Inhibition of Oligodendrocyte Apoptosis by Sublytic C5b-9 Is Associated with Enhanced Synthesis of Bcl-2 and Mediated by Inhibition of Caspase-3 Activation

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We have previously shown that generation of sublytic C5b-9, the membrane attack complex of complement, induces oligodendrocytes to enter cell cycle and reduces apoptotic cell death in vitro. In the present study, the cellular factors involved in apoptosis of oligodendrocyte progenitor cells and oligodendrocytes, and the inhibitory effect of C5b-9 on apoptotic process were investigated. Oligodendrocyte progenitor cells identified by mAb A2B5 that were isolated from neonatal rat brains were differentiated into oligodendrocytes in serum-free defined medium. The differentiation, which occurs simultaneously with apoptotic cell death, was associated with a rapid loss of bcl-2 mRNA and increased expression of caspase-3 mRNA. Activation of caspase-3 in differentiating cells was demonstrated by the generation of 17- and 12-kDa fragments of caspase-3 proenzyme and by cleavage of poly(ADP-ribose) polymerase, a specific caspase-3 substrate. Cell death associated with differentiation was inhibited by the caspase-3 inhibitor DEVD-CHO in a dose-dependent manner. Assembly of sublytic C5b-9 resulted in inhibition of caspase-3 activation. In addition, synthesis of BCL-2 protein in oligodendrocytes was significantly increased by C5b-9. The TNF-α-induced apoptosis of oligodendrocytes was also inhibited by C5b-9. These results indicate that up-regulation of BCL-2 protein and inhibition of caspase-3 activation are potential mechanisms by which C5b-9 increases survival of oligodendrocyte in vitro and possibly in vivo during inflammation and immune-mediated demyelination affecting the CNS.

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Abbreviations used in this paper: MS, multiple sclerosis; CD7, normal human serum immunochemically depleted of C7; DEVD-CHO, Asp-Glu-Val-Asp-Chloroethyl acetate (CE); DEVD, DEVD-CHO, Asp-Glu-Val-Asp-Chloroethyl acetate; EAE, experimental allergic encephalomyelitis; ERK, extracellular signal-related kinase; FGF, fibroblast growth factor; GC, galactocerebroside; MBP, myelin basic protein; MTS, methyl tetrazolium salt; NHS, normal human serum; O-2A, OLG progenitor cells identified by mAb A2B5; OLG, oligodendrocyte; PARP, poly(ADP-ribose) polymerase; PDGF, platelet-derived growth factor; PI-3, phosphatidylinositol-3; PLP, proteolipid protein; RT, room temperature; TCC, terminal complement complexes representing C5b-7, C5b-8, and C5b-9.
to C5b-9 (32). Therefore, C5b-9 can contribute to demyelination by directly damaging the myelin, even in the absence of myelin-specific Abs. In OLG, C5b-9 at a sublytic concentration induces cell cycle, as shown by activation of ERK1 and c-Jun N-terminal kinase 1, protooncogenes, and G1 progression to S phase (8, 33). Sublytic C5b-9 also induces phenotype changes in OLG by accelerating the decay of mRNA encoding myelin-specific genes (8, 34). While activating cell cycle, C5b-9 was also found to inhibit apoptosis of OLG associated with differentiation (8).

In this study, we have examined the differentiation-associated apoptosis of OLG in vitro by investigating involvements of caspase-3 and Bcl-2 as possible target sites regulated by C5b-9.

Materials and Methods

Determination of OLG from O-2A progenitor cells in culture

Primary O-2A progenitor cells were prepared according to Saneto and de Vellis (35). Glial cells were isolated from neonatal Sprague Dawley rat brains, as described in detail (35, 34). Dispersed glia cells are grown for 10 days as stratified mixed glial cultures. O-2A progenitors growing on surface of the mixed culture were isolated by a series of differential shaking. Cells were placed in OLG defined medium consisting of serum-free DMEM/Ham’s F-12 containing 50 ng/ml transferrin (Sigma, St. Louis, MO), 75 ng/ml insulin (Sigma), 75 μg/ml basic FGF (Collaborative Research, Lexington, MA), and 1 mM sodium pyruvate. O-2A cells isolated by serial shaking at the time of plating showed 2–3% cell death, as determined by trypan blue dye exclusion. Determination was stepwise, as shown by the expression of MBP and proteolipid protein (PLP) mRNA before the expression of galactocerebroside (GC) (17, 35). After 56 h in OLG defined medium, more than 85% of cells expressed GC, MBP, and PLP. Less than 5% of the MBP-negative cells were astrocytes and microglia, and the remaining cells were O-2A cells in different stages of differentiation. O-2A cells grown in a defined medium for 3 days are designated as OLG.

Determination of cell viability

Viability of O-2A cells during differentiation and the effect of C5b-9 on cell viability were determined by using CellTitre 96 Aqueous cell proliferation assay, according to the instruction supplied by Promega (Madison, WI). Cells were seeded on poly(t-lysine)-coated 96-well plates at 10^4 cells/well in 200 μl of OLG defined medium and cultured at 37°C. At the indicated time points, 40 μl methyl tetrazolium salt (MTS) solution was added to each well. Plates were kept at 37°C for additional 2 h, followed by determination of OD at 540 nm under a condition in which absorbance was in linear range. The results are expressed as percentage of dead cells ± SD, relative to the initial cell number.

Analysis of apoptosis

DNA strand break was detected in cells by TdT-dependent incorporation of dUTP (Apoptag, Oncor, Gaithersburg, MD). O-2A cells were cultured on plastic slide chambers for the indicated time period. Cells were fixed in buffered Formalin at room temperature (RT), then treated with TdT in the presence of digoxigenin-dUTP for 1 h at 37°C. After washing, cells were treated with peroxidase-conjugated anti-digoxigenin IgG/F(ab’)2 fragments for 1 h; then color was developed using diaminobenzidine as a substrate. Approximately 600 cells with clearly defined nucleus were examined in each sample by TUNEL staining. The number of cells showing apoptosis was counted by identifying TUNEL-positive nuclei. The percentage of apoptotic cells was then calculated using the following formula: (number of cells with TUNEL-positive nuclei/total number of cells examined) × 100. Results are expressed as mean percentage of cells with TUNEL-positive nuclei ± SD.

Activation of caspase complement and C5b-9 assembly

Normal human serum (NHS) pooled from several healthy donors was used as a source of serum complement. Rabbit antisera to GC was used to sensitize rat OLG. The specific anti-GC activity was assayed by treating GC-expressing OLG with trapped 45Rb aqueous marker with anti-serum, then measuring the released marker (31). Because anti-GC Abs are mostly IgM isotype, IgM fraction of the antisera was used in most experiments. A sublytic dose of Ab was predetermined by titrating anti-GC Ab using an excess of NHS (8, 34). To evaluate the effect of serum C5b-9, OLG sensitized with a dose of anti-GC Ab for 30 min at RT were incubated with a 1/20 dilution of NHS depleted of C7 (C7D) reconstituted with C7 (10 μg/ml). Alternatively, sensitized cells were treated with NHS (1/10) and NHS treated with K76 (Otuka Pharmaceutical, New York, NY) (NHS-K76) as a control (8, 34). K76 prevents C5b-9 assembly in serum by binding to C5 (36). Therefore, C7D and NHS-K76 allow complement activation to proceed up to C6 and C3, respectively. Purified human complement proteins C5-C9 were purchased from Quidel (San Diego, CA), and C5b6 complex was prepared from C5 and C6, as described (37). To assemble sublytic C5b-9 by using purified proteins, cells were incubated with C5b6 (30 μg) for 15 min, then with C7 (10 μg) for 5 min at RT, followed by addition of C8 (10 μg) and C9 (10 μg) in a final volume of 1 ml (8, 34). Cells were then incubated at 37°C for the indicated time periods.

Northern blot analysis

RNA was isolated from cells lysed with buffer containing guanidine isothiocyanate and 2-ME, and total RNA was purified by ultracentrifugation on 5.7 M CsCl (as described in Ref. 38). Poly(A)+ RNA was prepared from total RNA using Dynabead mRNA purification system (Dynal, Great Neck, NY). Poly(A)+ RNA was denatured and electrophoresed on 0.8% agarose-formaldehyde gels, then transferred to a nitrocellulose membrane. After baking for 2 h at 80°C, the membrane was hybridized with 32P-labeled cDNA probes. The probe binding was quantitated by measuring band densities of autoradiogram using Computing Densitometer (Molecular Dynamics, Sunnyvale, CA). Integrated volume of each band was calculated using the ImageQuant software (Molecular Dynamics), and the results are expressed by density ratio to actin. Caspase-3 cDNA probe was obtained by RT/PCR cloning of rat caspase-3 cDNA with specific forward (5'-GC AGGCTTAAGTGACCATTGCAACCAAC) and reverse (5’-GGCTCTA GACCCATGCTTCTTTAGTGA) primers designed according to rat CPP32 cDNA (39). The rat bcl-2 and bax cDNA were gifts from Dr. E. Podack (University of Miami) and Dr. S. Korsmeyer (Washington University, St. Louis, MO), respectively. The cDNA was labeled with α-32P]dCTP (Du Pont, Enfield Nuclear, Boston, MA) using reagents for DNA labeling from Pharmacia (Piscataway, NJ).

Western blot analysis of caspase-3, PARP protein, and BCL-2

The levels of caspase-3, PARP, and their cleavage products were determined by Western and immunoblot. Cells were lysed with RIPA buffer (30 mM Tris-HCl, pH 7.4, 0.1 M NaCl (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), as described (16). An equal amount of protein from each cell lysate was used directly for SDS-PAGE and Western blot, a method sufficient to detect caspase-3 proenzyme and high levels of the cleavage fragment. To detect cleaved fragments, cell lysates (100 μg) were immunoprecipitated with rabbit anti-caspase-3 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of protein A/G agarose at 4°C overnight. Cell lysates or immunoprecipitates were analyzed on 10% SDS-PAGE, then by Western blotting using the same rabbit anti-caspase-3 IgG. For PARP immunoprecipitates using polyclonal anti-PARP IgG (Boehringer Mannheim, Indianapolis, IN) were analyzed by 7% SDS-PAGE, and monoclonal anti-PARP IgG1 (Zymed, San Francisco, CA) was used for immunoblotting. This was followed by reaction with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology), then by enhanced chemiluminescence (ECL; Pierce, Rockford, IL). BCL-2 protein was determined similarly by immunoprecipitation of cell lysates. The BCL-2 Western blot reagents were from Oncogene (Cambridge, MA).

Effects of caspase-3 inhibitor on OLG viability

To test whether caspase-3 activity is required for differentiation-induced apoptosis, the cell-permeable caspase-3 inhibitor DEVD-CHO (Calbiochem, San Diego, CA) was used. O-2A cells were seeded in 96-well plates at 10^3 cells/well in 200 μl of OLG defined medium and cultured for 24 h. Cells were further incubated for 48 h in the presence of 0–100 μM of DEVD-CHO. Cell viability was then determined, as described earlier.

Effect of C5b-9 on OLG apoptosis induced by TNF-α

To test whether sublytic C5b-9 also protects OLG from apoptotic cell death induced by TNF-α (21, 22, 40). O-2A cells were differentiated in 96-well plates, then cells were exposed to sublytic NHS or NHS-K76 for 1 h. After addition of 100 ng/ml of human rTNF-α (R&D Systems, Minneapolis, MN), cells were incubated for 18 h at 37°C, and viability was determined.
RESULTS

Apoptotic cell death of O-2A progenitor cells during in vitro differentiation

Differentiation of OLG is associated with cell death in developing brains and during in vitro differentiation (17–19). As shown in Fig. 1A, cell death reached 36% at 48 h, and was increased further to 70% at 96 h. Many of these cells showed the characteristic features of apoptosis, including cell process retraction, chromatin condensation, and DNA cleavage with ladder formation (data not shown). By TUNEL stain, 31.3 ± 6.5% of cells were apoptotic after 48 h in OLG defined medium (Fig. 1B).

Bcl-2, Bax, and caspase-3 expression during OLG differentiation

The expression of caspase-3, bcl-2, and bax during OLG differentiation was examined. Northern blot analysis of poly(A)⁺ RNA showed a gradual increase in caspase-3 mRNA expression and a rapid decline of bcl-2 mRNA as early as 2 h (Fig. 2A–C). The changes in bcl-2 and caspase-3 mRNA level are associated with differentiation, as shown by the robust expression of PLP mRNA (Fig. 2A). The bax mRNA expression was reduced by 30% at 6 h, as shown by density ratios to actin, obtained by quantitative densitometry (Fig. 2C). The bax mRNA level after 3 days in differentiation medium, determined in a separate experiment, was similar to the initial level (data not shown). Western blot analysis of cell lysates showed an increase in 32-kDa caspase-3 proenzyme after 24 h (1 day) (Fig. 3, A and B), which correlated with increased expression of caspase-3 mRNA. The caspase-3 proenzyme began to decrease on day 3, as shown by the density ratio to β-actin on the same blot. Because caspase-3 activity requires the proenzyme cleavage to active subunits (41), the appearance of 17- and 12-kDa subunits was evaluated by anti-caspase-3 immunoprecipitation, followed by Western blot. The appearance of anti-caspase-3-reactive 17- and 12-kDa bands was identified on day 3 (Fig. 3C).

Decreased caspase-3 proenzyme with increased cleavage products on day 3 (Fig. 3C) is clearly evident in experiments when cell lysates were immunoprecipitated, then examined by Western blotting.

To determine whether caspase-3 activation is required for cell death, O-2A cells in medium for 24 h were cultured for additional 48 h with the cell-permeable caspase-3 inhibitor DEVD-CHO. Cell death was inhibited in a dose-dependent manner, with 50% protection at 25 μM and 100% at 100 μM of DEVD-CHO (Fig. 4). DMSO at concentrations used to resuspend DEVD-CHO was not toxic to the cell.

Inhibition of caspase-3 activity by C5b-9

To evaluate the antiapoptotic activity of C5b-9 previously shown (8), OLG exposed to serum C5b-9 were examined for caspase-3 cleavage. As shown in Fig. 5A, a prominent 17-kDa cleavage product was seen in control cells treated with C7D for 18 h, which was inhibited by addition of C7 to C7D. Because the data were obtained by direct analysis of the cell lysates by SDS-PAGE/Western blotting, presence of the cleavage fragment in unstimulated cells was not detected. When cell lysates were immunoprecipitated first, then examined by Western/immunoblot (Fig. 5B), the caspase-3 cleavage fragment increased with time in cells exposed to NHS-K76. This increase was inhibited when C5b-9 assembly was allowed in NHS, in contrast to NHS-K76. To exclude a possibility that C5b-9 may have enhanced the serum effect on caspase-3, identical experiments were performed by treating cells with C5b-9 assembled using purified proteins (Fig. 5C). A cleavage fragment of caspase-3 was detected in unstimulated OLG. This 17-kDa band increased with time in control cells exposed to C5b6, C8, and C9 without C7. However, the cleavage product was barely detected in cells exposed to C5b-9. We have also examined the effect of C5b-9 on cleavage of PARP, a specific substrate for caspase-3 (41, 42).
On Western blotting, the 89-kDa fragment of PARP protein was detected in unstimulated OLG. The PARP cleavage was significantly reduced in cells treated with serum C5b-9, compared with the level of NHS-K76 (Fig. 6).

Expression of BCL-2 in OLG exposed to C5b-9
In view of the ability of C5b-9 to inhibit caspase-3 activation, the steps upstream to caspase-3 activation that may be affected by C5b-9 were explored. Caspase-3 can be activated by caspase-9 through mitochondrial pathway or by caspase-8 in death receptor-dependent pathway (43). We have analyzed the effect of C5b-9 on Bcl-2, a potent antiapoptotic factor, which inhibits caspase-3 activation by regulation of mitochondrial pathway (44). The expression of bcl-2 mRNA was not affected by C5b-9, as shown in Fig. 7B. However, C5b-9 significantly increased the level of BCL-2 protein within 4 h, and to the maximum level at 8 h (Fig. 7A). BCL-2 protein was not detected in unstimulated OLG and in OLG treated with control C5b6.
Protection of TNF-α-induced cell death by C5b-9

We have examined whether C5b-9 also protects OLG from apoptosis induced by other factors. TNF-α was tested, since TNF-α induces apoptotic cell death in OLG both in vivo and in vitro (21, 22, 45). In our system, 100 ng/ml of TNF-α induced 50% cell death after 18 h (Fig. 8A). Pretreatment with NHS, but not with NHS-K76, protected OLG from cell death (Fig. 8A). The cleavage product of caspase-3 proenzyme, which was increased by TNF-α, was abolished in OLG treated with NHS (Fig. 8B).

Discussion

The C5b-9 complex is a pleiotropic effector generated during inflammation and immune response. When inserted into the target cell membrane, C5b-9, depending on doses, causes cell death or cell activation (4, 5). We have previously shown that at a sublytic concentration, C5b-9 enhances OLG survival in vitro through inhibition of apoptosis (8). C5a and C5b-9 have been implicated in inflammation and immune response. When inserted into the target cell membrane, C5b-9, depending on doses, causes cell death or cell activation (4, 5). We have previously shown that at a sublytic concentration, C5b-9 enhances OLG survival in vitro through inhibition of apoptosis (8). C5a and C5b-9 have been implicated in inflammation and immune response. When inserted into the target cell membrane, C5b-9, depending on doses, causes cell death or cell activation (4, 5). We have previously shown that at a sublytic concentration, C5b-9 enhances OLG survival in vitro through inhibition of apoptosis (8). 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was abrogated by C5b-9, as shown by inhibition of caspase-3 proenzyme cleavage into its active subunits. This finding was consistent with the inhibition of PARP cleavage, a substrate for activated caspase-3. Regulation of BCL-2 expression was examined as a possible step upstream to the caspase-3 affected by C5b-9. In OLG, bcl-2 mRNA was expressed at a very low level without detectable protein, as examined by sensitive methods, such as the use of poly(A) RNA for Northern blot and analysis of cell lysates by immunoprecipitation and Western immunoblot. Interestingly, C5b-9 was able to increase BCL-2 protein without significantly affecting the mRNA level, suggesting a possible role of C5b-9 in posttranscriptional regulation of Bcl-2. Detection of bcl-2 mRNA in the absence of BCL-2-2 protein in germinal center B cells (55) and in a trophoblastic tumor cell line when induced to differentiate (56) also suggested a step of translational regulation of Bcl-2. A specific cis element within the promoter has been identified as a regulatory site involved in the translational control of bcl-2 gene (57). How BCL-2 synthesis is regulated by C5b-9 remains unclear. We have shown that sublytic C5b-9 induces ERK1 pathway, and this is through activation of phosphatidylinositol-3 kinase (PI-3) kinase (9, 10, 33). In OLG, ERK1 activated by C5b-9 is responsible for enhanced DNA synthesis (33), and C5b-9 increased the p70 S6 kinase activity (33), a ribosomal kinase responsible for protein synthesis (58). PI-3 kinase has been shown to inhibit apoptosis, and this is thought to be through activation of Akt kinase and by increasing BCL-2 (59, 60). In postmitotic cells such as OLG, C5b-9, instead of inducing proliferation, may enhance cell survival. The putative antiapoptotic signaling generated by C5b-9 may include PI-3 kinase. C5b-9, by increasing BCL-2, may stabilize mitochondrial inner membrane permeability, or inhibit the interaction of BAX with outer membrane proteins (61). BCL-2 prevents cytochrome c release and inhibits activation of caspase-9 and caspase-3 (54). Therefore, up-regulation of BCL-2 protein by C5b-9 in OLG may precede the inhibition of caspase-3 activation. C5b-9 also inhibited cell death and caspase-3 activation induced by TNF-α. TNF-α induces apoptosis via caspase-8 through the recruitment of TRADD/FADD (TNFR-associated death domain/Fas-associated death domain) proteins to the TNFR1 (62). However, TNF-α also generates ceramide, which induces caspase-9 activation and apoptosis, in a caspase-8-independent manner (49, 63). We can speculate that C5b-9 inhibits OLG apoptosis induced by differentiation and by TNF-α, and this is mediated through up-regulation of BCL-2 protein and inhibition of caspase-9 and caspase-3.

Our finding that C5b-9 rescues OLG from differentiation-induced apoptosis and apoptosis caused by TNF-α may have a biological significance in inflammatory and immune-mediated demyelination. Apoptosis of OLG has been observed in EAE and MS (64, 65), IFN-γ, cuprizone, and HTLV-1, known to induce demyelination in vivo, also induce OLG apoptosis (66–68). Therefore, an understanding of mechanisms leading to and preventing apoptosis of OLG and its progenitor cells is critically important to develop rational approaches to enhance OLG survival and remyelination.

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