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Association of Increased Bcl-2 Expression with Rescue from Tumor Necrosis Factor-α-Induced Cell Death in the Oligodendrocyte Cell Line OLN-93

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Abstract: The present study investigated the effects of flupirtine (Katadolon) on tumor necrosis factor (TNF)-α-mediated cell death and Bcl-2 expression in the permanent rat oligodendrocyte cell line OLN-93 (OLN cells). TNF-α (500 U/ml) induced apoptosis of OLN cells, which was confirmed by DNA fragmentation using an in situ end-labeling technique and ultrastructural analysis. Flupirtine significantly reduced the rate of spontaneous cell death of OLN cells already at low concentrations; TNF-α-mediated apoptosis was suppressed only with higher concentrations of flupirtine (100 μ*M*). Expression of Bcl-2 protein and mRNA in OLN cells was detected by immunocytochemistry, western blot, and RT-PCR. Quantitative analysis of western blots revealed an ~2.5-fold upregulation of Bcl-2 protein during TNF-α treatment. Furthermore, addition of 10 or 100 μ*M* flupirtine before incubation with TNF-α led to an approximately threefold increase of Bcl-2 expression. Exposure of OLN cells to flupirtine alone moderately augmented the expression of Bcl-2 protein. Our data demonstrate that flupirtine upregulates the expression of Bcl-2 protein in OLN cells; this Bcl-2 induction is associated with a reduced rate of TNF-α-induced cell death. **Key Words:** OLN cells—Apoptosis—Bcl-2—Flupirtine—Multiple sclerosis.
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Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS leading to selective destruction of myelin sheaths and/or oligodendrocytes (Lassmann, 1983). The mechanisms behind demyelination or remyelination, however, are poorly understood, although a heterogeneous pathogenesis is suggested owing to detailed investigations of oligodendrocyte pathology in demyelinating lesions (Lucchinetti et al., 1996, 1999). In particular, it is not yet clear whether oligodendrocytes are primarily affected in the disease or whether they are destroyed together with myelin during active demyelination (Itoyama et al., 1980; Brück et al., 1994; Ozawa et al., 1994; Raine, 1994). Oligodendrocyte death is a prominent feature in MS lesions. It is, however, not yet clear whether oligodendrocytes die via apoptosis or necrosis, which are different mechanisms of cell death (Selmaj et al., 1991; Lucchinetti et al., 1996). Dowling et al. (1997) observed that 14–40% of all degenerating cells in MS lesions are of oligodendroglial lineage and that most of these cells were dying by apoptosis. Exposure to heat-treated cerebrospinal fluid from MS patients caused apoptotic death of astrocytes and oligodendrocytes (Menard et al., 1998). On the other hand, Bonetti and Raine (1997) found that oligodendrocytes express cell death-related molecules such as tumor necrosis factor (TNF) receptors but show no evidence of apoptosis. TNF is a key protein in inducing oligodendrocyte death. It mediates apoptosis of oligodendrocytes in vitro (Selmaj and Raine, 1988), and its overexpression in the CNS leads to demyelination and oligodendrocyte death (Akassoglou et al., 1998). A recent study revealed a strong association of TNF-α mRNA expression and active demyelination in MS lesions (Bitsch et al., 2000).

Apoptosis or programmed cell death (PCD) is an active process of normal cell death during development and also occurs as a cytotoxic consequence of several stimuli, such as cytokines or irradiation (Schwartz and Osborne, 1993). Apoptotic cell death is accompanied by nuclear changes that include oligonucleosomal DNA fragmenta-

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Abbreviations used: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MS, multiple sclerosis; OLN cells, rat oligodendrocyte cell line OLN-93; PCD, programmed cell death; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

tion and chromatin condensation into a dense crescent-shaped aggregate that marginates along the nuclear envelope and leads to cell shrinkage and membrane blebbing (Lo et al., 1995). Several proteins are involved in the regulation of apoptosis. Bcl-2 is suggested to play an important role in protecting cells from PCD. It was first described in a B cell lymphoma in which it is overexpressed; it is located in the endoplasmatic reticulum and in the inner and outer mitochondrial membranes (Tsujimoto and Croce, 1986; Hawkins and Vaux, 1994; Reed, 1994). Bcl-2 seems to be involved in the apoptotic pathway by inhibiting damage of lipid membranes and cell organelles through oxygen radicals or by affecting the cell cycle (Hockenbery et al., 1990); it was shown to be expressed by oligodendrocytes in chronic active and silent MS lesions (Bonetti and Raine, 1997) and to correlate positively with the survival of oligodendrocytes after a demyelinating attack (Kuhlmann et al., 1999).

Flupirtine is a centrally acting nonopioid analgesic that displays cytoprotective activity in cultured neurons induced to undergo apoptosis and reduces ischemic damage in rats (Block et al., 1997). Flupirtine has been shown to induce and up-regulate the neuronal expression of Bcl-2 protein in vitro in different experiments (Müller et al., 1997; Perovic et al., 1997). Oligodendrocytes are specifically sensitive to TNF-α and undergo PCD that might involve Bcl-2 regulation. To elucidate whether flupirtine exerts protective effects also on glial cells via an up-regulation of Bcl-2, we have used the rat oligodendroglia cell line OLN-93 (OLN cells) (Richter-Landsberg and Heinrich, 1996). These cells show characteristics of immature oligodendrocytes and provide a model system for the study of cells of oligodendroglia origin.

MATERIALS AND METHODS

Cell line

OLN-93, a permanent oligodendroglia cell line derived from primary Wistar rat brain glial cultures, was used (Richter-Landsberg and Heinrich, 1996). These cells morphologically resemble bipolar O-2A progenitors but are differentiated into myelin basic protein-, myelin-associated glycoprotein-, and proteolipid protein-expressing oligodendrocytes.

Cells were kept at 37°C and 10% CO_2 in Dulbecco's modified Eagle's medium (Seromed, Berlin, Germany) containing 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Culture medium was changed three times a week. For the experiments described below, OLN cells were exposed to 500 U/ml recombinant human TNF-α (Genzyme, Cambridge, MA, U.S.A.) for 96 h. For cotreatment with flupirtine, OLN cells were preincubated for 2 h with increasing concentrations (0, 1, 5, 10, 50, and 100 μ*M*) of flupirtine (Katadolon; Asta Medica, Frankfurt, Germany). OLN cells cultured in the absence of any treatment served as controls.

Immunocytochemistry

Cells (10^6/ml) were grown on Nunc (Wiesbaden Germany) Lab-Tek Chamber Slides in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum alone or supplemented with 10 μ*M* flupirtine. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature and washed in phosphate-buffered saline. Endogenous peroxidase activity was blocked with 3% H_2O_2. Cells were permeabilized in 0.2% Triton X-100 diluted in 5% bovine serum albumin for 20 min. Rabbit anti-Bcl-2 antibody (Santa Cruz, Heidelberg, Germany; diluted 1:100) was applied for 2 h at room temperature. A peroxidase-antiperoxidase technique was used and visualized with a DAB-Metal-Enhancement Kit (Pierce, Germany). In negative controls, the primary antibody was omitted.

Western blot analysis

Cellular monolayers were washed with phosphate-buffered saline and scraped off in sample buffer [1% sodium dodecyl sulfate, 10% glycerin, 1% β-mercaptoethanol, and 12.5% Tris (0.5 *M*, pH 6.8) in double-distilled water] and boiled for 5 min. Total protein content was determined by photometric quantification. For quantification of Bcl-2 protein, 5 μg of control Bcl-2 protein (Santa Cruz) was loaded.

For immunoblotting, total cellular extracts (80 μg of protein per lane) were separated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12.5% polyacrylamide gels and transferred to nitrocellulose membranes according to the technique of Towbin et al. (1979). The blots were washed and incubated with rabbit anti-Bcl-2 antibody (Santa Cruz; 1:1,500, 90 min at room temperature) followed by the secondary antibody (mouse anti-rabbit IgG; DAKO; 1:3,000, 1 h at room temperature). After application of the peroxidase–antiperoxidase complex (DAKO; 1:3,000, 30 min at 37°C), the blots were washed and visualized by enhanced chemiluminescence according to the manufacturer's protocol (Amersham, Little Chalfont, Bucks, U.K.). Western blots were performed from three independent experiments. The blots were scanned, and the optical density of Bcl-2 bands on western blots was recorded using computer-aided software (Adobe Photoshop). The luminescence of bands in scanned western blots was evaluated by analyzing histograms for each band. The density of the bands is given in arbitrary units as mean values with SD.

RNA isolation and PCR

Total RNA extraction was performed with the RNeasy Total RNA Purification Kit from Qiagen (Hilden, Germany). One microgram of RNA was reverse-transcribed using 40 units of Moloney murine leukemia virus reverse transcriptase (Boehringer, Mannheim, Germany), and the resulting cDNA was amplified by PCR for 40 reaction cycles (95°C for 1 min, 58.5°C for 1 min, 72°C for 1 min). Cycle dependency tests were performed to ensure capture of the PCR products in the linear range of the amplification reaction. The primers used were as follows: rat Bcl-2, forward 5′ AGG GGG AAA CAC CAG AAT C 3′, reverse 5′ TGG AAG GAG AAG ATG CCA G 3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′ ACC ACA GTC CAT GCC ATC AC 3′, reverse 5′ TCC ACC ACC CTG TTG CTG TA 3′. The PCR mixture consisted of 4 μl of cDNA, 1 μl of each primer (20 pmol/μl), 0.2 m*M* deoxynucleotide triphosphates, and 2.5 U *Taq* polymerase in a total volume of 100 μl (for GAPDH, 50 μl), resulting in a fragment of 331 (rat Bcl-2) or 451 (GAPDH), respectively. The amplification product was visualized on a 1.5% agarose gel.

Sequence analysis

The sequence analysis of the PCR product was performed with a purified PCR product (QIAquick PCR Purification Kit; Qiagen). The rat Bcl-2 primers for the tailing PCR cycling

(95°C for 2 min; three cycles of 95°C for 20 s, 57°C for 20 s, 70°C for 30 s; 12 cycles of 95°C for 20 s, 70°C for 1 min) were as follows: forward 5′ TGT AAA ACG ACG GCC AGT AGG GGG AAA CAC CAG AAT CAA GT 3′, reverse 5′ CAG GAA ACA GCT ATG ACC TGG AAG GAG AAG ATG CCA GGG GT 3′. The sequence reaction was performed in 30 cycles (95°C for 2 min; 30 cycles of 95°C for 15 s, 57°C for 15 s, 70°C for 15 s). Each lane of a 4.3% gel (Licor System) was loaded with 3 μl.

Apoptosis assay

To assess the extent of apoptosis in situ, the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique was applied to OLN cells with or without preincubation with flupirtine (1, 5, 10, 50, and 100 μM) cultured in the presence or absence of TNF-α (500 U) as described in detail above. According to the manufacturer's instructions (Boehringer Mannheim), cells were incubated for 1 h at 37°C with the tailing mixture (containing double-distilled water, tailing buffer, 2.5 mM $CoCl_2$, 10 pmol of digoxigenin DNA, and 25 units of terminal transferase). After washing in Tris-buffered saline, OLN cells were incubated for 1 h at room temperature with an alkaline phosphatase-conjugated anti-digoxigenin antibody (diluted 1:250 in 10% fetal calf serum). The reaction was visualized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The number of OLN cells with DNA fragmentation was calculated from the total number of cells within the culture flask. A minimum of 100 cells was evaluated in each culture flask.

Electron microscopy

OLN cells were cultured in 12-well plates and exposed to TNF-α for 96 h. The cells were fixed in 3% glutaraldehyde (2 h, 4°C) and in 1% OsO_4 (1 h, 4°C). After washing in phosphate-buffered saline, cells were dehydrated in an ascending ethanol series (10 min in 50%, 1 h in 70%, 10 min in 80%, 10 min in 96%, 15 min in 100% ethanol, and 20 min in propylene oxide) and embedded in Araldite (incubation for 35 min in 1:1 and 2:1 Araldite–propylene oxide solution and finally 1 h in pure Araldite). Thin sections were placed onto copper grids for electron microscopy and contrasted with lead citrate. Electron microscopy was performed with an EM10 electron microscope (Zeiss).

Statistical analysis

Student's t test and Mann–Whitney U test were used for statistical analysis.

RESULTS

Bcl-2 protein expression

Immunocytochemistry showed Bcl-2-positive OLN cells with staining of the cytoplasm and the nuclear membrane (Fig. 1a). For quantitative determination of Bcl-2 protein expression, a western blot analysis was performed. OLN cells were incubated for 2 h with different concentrations of flupirtine (1, 5, 10, 50, and 100 μM), with TNF-α (500 U/ml) alone or a combination of both as described above. Cells without any treatment were used as controls. The Bcl-2 control protein migrated with a molecular mass of 26 kDa; each probe revealed a band of the correct size (~26 kDa). Flupirtine alone caused a concentration-dependent induction of Bcl-2 protein in OLN cells (Fig. 2a). Also, Bcl-2 protein was induced by TNF-α alone and even stronger after the combined treatment with TNF-α and flupirtine (Fig. 2b). Quantitative evaluation showed that 100 μM flupirtine caused an ~1.6-fold increase in Bcl-2 expression. An almost 2.5-fold Bcl-2 induction was observed after treatment with 500 U/ml TNF-α. The strongest induction was seen after combined treatment with flupirtine (10 and 100 μM) and 500 U/ml TNF-α, corresponding to an approximately threefold increase compared with untreated cells (Fig. 3). These data indicate that Bcl-2 expression in OLN cells was moderately induced by flupirtine. TNF-α alone led to strong Bcl-2 expression, which further increased during combined treatment with these substances.

Bcl-2 mRNA expression

The presence of Bcl-2 mRNA in OLN cells was confirmed at the transcriptional level with the RT-PCR technique. GAPDH was used as a housekeeping gene for the control. Bcl-2 mRNA was consistently expressed in OLN cells treated with different flupirtine concentrations (1, 5, 10, 50, and 100 μM) alone or in combination with TNF-α. The expected Bcl-2 PCR product (331 bp long) was present in all samples tested (Fig. 4).

Sequencing of the Bcl-2 PCR product identified the examined cDNA as the rat *bcl-2* gene. The rat Bcl-2 mRNA consists of 1,179 bases. The present study sequenced between base 58 and 390 (331 bases). The obtained sequence was identical to the known sequence of the *Rattus norvegicus* apart from two point mutations that were not located in the coding region.

Rate of oligodendrocyte apoptosis

The TUNEL technique was used to assess the rate of apoptotic OLN cells after treatment with different concentrations of flupirtine (1, 5, 10, 50, and 100 μM) alone or followed by incubation with 500 U of TNF-α for 96 h. TUNEL-positive OLN cells were found in each sample tested. In comparison with control and flupirtine-treated cells, however, TNF-α most effectively induced apoptosis. Flupirtine significantly reduced the spontaneous cell death rate already at low concentrations of 1 μM. TNF-α-induced apoptosis was also prevented by flupirtine; however, higher concentrations of flupirtine were required to rescue OLN cells from cell death (Figs. 1b and c and 5).

The nature of the cell death, apoptotic or necrotic, was further investigated by electron microscopy. Ultrastructurally, the cells revealed the characteristic morphological signs of apoptosis such as chromatin condensation and the formation of apoptotic bodies. This was especially observed in OLN cells stimulated with TNF-α (Fig. 6).

DISCUSSION

The pathogenesis of demyelination and the fate of oligodendrocytes in MS are closely connected. The patterns of oligodendrocyte destruction, preservation, or proliferation are still a matter of debate. Different immu-

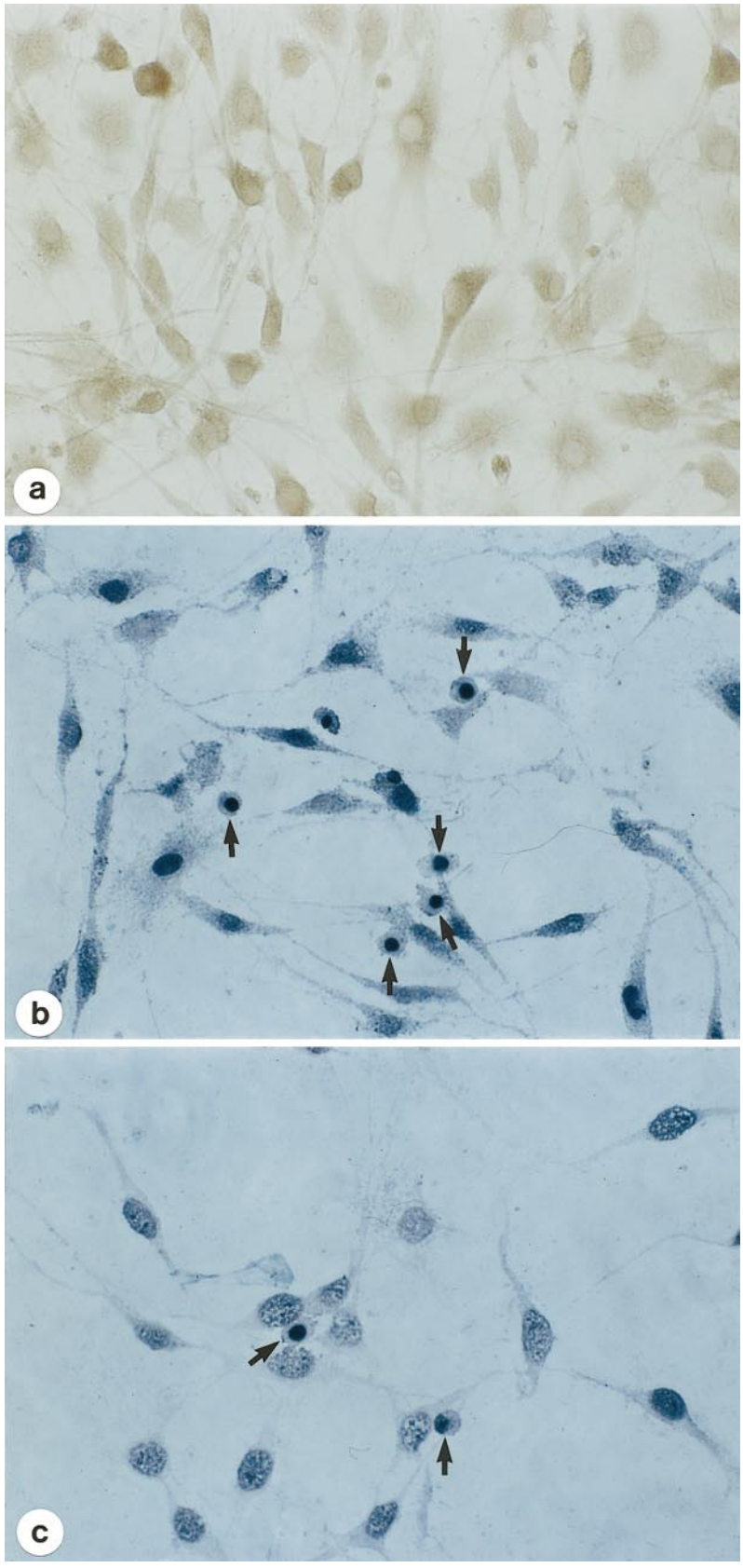

FIG. 1. a: Immunocytochemistry for Bcl-2 in OLN cells. The cultured cells express Bcl-2 (immunocytochemistry for Bcl-2). **b:** TUNEL staining of OLN cells in the presence of 500 U/ml TNF-α. There are numerous cells with DNA fragmentation (arrows) showing condensed, apoptotic nuclei. **c:** In the presence of 500 U/ml TNF-α and 100 μM flupirtine, the number of OLN cells with DNA fragmentation is significantly reduced. Original magnification $\times$40.

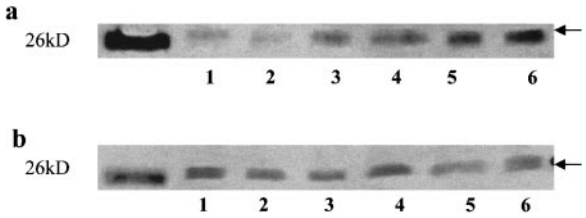

FIG. 2. Western blot for Bcl-2 protein: first unlabeled lane, Bcl-2 control protein; lane 1, no flupirtine; lane 2, 1 μM flupirtine; lane 3, 5 μM flupirtine; lane 4, 10 μM flupirtine; lane 5, 50 μM flupirtine; and lane 6, 100 μM flupirtine. **a:** Flupirtine induced a moderate increase in Bcl-2 protein expression. **b:** Combined treatment with 500 U/ml TNF-α led to a much higher Bcl-2 protein expression.

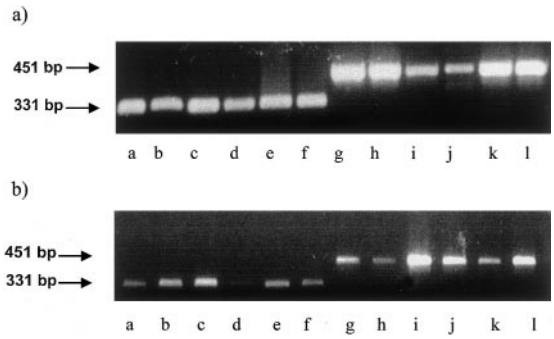

FIG. 4. PCR for Bcl-2 (lanes a–f) and GAPDH mRNA (lanes g–l) results in amplification of the expected 331-bp product for Bcl-2 and the 451-bp product for GAPDH. There is a consistent expression of Bcl-2 mRNA in OLN cells treated with different concentrations of flupirtine in the (**a**) absence or (**b**) presence of 500 U/ml TNF-α.

nological or toxic mechanisms have been suggested to be involved in oligodendrocyte destruction, including the proinflammatory cytokine TNF-α. OLN cells may be used as a model to investigate the oligodendroglia cell lineage. The present study provides evidence for an important role of the antiapoptotic protein Bcl-2 in the rescue of OLN cells from TNF-α-induced apoptosis.

Cell death is a key event in various different cell processes. Apoptosis as a physiological event has been shown to be one of the two important cell death mechanisms and is regulated by a wide range of different stimuli as well as proapoptotic and antiapoptotic genes. The Fas/APO-1 (CD 95) system and the tumor suppressor gene p53 are typical members of the family of apoptosis-inducing genes, whereas Bcl-2 is the prototype of an antiapoptotic protein. Bcl-2 inhibits apoptosis and promotes cell survival (Hockenbery et al., 1990; Vaux, 1998). The antiapoptotic activity is achieved by suppressing the apoptosis-inducing function of Bax by forming heterodimers with the Bax protein (Oltvai et al., 1993). It has been shown that Bcl-2 up-regulation in neurons reduces apoptosis during physiological cell death (Zanjani et al., 1996).

There is still considerable controversy on the role of apoptosis in the elimination of oligodendrocytes from demyelinating CNS lesions. Local TNF/p55TNF receptor signaling is capable of inducing oligodendrocyte apoptosis and demyelination in transgenic mice (Akassoglou et al., 1998). After intracerebral infection of Lewis rats with JHM coronavirus, a chronic inflammatory demyelinating disease is induced, which in many aspects mimics the pathology of MS. At later stages after infection, the virus antigen was nearly completely cleared from the lesions, and oligodendrocytes were mainly destroyed by apoptosis (Barac-Latas et al., 1997). Similar observations were made in canine distemper virus demyelinating encephalitis (Schobesberger et al., 1999) as well as in an experimental model of myelin-associated glycoprotein deficiency (Lassmann et al., 1997). In and around MS lesions, molecules belonging to the apoptotic cascade have been shown to be expressed by oligodendrocytes (Bonetti and Raine, 1997). Whether the expression of these molecules is associated with the presence of oligodendrocyte apoptosis, however, is still controversial (Lucchinetti et al., 1996; Bonetti and Raine, 1997; Dowling et al., 1997, 1999).

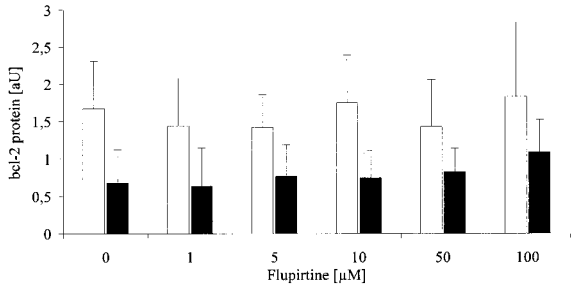

FIG. 3. Quantitative determination of amount of Bcl-2 protein after treatment with increasing concentrations of flupirtine in the presence (open columns) or absence (solid columns) of 500 U/ml TNF-α. TNF-α and flupirtine alone induced Bcl-2 expression in OLN cells, but the combination of both led to a much stronger Bcl-2 induction. aU, arbitrary units.

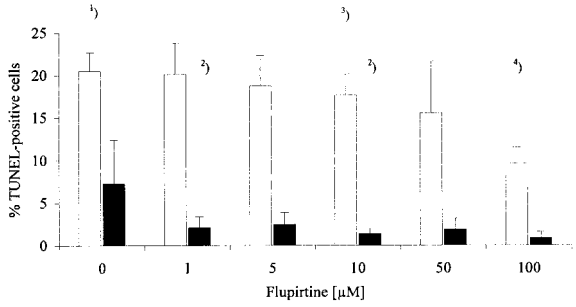

FIG. 5. Percentage of OLN cells with DNA fragmentation during treatment with increasing concentrations of flupirtine in the presence (open columns) or absence (solid columns) of 500 U/ml TNF-α. Data are mean $\pm$ SD (bars) values. Flupirtine significantly reduced the spontaneous as well as the TNF-α-induced apoptosis of OLN cells. [1] $p < 0.0005$, [2] $p < 0.05$ by Mann–Whitney U test, significant versus cells without any treatment; [3] $p < 0.05$, [4] $p < 0.0001$ by Student's t test, significant versus cells treated with 500 U/ml TNF-α only.

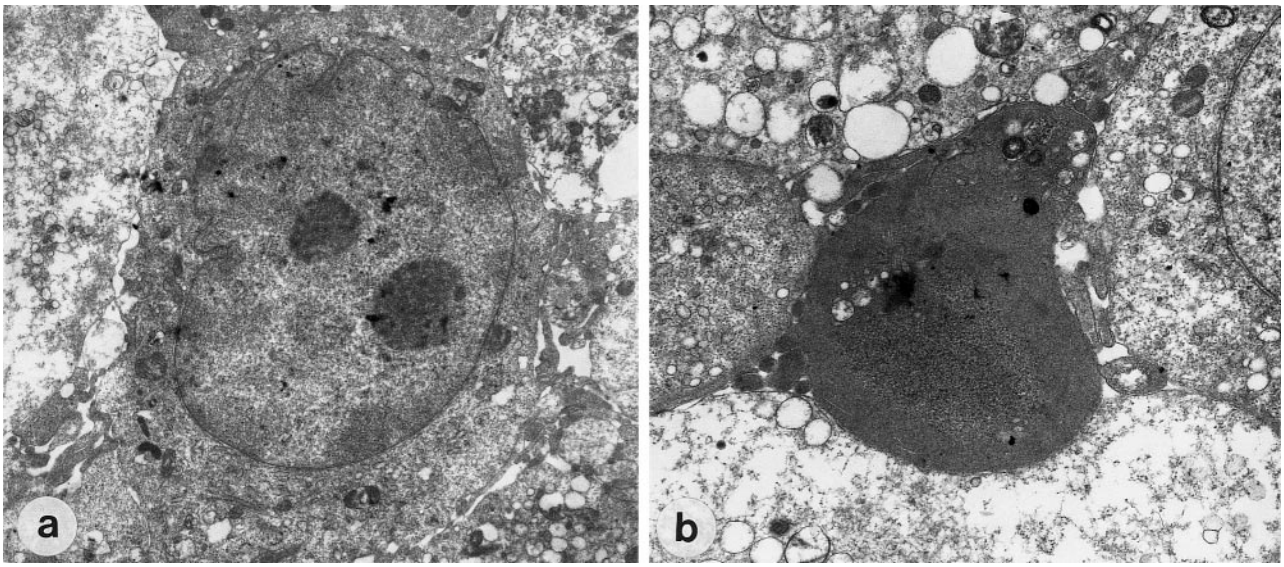

FIG. 6. Ultrastructure of untreated and TNF-α-treated OLN cells. **a:** Normal-appearing nucleus in untreated control cultures. **b:** Condensed, apoptotic nucleus in a culture treated with 500 U/ml TNF-α. Magnification ×6,300.

The fact that oligodendrocytes can be driven into apoptosis in vitro, in contrast, is undoubted, and TNF-α seems to play a critical role in this process (Selmaj and Raine, 1988; Selmaj et al., 1991). The protooncogene *p53* induces oligodendrocyte death after induction by TNF-α (Eizenberg et al., 1996; Ladiwala et al., 1999), and TNF-α potentiates interferon-γ-induced cell death in oligodendrocyte progenitors (Andrews et al., 1998). The final execution phase of TNF-α-induced oligodendrocyte death seems to be mediated by the ICE/CED-3 family of caspases (Hisahara et al., 1997; Gu et al., 1999). In our study, flupirtine up-regulated Bcl-2 when given in high concentrations (100 *μM*). The greatest increase in Bcl-2 protein was observed when TNF-α was applied in combination with 10 or 100 *μM* flupirtine. Flupirtine treatment protected oligodendrocytes from TNF-α-mediated apoptosis. However, only the 100 *μM* concentration of flupirtine produced a highly significant reduction of oligodendrocyte apoptosis. This may be due to the fact that flupirtine does not selectively affect Bcl-2 protein expression, but also modifies other proteins of the apoptosis cascade. Besides up-regulation of Bcl-2, flupirtine is known to decrease levels of ICH-1, which is caspase 2 (Osborne et al., 1997). It is interesting that caspase 2 and 3 expression was demonstrated in oligodendrocytes undergoing apoptosis (Gu et al., 1999). A recent study identified the inhibition of caspase 3 and up-regulation of Bcl-2 as mechanisms to rescue oligodendrocytes from TNF-α-mediated apoptosis (Soane et al., 1999). Thus, flupirtine may act at different levels of the apoptotic cascade and interfere with the balance of proapoptotic and antiapoptotic signals.

The present study provides evidence that Bcl-2 is expressed constitutively by OLN cells and that the level of Bcl-2 expression depends on the stimulation with different concentrations of flupirtine and/or TNF-α. Flupirtine increases the expression of Bcl-2 and protects neurons from β-amyloid-induced apoptosis or prion fragment-mediated neurotoxicity (Müller et al., 1997; Perovic et al., 1997). In the present study, flupirtine alone or in addition to TNF-α up-regulated Bcl-2 protein and rescued oligodendrocytes from TNF-α-induced cell death. The induction of Bcl-2 expression may therefore be an interesting therapeutic target in demyelinating diseases to support oligodendrocyte survival. The question whether Bcl-2 plays a role in the survival of oligodendrocytes in MS lesions was recently studied (Kuhlmann et al., 1999). As already shown by others (Bonetti and Raine, 1997), Bcl-2 is present in MS plaques; our study, however, found a clear association among Bcl-2 expression, oligodendrocyte survival, and remyelination in MS plaques (Kuhlmann et al., 1999). Bcl-2-associated mechanisms may belong to a group of different effectors that support survival of oligodendrocytes and include the activation of tyrosine kinase or cytokine receptors (Casaccia-Bonnefil, 2000). New therapeutic strategies may develop from these experiments, preventing loss of oligodendrocytes from demyelinating lesions and supporting myelin regeneration.

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