fractions were analyzed for cyto-c by SDS-PAGE and immunoblotting with a mouse monoclonal anti-cyto-c IgG (PharMingen, San Diego, CA). Blots were also probed for actin and a mitochondrial membrane protein cyto-c oxidase subunit IV (cox IV) using antiactin IgG (Santa Cruz Biotechnology) and anti-cox IV IgG (Molecular Probes, Eugene, OR), respectively. Actin served as control for supernatant, and cox IV was used as a pellet control. The immunoreactive bands were detected using peroxidase-conjugated secondary Ab and then enhanced chemiluminescence (Pierce). The radiographic band density was measured using UN-SCAN-IT software (Silk Scientific, Orem, UT), and results are shown as density ratios to actin or cox IV.

The level of cyto-c released from the permeabilized cell cytosol was also determined by ELISA using the cyto-c immunoassay kit (R&D Systems, Minneapolis, MN). Equal amounts of protein were assayed for cyto-c, as described in the manufacturer's instructions. Results are expressed as picograms cyto-c per microgram total supernatant protein.

Analysis of caspase-9 activation by Western blot

After exposure to C5b-9 or C5b6, cells were cultured for 18 h in defined medium and then lysed in RIPA buffer. Lysates were examined for caspase-9 cleavage products by immunoblotting using an Ab specific for the 37- and 17-kDa caspase-9 fragments (NEB, Beverly, MA).

Analysis of Akt and Bad phosphorylation

Phosphorylation of Akt and Bad was examined using NEB assay. After exposure to C5b-9 or C5b6 for the indicated time periods, cell lysates were immunoprecipitated with goat anti-Akt1 IgG (Santa Cruz Biotechnology), rabbit anti-Bad IgG, rabbit IgG to Ser¹¹² phosphorylated Bad (NEB), and goat anti-actin IgG together with protein A/G agarose. The beads were washed with lysis buffer and then boiled in Laemmli sample buffer. Eluted protein was then examined by SDS-PAGE (7.5% for Akt and 12% for Bad) and immunoblotted using Ab to phosphorylated Akt1 at Ser⁴⁷³ (NEB) and Bad at Ser¹¹² or Ser¹³⁶ (NEB). Results are expressed as density ratios to actin. In some experiments, cells were pretreated with pertussis toxin (PTX; List Biological, Campbell, CA) for 4 h to inhibit G_{iα} or with LY294002 (Biomol, Plymouth, PA) for 1 h to inhibit PI-3K.

Determination of Bad complexed with Bcl-x_L

Cell lysates (100 μg protein) were immunoprecipitated with rabbit anti-Bcl-x_L IgG (Santa Cruz Biotechnology) before immunoblotting. The presence of Bad in anti-Bcl-x_L immunoprecipitates was evaluated by 10% SDS-PAGE and immunoblotting using anti-Bad IgG (Santa Cruz Biotechnology). The same blot was then examined for Bcl-x_L, and the results are shown as Bad:Bcl-x_L density ratios.

Results

Apoptosis of OLG is associated with a decrease in PI-3K and Akt1 activities

In defined medium, OPC cease to proliferate and begin to differentiate into OLG. The lack of serum growth factors also initiates the process of apoptosis in these cells (1–5). After 56 h in defined medium, ~40% of cells survive as differentiated OLG (29). We examined the PI-3K and Akt1 activities in OPC in defined medium for up to 72 h (Fig. 1). The rate of decline in PI-3K and Akt activities, determined in separate experiments, are presented together in Fig. 1. PI-3K and Akt1 activities declined rapidly within 3–6 h in viable cells. The rate of their decline was similar to the loss of Bcl-2 mRNA expression under identical conditions (29). These findings suggested that serum deprivation results in rapid loss of signal kinases required for survival, and also Bcl-2 gene expression.

Apoptosis of OLG is associated with an increase in cyto-c release and caspase-9 activation

We have measured the release of cyto-c and the activation of caspase-9 in OPC in defined medium. We have detected cytoplasmic cyto-c after 24 h, and the released cyto-c level was increased significantly at 72 h. Increased cyto-c in supernatants was associated with decreased cyto-c in pellet fractions (Fig. 2A). The supernatant was free of mitochondrial contamination, as shown by the absence of cox IV in digitonin-treated samples. The absence of cyto-c in digitonin-permeabilized supernatant of the control cells (Fig. 2A, $t = 0$ point) also indicated that the cyto-c was released from the mitochondria affected by apoptosis, not mitochondria permeabilized by digitonin. The mitochondrial involvement was further supported by the activation of caspase-9, as shown by the generation of a 37-kDa caspase-9 cleavage fragment (Fig. 2B), a process requiring cyto-c release (50).

Sublytic C5b-9 inhibits cyto-c release and caspase-9 activation

Mitochondria play a central role in apoptosis induced by growth factor deprivation and stress injuries, such as those following UV irradiation and protein synthesis inhibition (41, 50). Mitochondria are also involved in apoptosis induced by cytokines such as TNF- α (51). We previously showed that sublytic C5b-9 rescued OLG from apoptosis induced in vitro by serum deprivation and by TNF- α , and this rescue was associated with inhibition of caspase-3 activation (29). Because the mitochondrial pathway of apoptosis was clearly implicated in death of OLG cultured in defined medium (Figs. 1 and 2), the effects of C5b-9 on cyto-c and caspase-9 were investigated. OLG differentiated from OPC for 56 h were

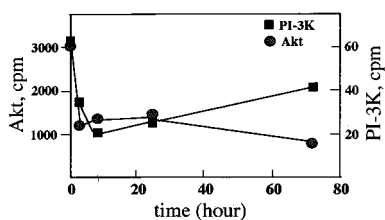


FIGURE 1. Down-regulation of PI-3K and Akt activities during OLG differentiation. OPC were cultured in defined medium and lysed at varying time points. Anti-PI-3K immunoprecipitates were examined for PI-3K activity by assessing phosphorylated phosphatidylinositol by TLC. Radioactive spots corresponding to PI-3K activity were excised and counted. In separate experiments, anti-Akt1 immunoprecipitates were examined by in vitro kinase assay using an Akt-specific peptide substrate. Data from two separate experiments are shown.

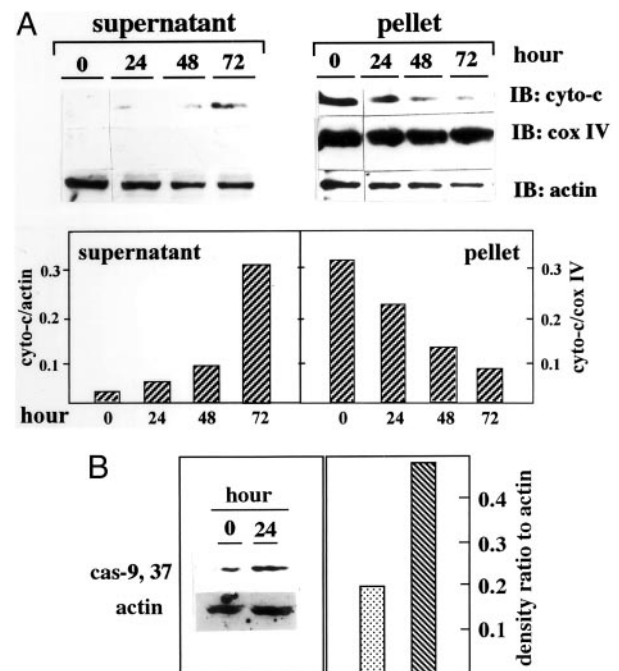


FIGURE 2. Release of cyto-c and activation of caspase 9 in apoptotic OLG. *A*, OPC cultured in defined medium for the indicated time points were collected by trypsinization. After permeabilization of cells with digitonin, the supernatants and pellet fractions were separated, and each sample (25 μ g protein) was examined for cyto-c by immunoblotting using monoclonal anti-cyto-c IgG. Antiactin and anti-cox IV IgG were used as controls. Results are shown as density ratios of cyto-c (15 kDa) to cox IV for pellets and to actin for supernatants. Actin is released by digitonin treatment. A representative result of three experiments is shown. *B*, OPC cultured in defined medium for 0 and 24 h were lysed and examined by immunoblot using a rabbit IgG to the 37-kDa cleavage product of caspase-9. The same blot was probed for actin. Results are shown as density ratios to actin.

further cultured in defined medium for 18 h, as previously described (29, 31). OLG death occurring during this 18 h was ~45% (see Fig. 6). Using this system, the effect of sublytic C5b-9 on cyto-c release was measured. The sublytic dose of C5b-9 was determined by trypan blue stain after treating OLG with limiting C5b6 and excess C7, C8, and C9 (Fig. 3). As shown in Fig. 4A, C5b-9, but not C5b6 (30 μ g or 18 U), caused >60% inhibition of cyto-c release. An increase in cyto-c was seen in the corresponding pellets. Similar results were obtained (63% inhibition) when cyto-c

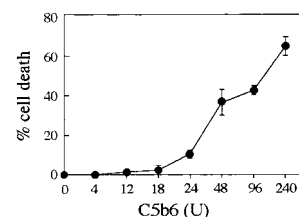


FIGURE 3. Determination of C5b-9 sublytic dose on OLG. OLG in 24-well plates were treated with limiting C5b6 and an excess of C7 (10 μ g), C8 (10 μ g), and C9 (15 μ g), as described in *Materials and Methods*. After 60 min at 37°C, cell death was determined by staining with trypan blue and counting >600 cells/sample. Results are shown as percent cell death \pm SE of two separate experiments performed in duplicate. By protein analysis and hemolytic assay, 30 μ g C5b6 prepared by us (30) and used as a sublytic dose are equivalent to 18 U C5b-6 obtained from Advanced Research Technologies.

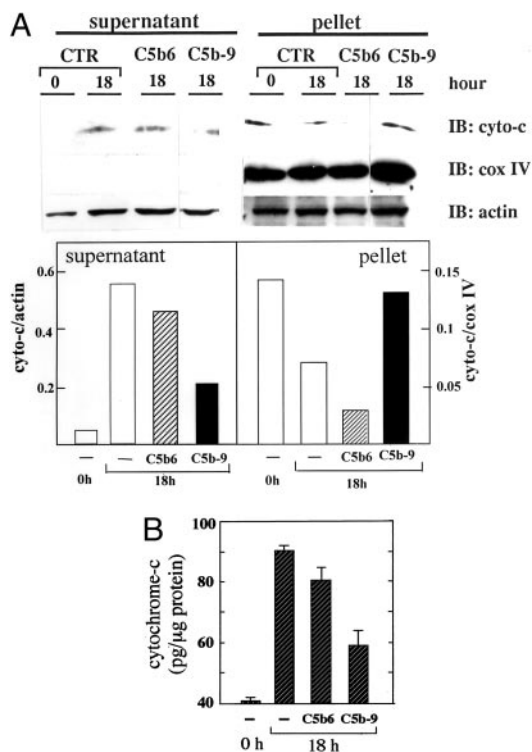


FIGURE 4. Effect of C5b-9 on cyto-c release in OLG. *A*, OLG differentiated from OPC for 56 h were exposed to a sublytic dose of C5b-9, as described in *Materials and Methods*. After 18 h, cells were permeabilized with digitonin, and cyto-c release was assessed by immunoblotting. Controls (CTR) included cells treated with C5b6 or medium. Results are also shown as density ratios to actin and to cox IV, for supernatants and pellets, respectively. *B*, Experiments identical with those in *A* were conducted. Cyto-c in the supernatants, released from permeabilized cells, was assayed by ELISA, as described in *Materials and Methods*. Results are expressed as picograms cyto-c per microgram total protein.

release was measured by ELISA (Fig. 4*B*). To evaluate the caspase-9 activation, the presence of the 17-kDa caspase-9 fragment was examined in C5b-9-treated cells (Fig. 5). C5b-9, but not C5b6, totally abrogated the increase in the 17-kDa fragment during the 18-h period. These findings indicated that C5b-9 inhibits the mitochondrial pathway of apoptosis, in which caspase-9 activated by cytosolic cyto-c/Apaf complex plays an essential role in caspase-3-dependent cell death (41, 50).

The PI-3 kinase inhibitor LY294002 reverses the C5b-9 rescue of OLG apoptosis

Both PI-3K and mitogen-activated protein kinase pathways promote cell survival through regulation of the Bcl-2 family of proteins (36–40). We examined the role of PI-3K and ERK pathways in OLG survival induced by C5b-9. OLG derived from OPC in defined medium for 56 h were treated with PI-3K inhibitor (10 μ M LY294002) or MEK1 inhibitors (25 μ M PD98059 or 10 μ M U0126) for 18 h. Cell survival, determined by the MTS assay, showed 46% reduction in control cells in defined medium after 18 h. As shown by Fig. 6, 75% of cell death was reversed by C5b-9. This reversal is statistically significant ($p < 0.05$). The protective effect of C5b-9 was totally abrogated by LY294002 (Fig. 6) and partially abrogated by inhibiting MEK1 activity (data not shown). These data suggested that PI-3K, but not the ERK pathway, is the primary signal pathway to rescue OLG from apoptosis. Alternatively, both the PI-3K and ERK1 pathways may be required, because ERK1 activity can be inhibited by PI-3K inhib-

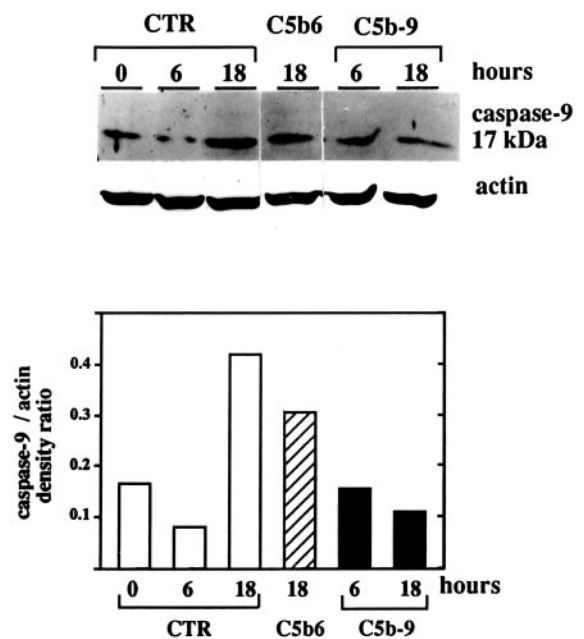


FIGURE 5. Effect of C5b-9 on caspase-9 activation in OLG. OLG were exposed to C5b-9 or C5b6 for 6 and 18 h. Cells were lysed and examined for the 17-kDa caspase-9 fragment by immunoblotting. Results are also shown as density ratios to actin. CTR, control.

itors (33, 52). The cell survival reduced below the control level (Fig. 6, CTR, 18 h) by LY294002 may be due to PI-3K activation by the low level of fibroblast growth factor in defined medium.

C5b-9 induces activation of PI-3K and Akt

Because OLG survival induced by C5b-9 was inhibited by PI-3K inhibitor, the ability of C5b-9 to activate PI-3K in OLG was assessed. C5b-9 increased PI-3K activity to a maximum of 5-fold (Fig. 7*A*), similar to the effect of C5b-9 in aortic smooth muscle cells (33). PI-3K and Akt activities decreased with an induction of OPC/OLG apoptosis (Fig. 1). Because Akt is considered to be the critical kinase in PI-3K-dependent survival, Akt activation by C5b-9 was determined by *in vitro* kinase assay and by assessing the phosphorylated Akt at Ser⁴⁷³. Akt activity reached a maximum of 3.4-fold at 10 min and remained elevated at 40 min (Fig. 7*B*). Akt phosphorylation at Ser⁴⁷³ increased 2.5-fold over the untreated or C5b6 controls at 30 min (data now shown).

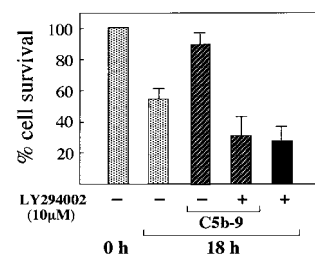


FIGURE 6. Effect of PI-3K inhibition on C5b-9 rescue of OLG apoptosis. OPC plated in 96-well plates at 10^5 cells/well were cultured in defined medium for 56 h. Cells were treated with LY294002 (10 μ M) for 1 h and then exposed to C5b-9. After 18 h, cell survival was assessed by MTS assay. The number of live cells at the beginning of the experiment (CTR, 0 h) was considered to be 100% viable. Results of four separate experiments performed in triplicate are expressed as percent surviving cells \pm SE, relative to CTR value at 0 h.

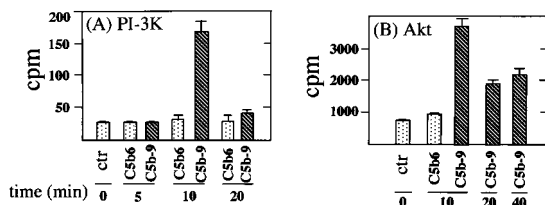


FIGURE 7. Activation of PI-3K and Akt by C5b-9. A, OLG were treated with C5b-9 or C5b6 for the indicated time periods. PI-3K activity was determined as described in Fig. 1. Results from triplicate samples are shown as cpm ± SE. B, OLG, treated with C5b-9 or C5b-6 for the indicated time period, were examined for Akt activity as described in Fig. 1.

C5b-9 induces Bad phosphorylation at Ser¹¹² and Ser¹³⁶

Phosphorylation of Bad at Ser¹³⁶ by Akt is a critical step in PI-3K-dependent cell survival (39). Phosphorylation of other sites, such as Ser¹¹² and Ser¹⁵⁵, by other kinases is also important in down-regulating Bad activity (38, 53–57). Phosphorylation of these sites neutralizes Bad by promoting the dissociation of Bad/Bcl-x_L complex and by inducing the cytoplasmic sequestration of Bad by binding the 14-3-3 family of proteins (40). In our system, phosphorylation at Ser¹¹², undetected in control cells, was increased 4-fold by C5b-9 (Fig. 8A). Phosphorylation of Bad at

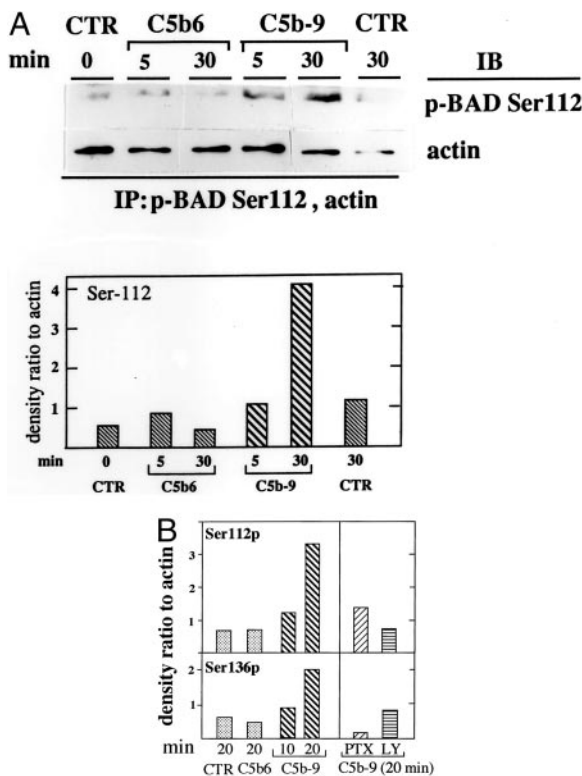


FIGURE 8. C5b-9 induces Bad phosphorylation at Ser¹¹² and Ser¹³⁶. A, OLG treated with C5b-9 or C5b6 for 5 and 30 min were lysed. Lysates (100 μg protein) were immunoprecipitated with rabbit IgG to Bad phosphorylated at Ser¹¹² and goat IgG to actin. Samples were analyzed by immunoblotting using the same phospho-specific anti-Bad. The blot was probed again for actin. Results are also shown as density ratios to actin. B, Effects of LY294002 (LY; 10 μM) and PTX (100 ng/ml) were tested on C5b-9-induced phosphorylation of Bad at Ser¹¹² and Ser¹³⁶, by preincubating cells with each of these inhibitors. After exposure to C5b-9 or C5b-6 for the indicated period, cell lysates were immunoprecipitated as in A and then blotted with Abs to Bad phosphorylated at Ser¹¹² or Ser¹³⁶, and to actin, as above. Representative results of two experiments are shown as density ratios to actin. CTR, control.

Ser¹³⁶ was increased 3-fold at 20 min over the untreated or C5b6 control levels (Fig. 8B). LY294002 and PTX were effective in inhibiting phosphorylation of Ser¹³⁶ and Ser¹¹². Therefore, phosphorylation of Bad at Ser¹¹² and Ser¹³⁶ by C5b-9 was induced by a Gi/PI-3K-dependent pathway.

Bad/Bcl-x_L association is decreased in OLG exposed to C5b-9

Phosphorylation of Bad is associated with loss of proapoptotic activity upon its release from the Bad/Bcl-x_L complex (40, 54–57). To evaluate the functional significance of Bad phosphorylation in OLG, we examined the effect of C5b-9 on the binding of Bad to Bcl-x_L. C5b-9 reduced the level of the Bad/Bcl-x_L complex by 60% at 18 h, and this was reversed by PTX (Fig. 9).

Discussion

Growth factors such as PDGF, basic fibroblast growth factor, and IL-3 inhibit apoptosis by inducing survival kinase activity (36, 41). The PI-3K/Akt and MEK/ERK pathways activated by these growth factors play a significant role in posttranslational regulation of pro- and antiapoptotic proteins that regulate the functional integrity of the mitochondria. Because sublytic C5b-9 reverses the caspase-3-dependent apoptotic death of OLG (29), we investigated the possible involvement of the mitochondrial pathway of apoptosis and the mechanisms by which C5b-9 rescue OLG apoptosis. We showed for the first time that apoptosis of OLG and their progenitor cells is associated with cyto-c release and caspase-9 activation. These events were inversely correlated with PI-3K and Akt activities, suggesting a role of these kinases in OLG survival. Therefore, we evaluated the effect of C5b-9 on the mitochondrial pathway in OLG and the role of PI-3K/Akt pathway in rescuing OLG from apoptosis. At a sublytic dose, C5b-9 was able to inhibit the cyto-c release and caspase-9 activation in OLG. C5b-9 also increased PI-3K and Akt1 activities, and the PI-3K inhibitor LY294002 completely abrogated the C5b-9 rescue of OLG apoptosis. These findings indicate that the mitochondrial pathway of apoptosis is abrogated by C5b-9 through activation of PI-3K.

Bad protein, which belongs to the BH3-only proteins, is essential in initiating the mitochondrial apoptosis (58). We examined Bad as a substrate for the PI-3K/Akt pathway in C5b-9-mediated OLG survival. C5b-9 induced Bad phosphorylation at Ser¹¹² and enhanced Ser¹³⁶ phosphorylation in a PI-3K- and Gi-dependent manner. The fact that the same dose of LY294002 reversed the C5b-9 rescue of OLG death and effectively blocked the phosphorylation of Bad at both serine residues strongly suggests that Bad is coordinately regulated by C5b-9 through activation of PI-3K. Similarly, growth factors can induce concomitant phosphorylation of Bad at multiple Serine residues, including Ser¹¹², Ser¹³⁶, and Ser¹⁵⁵ (55). Whereas Akt phosphorylates Bad primarily at Ser¹³⁶,

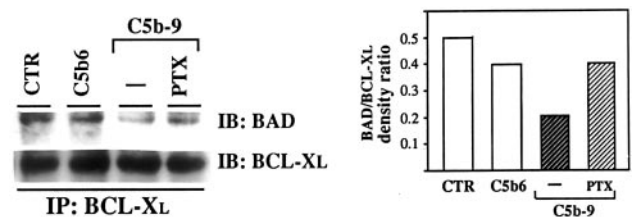


FIGURE 9. C5b-9 decreases Bad/Bcl-x_L complex formation in OLG. OLG, with or without pretreatment with PTX, were exposed to C5b-9. After 18 h, cells were lysed and immunoprecipitated with rabbit IgG to Bcl-x_L. The presence of Bad in the anti-Bcl-x_L immunoprecipitate was evaluated by immunoblotting with rabbit IgG to Bad. Blots were also probed for Bcl-x_L. Results are also shown as Bad to Bcl-x_L density ratios. CTR, control.

but not Ser¹¹² (39), Ser¹¹² is phosphorylated by other kinases, such as mitochondrial protein kinase A, p65 p21-activated kinase 1, and RSK1 (38, 53–58). RSK1 is activated by C5b-9 through ERK1 (data not shown), and ERK1 can be inhibited by PI-3K inhibitors (33). Therefore, regulation of Bad by C5b-9 may also involve an activation of kinases, other than Akt1, downstream of PI-3K. Protection of OLG from apoptosis through regulation of Bad may be mediated by inhibition of Bad/Bcl-x_L complex assembly, thus increasing the Bcl-x_L activity.

We previously showed that C5b-9 increases Bcl-2 synthesis in OLG (29), a process that may also be regulated by PI-3K through p70 S6 kinase which is required for protein translation. Therefore, an increase in Bcl-2 protein in OLG may increase the Bcl-2/Bax complex formation. This mechanism is thought to neutralize the proapoptotic Bax, as proposed by the rheostat model (41). Thus, full protection of OLG from apoptotic death may require both the neutralization of proapoptotic activities and enhanced activities of Bcl-2 and Bcl-x_L.

In situ activation and assembly of C5b-9 occur in MS and other inflammatory and degenerative CNS disorders, as demonstrated by the presence of C5b-9 in spinal fluids and affected tissues (14–20, 59). We propose that C5b-9 may have a beneficial role by rescuing OLG from apoptotic death, thus promoting their survival and remyelination.

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