Overexpression of the apoptosis inhibitor FLIP in T cells correlates with disease activity in multiple sclerosis

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Abstract

The cellular caspase-inhibitory protein FLIP has been recently identified as a potent regulator of T lymphocyte susceptibility to Fas-mediated programmed cell death (apoptosis). Since impairment of apoptosis may be involved in multiple sclerosis (MS), we investigated the dynamics of cellular FLIP in unstimulated and activated T lymphocytes from MS patients, inflammatory and non-inflammatory neurological disorders, and healthy subjects. Cellular expression of the long and short forms of FLIP protein was similar in unstimulated T cells from MS patients and controls, but was significantly higher in activated T cells from patients with clinically active MS. This high FLIP expression in active MS correlated with cellular resistance to Fas-mediated apoptosis. In contrast, cellular expression of the anti-apoptotic protein Bcl-2 did not differ between active and stable disease, and was relatively similar between the MS group and controls. These findings suggest that cellular overexpression of the anti-apoptotic protein FLIP is a feature of clinically active multiple sclerosis.

Keywords: Multiple sclerosis; Disease activity; Apoptosis; FLIP

1. Introduction

The pathogenesis of multiple sclerosis (MS) is thought to involve autoimmune recognition of self-antigens by autoreactive T lymphocytes (Martin and McFarland, 1995). The cause of this breakdown of immune tolerance to self-antigens is unknown, but probably involves impairment of activation-induced programmed cell death (apoptosis) of autoreactive T lymphocytes (Pender, 1998). In normal subjects, apoptosis regulates the elimination of activated, potentially pathogenic lymphocytes from the circulation and tissues (Mountz et al., 1996) through a subgroup of the tumor necrosis factor receptor family, the death receptors (Scaffidi et al., 1999a). The best-characterized member of the death receptor family is the cell surface receptor Fas (CD95/Apo-1) (Chinnaiyan and Dixit, 1997). There is emerging evidence that Fas-mediated apoptosis is involved in the regulation of autoimmune responses in experimental models of MS (Suvannavejh et al., 2000). Similarly, impairment of the Fas system may be involved in the pathogenesis of MS since increased expression of Fas and its ligand has been reported in MS plaques (D’Souza et al., 1996; Dowling et al., 1996), and high circulating levels of the soluble form of Fas protein have been detected in MS patients (Iinoue et al., 1997; Ciusani et al., 1998). Moreover, recent reports suggest that activated lymphocytes from MS patients are less sensitive to Fas-mediated apoptosis than cells from healthy individuals (Comi et al., 1999; Macchi et al., 1999; Zang et al., 1999). This reduced susceptibility to apoptosis in MS has been partly attributed to elevated levels of soluble Fas, which may block cell death by antagonizing the activity of the membrane-bound receptor (Zipp et al., 1998b). However, serum levels of Fas are not necessarily elevated in MS patients (Bansil et al., 1997) and Fas expression in lymphocytes is usually within the normal range (Zipp et al., 1998a), suggesting that other inhibitors of apoptotic-signalling pathways may exist in MS.

The sensitivity to Fas-mediated apoptosis can be modulated by the overexpression of anti-apoptotic molecules, such as the Bcl-2 family of regulatory proteins (Hawkins and Vaux, 1997). In addition, the novel anti-apoptotic protein FLIP (also known as Casper, I-FLICE, CLARP, FLAME-1, CASH, MRIT or Usurpin) (Thome et al., 1997) has been recently identified in human peripheral lymphocytes and tissue, and is thought to regulate apoptosis...
induced by Fas and all other known human death receptors (Irmler et al., 1997). Although its precise role is still being elucidated, FLIP protein appears to control the fate of mature T lymphocytes in the periphery, as it is expressed during the early stages of cell activation, but disappears when the cells become susceptible to Fas-mediated apoptosis (Irmler et al., 1997; Wallach, 1997; Scaffidi et al., 1999b).

The importance of FLIP in regulating cellular homeostasis is illustrated by the detection of high levels in melanoma cell lines and other malignant tumors (Irmler et al., 1997). Similarly, overexpression of FLIP in T lymphocytes and other mononuclear cells confers resistance to apoptosis (Wallach, 1997; Scaffidi et al., 1999b) and may contribute to the development of autoimmune diseases (Algaciras-Schimmich et al., 1999; Perlman et al., 1999). On the basis of these observations, we sought to evaluate FLIP expression in both resting and activated T lymphocytes from MS patients, and assess its clinical significance by correlating cellular levels with disease activity, susceptibility to Fas-mediated apoptosis and the expression of Fas receptor protein. Results presented here indicate that FLIP protein is over-expressed in activated T cells from patients with clinically active MS and correlates with cellular resistance to apoptosis, information of relevance to the pathogenesis of MS.

2. Patients and methods

2.1. Patients and controls

Heparinized blood was obtained from 28 patients with clinically definite MS, who were recruited from patients attending the MS Research Clinic at Guy’s Hospital, London. All had the relapsing-remitting form of the disease, and none had received immunosuppressive therapy or steroid treatment in the 6 months before blood collection. Their mean disease duration was 6.8 years (range, 1.5–14.6 years), and the mean Expanded Disability Status Scale (EDSS) was 3.4. All patients underwent gadolinium-enhanced magnetic resonance imaging (MRI), detailed clinical assessment, and scoring of EDSS at study entry. Patients were classified according to both clinical and MRI features into two groups: clinically active (18 patients) and stable MS (10 patients). The criteria for active disease were (i) the onset of clinical exacerbations within 3 weeks of blood collection with a history of at least two clearly identified clinical relapses during the preceding 2 years, and (ii) the presence of one or more enhancing lesions on cranial MRI within 2 weeks of blood collection. The blood samples from the active MS group were collected before the administration of steroid therapy. The criteria for stable MS were (i) no clinical relapses or significant decline in the EDSS score for at least 18 months from sample drawing, and (ii) no enhancing lesions on the cranial MRIs that were performed within 10 days of blood collection. The study also recruited 18 patients with inflammatory and 28 patients with non-inflammatory neurological disorders to serve as disease controls (Table 1). The inflammatory group included eight patients with lupus myelopathy, four with Guillain–Barré syndrome, two with tuberculous meningitis, two with herpes simplex encephalitis, a patient with neuro-sarcoid and a patient with stiff man syndrome. Samples from the inflammatory group were obtained for diagnostic purposes before the administration of specific treatment, except for four patients with lupus who were receiving chloroquine, salicylates or warfarin during sample collection. The non-inflammatory group consisted of eight patients with brain tumors, six with cranial trauma, four with secondary generalised epilepsy, four with stroke, four with amyotrophic lateral sclerosis, and two patients with hydrocephalus. Heparinized blood samples were also obtained from 12 healthy individuals, seven of whom had presented with non-specific headache, to serve as normal controls.

2.2. Cell preparation and induction of apoptosis

T lymphocytes were isolated by centrifugation on a Ficoll-Hypaque density gradient (Pharmacia Biotech, UK) followed by two cycles of plastic adherence to deplete monocytes. Cell viability at the time of cell isolation was determined by trypan blue dye exclusion assay. For activation studies, unstimulated (resting) lymphocytes (day 0) were cultured at 10^6 cells/ml with 1 μg/ml phytohemagglutinin (PHA) then maintained in continuous presence of PHA and 100 units/ml interleukin-2 for 7 days. Fas-mediated apoptosis was induced on day 7, when activated cells were incubated with a blocking anti-Fas agonist as described earlier (Rieux-Laurot et al., 1995). Fas-independent apoptosis of activated cells was induced by incubation for 24 h with 10 μM methotrexate (Sigma, UK) or culture medium as described in detail elsewhere (Genestier et al., 1998). Apoptosis was quantified according to published protocols (Magaud et al., 1988) using commercial cellular DNA fragmentation immunoassays (Boehringer Mannheim, Germany), and confirmed with the detection of apoptotic morphology by staining with the fluorochrome Hoechst 33258.
2.3. Quantification of cellular FLIP and Western blots

The amount of short (FLIP_S) and long (FLIP_L) isoforms of FLIP protein in cellular lysates was measured by analysing signals obtained from Western blots as recently described (Scafidi et al., 1999b). Cellular equivalents of 5 μg of protein were separated by 12% SDS–polyacrylamide gel electrophoresis, blotted onto nylon membranes, blocked then incubated with primary antibodies to FLIP_S, FLIP_L (C-19 and F-20, Santa Cruz Biotechnology, CA), and Bcl-2 (OP91, Oncogene, MA) antibodies according to published protocols (Scafidi et al., 1999b). Developed blots were analysed by a computerised densitometer (Bio Vision, UK) and Phoretix Version 4.0 Analysis software (Bjornsson, 1998). Primary antibodies to β-actin (Biogenesis, UK) were also used to confirm equal protein loading. To further quantify cellular contents of FLIP_S and FLIP_L in individual patients, we used a dot blot immunoassay utilizing cellular lysates, primary antibodies to human FLIP isoforms (Santa Cruz Biotechnology, CA) and nylon membranes (Boehringer Mannheim, Germany), as described in detail elsewhere (Sharief, 2000b). In brief, cellular lysate equivalents of 5 μg of protein were blotted onto nylon membranes, blocked then incubated with primary antibodies. Following incubation with HRP-conjugated secondary antibody and color development, the membranes were scanned in the reflectance-color mode of a computerised densitometer (Bio Vision, UK) and quantified using Phoretix analysis software. In earlier experiments, there was a strong correlation between cellular FLIP levels as determined by the dot-blot assay (Sharief, 2000b) and corresponding levels measured by Western blots (r=0.84, P<0.001).

2.4. Measurement of cellular Bcl-2 and Fas

Quantification of Fas in cell lysates was performed using a commercial immunoassay (Oncogene Research, Calbiochem, UK) according to the manufacturer’s instructions. Cellular Bcl-2 protein content was also measured by a quantitative immunoassay (Calbiochem, UK).

2.5. Data analysis

Values were compared, as appropriate, using paired t-test, ANOVA, Kruskal–Wallis test followed by multiple comparisons, and Pearson’s correlation test. Statistical analyses were performed with SPSS/PC+ software package (Chicago, IL).

3. Results

3.1. Cellular expression of FLIP_S and FLIP_L

There was strong expression of both isoforms of cellular FLIP in resting T lymphocytes from all clinical groups. However, activated T cells from patients with clinically active MS, but not from stable MS or the control groups, were still expressing cellular FLIP despite prolonged culture in the presence of IL-2 (Fig. 1).

To further investigate the dynamics of cellular FLIP during lymphocyte activation, we serially monitored the expression of FLIP isoforms in activated T cells during culture. There was a progressive downregulation of cellular FLIP_S in patients with stable MS and the control groups between days 1 and 7 of culture in the presence of IL-2, whereas cells from active MS patients expressed higher levels of FLIP_L during the same period (Fig. 2A). By day 7 of culture, abnormally high cellular levels of FLIP_S, i.e., higher than the cut-off level (mean±2 standard deviations) in healthy individuals, were detected in 15 of 18 patients with active MS, but in only four of 10 patients with stable MS (P<0.03) and three of 18 inflammatory controls (two with lupus myelopathy and a patient with Guillain–Barré syndrome; P<0.01). Similarly, the expression of FLIP_L in activated T cells on day 7 was significantly higher in 14 of 18 patients with active MS compared to three of 10 patients with stable MS (P<0.01; Fig. 2B) and two of 18 inflammatory controls (the same two patients with lupus myelopathy who had raised FLIP_S; P<0.01). The three stable MS patients with high cellular FLIP_L are the same three who had raised FLIP_S expression.

Table 1. Cellular expression of FLIP isoforms in activated T cells and the EDSS score (r=0.22) and MRI lesion load (r=0.19), but this failed to reach statistical significance.
significance. However, there was an inverse correlation between disease duration and the expression of FLIP$_S$ and FLIP$_L$ ($r=-0.24$, and $r=-0.29$, respectively, $P<0.05$).

### 3.2. Expression of Bcl-2 and Fas

In contrast to the high cellular expression of FLIP, the expression of Bcl-2 in activated T cells from patients with active MS (mean±S.E.M.$=183±15$ Units/mg protein) was not significantly different from Bcl-2 expression in stable MS (149±22), inflammatory (158±10) or non-inflammatory neurological controls (174±20). Similarly, cellular expression of Fas protein following activation was equally upregulated in T cells from active MS (5.1±0.43 Units/mg protein), stable MS (4.9±0.34) and inflammatory (4.2±0.41) or non-inflammatory (4.7±0.37) neurological controls. In patients with active MS, cellular expression of Bcl-2 protein or Fas receptor did not correlate with EDSS score, MRI lesion load or disease duration.

### 3.3. Relationship between cellular FLIP and susceptibility to Fas-mediated apoptosis

Activated T cells from patients with active MS were more resistant to Fas-mediated apoptosis than cells from stable MS or the control groups (Fig. 3). Microscopic

![Figure 2](image-url)

**Fig. 2.** Cellular expression of short (A) and long (B) forms of FLIP in T cells immediately ex vivo (day 0) and serially during culture in continuous presence of mitogen and interleukin-2. Cellular FLIP expression in healthy individuals was similar to the non-inflammatory group (data not shown). Results depict means±S.E.M. of data from individual patients, and the Y axes represent densitometric units. *$P<0.05$ and **$P<0.01$ compared to patients with stable MS and the control groups.

![Figure 3](image-url)

**Fig. 3.** Fas-mediated apoptosis of activated T cells from patients with active MS, showing relative resistance to cell death as compared to stable MS and the neurological control groups. Results are expressed as means±S.E.M. of data from individual patients. *$P<0.05$ compared to stable MS and the control groups.
evaluation following fluorochrome staining confirmed that the number of cells with an apoptotic phenotype was significantly lower in the active MS samples (mean±S.D.=18±3%) when compared to samples from stable MS (23±5%; P=0.06) or the two neurological control groups (29±6%; P<0.05). The potential inhibitory activity of high cellular FLIP expression on this observed resistance to Fas-mediated cell death in patients with active MS prompted us to correlate FLIP expression in activated T cells with their susceptibility to Fas-mediated apoptosis. Results showed that cells expressing abnormally high levels of FLIP protein were more resistant to Fas-mediated apoptosis than cells with low FLIP expression (Fig. 4).

3.4. Relationship between cellular FLIP and Fas-independent apoptosis

Fas-independent apoptosis was investigated in activated T cells from a subgroup of 10 patients with active MS and eight healthy controls. The cellular apoptotic response in the active MS group (mean±S.D. DNA fragmentation=0.9±0.21 A405 nm) was significantly lower than cellular apoptosis of the control group (1.4±0.37; P<0.01). The results, which were confirmed by morphological analysis, are consistent with recent findings that Fas-independent apoptosis is impaired in T cells from MS patients (Sharief, 2000a). However, the relationship between Fas-independent apoptosis of T cells from MS patients and cellular expression of FLIP was not statistically significant (P=0.09 and P=1.03, respectively).

4. Discussion

Our results indicate that the recently identified suppressor of apoptosis FLIP is over-expressed in activated T cells from patients with clinically active MS when compared to stable MS or other neurological disorders. Unlike the Bcl-2 family of oncogenic proteins which are potent inhibitors of apoptosis induced by growth factors withdrawal and γ-irradiation (Hawkins and Vaux, 1994, 1997), cellular FLIP primarily blocks apoptotic signals induced by death receptors (Kataoka et al., 1998).

Death receptor-induced apoptosis is an important event in tissue homeostasis as exemplified in patients with defective lymphocyte apoptosis who develop lymphadenopathy, splenomegaly, and systemic autoimmunity (Vaishnaw et al., 1999). Therefore, a tight regulation of the balance between susceptibility and resistance of cells towards apoptosis is required. In T lymphocytes, this resistance is transient to avoid premature cell death and to allow T cell-mediated help or cytotoxicity. Activated T cells are then eliminated through activation-induced apoptosis (Moulian and Berrih-Aknin, 1998), a process that limits the expansion of the immune response in vivo by deleting activated lymphocytes that are no longer needed. A relatively similar state occurs in vitro, where short-term activated (day 1) T cells are resistant, but T cells additionally cultured in the presence of IL-2 for longer periods are sensitive to Fas-mediated apoptosis (Peter et al., 1997). In contrast, T cells form patients with some autoimmune diseases seem to have lost the capacity to undergo activation-induced apoptosis, either as a result of loss of certain death receptors or through impairment of signalling pathways (Mountz et al., 1996; Beutler and Bazzoni, 1998).

The potent inhibitory activity of FLIP on death receptor signalling prompted us to investigate T cell expression of this protein in MS patients and other neurological disorders, and explore the relationship between FLIP expression and the susceptibility of T cells to Fas-mediated cell death. Results presented here indicate that high cellular FLIP expression in activated T cells from MS patients is closely associated with disease activity and T cell resistance to Fas-mediated apoptosis. On the other hand, disease activity and the susceptibility of T cells to Fas-induced apoptosis appear to be independent of cellular expression of Bcl-2 protein. We have specifically evaluated Bcl-2
expression because of its unique role amongst oncogenic proteins, as it enhances lymphoid cell survival by interfering with apoptosis rather than promoting cell proliferation (Hawkins and Vaux, 1997). Bcl-2 protein is usually over-expressed in T cells from patients with some autoimmune diseases (Aringer et al., 1994; Ohsako et al., 1994; Zipp et al., 1997), and this overexpression appears to correlate with clinical disease activity (Ohsako et al., 1994). However, Bcl-2 expression in this study was similar between active and stable MS, and indeed between MS patients and the control groups, suggesting that Bcl-2 is not responsible for the observed differences in susceptibility to apoptosis. This finding is consistent with recent reports that overexpression of Bcl-2 did not protect T cells from Fas-mediated cell death (Strasser et al., 1995; Inaba et al., 1999). Similarly, the impairment of Fas-mediated apoptosis of T cells from MS patients is in agreement with earlier studies (Comi et al., 1999; Macchi et al., 1999). We did not assess FLIP expression in T cell subgroups, but it will be of interest to determine whether there is differential expression amongst T cells reactive to MBP or other myelin antigens in patients with active MS. Similarly, further studies are required to assess FLIP expression in T cells from patients with other clinical types of MS, particularly those with secondary progressive disease.

In addition to its regulatory effect on activated T cells, FLIP may also inhibit cytotoxic T cells and regulate systemic B cell survival (Kataoka et al., 1998; Wang et al., 2000). Therefore, it seems reasonable to hypothesize that high cellular FLIP expression in MS patients may contribute to disease activity by extending the viability of potentially pathogenic, autoreactive cells and regulating their resistance to apoptosis in vivo. Alternatively, high cellular expression of FLIP may not modulate the susceptibility to apoptosis per se, but rather reflects a more complex cellular response in MS. Indeed, the lack of a significant correlation between cellular FLIP and resistance to Fas-independent apoptosis suggests that other anti-apoptotic molecules may have contributed to the observed differences in susceptibility to apoptosis between the clinical groups. For example, Bcl-x may be upregulated in activated T cells (Broome et al., 1995). Similarly, the inhibitor of the apoptosis protein (IAP) family, cellular IAP-1 and IAP-2 (Roy et al., 1997), X-linked inhibitor of apoptosis (Yamaguchi et al., 1999) and survivin (Ambrosini et al., 1997), which could be induced in activated T cells (Kobayashi et al., 1999), may inhibit some caspases that mediate Fas-mediated cell death (Enari et al., 1996). The IAP family of proteins are important negative regulators of apoptosis and their overexpression renders cells resistant to a wide variety of apoptotic stimuli, including Fas-independent pathways (Deveraux and Reed, 1999). Further studies are underway to examine whether these anti-apoptotic molecules are involved in modulating the susceptibility of T cells from MS patients to Fas-independent apoptosis. Nonetheless, our earlier observations (Sharief, 2000a) and data presented here indicate that both of the major cell death mechanisms are impaired in activated T cells from MS patients.

In conclusion, the resistance to apoptosis of activated T lymphocytes from patients with clinically active MS appears to correlate with high cellular expression of FLIP protein, but not with the expression of Bcl-2 or Fas receptor protein. The polygenic nature of MS suggests that the overexpression of FLIP protein may not be a primary pathogenic mechanism. Nonetheless, findings presented here may provide more insight into the role of FLIP in this disease.

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