TNF-induced death of adult human oligodendrocytes is mediated by c-jun NH2-terminal kinase-3

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Summary

Tumour necrosis factor (TNF) induces death of oligodendrocytes, the putative cell target in multiple sclerosis. We defined that the intracellular transduction pathway involved in TNF-induced death of human adult oligodendrocytes (hOLs) is dependent on c-jun NH2-terminal kinase (JNK) activation, but not the other mitogen-activated protein kinase (MAPK), p38. JNK activation, measured by c-jun phosphorylation and induction of the phosphorylated form of JNK, was enhanced, prolonged and correlated with cell death in hOLs exposed to TNF. Comparative autoradiographic analysis revealed that JNK-3, but not JNK-1 or JNK-2, is responsible for prolonged JNK activation in TNF exposed hOLs. Expression of a dominant-negative mutant of JNK upstream kinase, MKK4/SEK1, inhibited apoptosis induced by TNF, whereas expression of a constitutive active mutant of MEKK1, an upstream kinase to JNK, accelerates TNF-induced apoptosis. JNK activation occurred prior to changes of mitochondrial membrane potential in hOLs exposed to TNF. These results demonstrate that TNF-induced death in adult hOLs depends on prolonged JNK-3 activation, and that this apoptosis requires the mitochondrial dysfunction that occurs after JNK activation. This is the first evidence that a JNK-3 isoform is involved in oligodendrocyte death and might have significant importance in designing new molecules to protect hOLs demise in multiple sclerosis.

Keywords: TNF; JNK; cell death; oligodendrocytes; multiple sclerosis

Abbreviations: ERK = extracellular signal-related kinase; FADD = Fasassociated death domain protein; FITC = fluorescein isothiocyanate; GFP = green fluorescent protein; hOL = human adult oligodendrocyte; JNK = c-jun NH2-terminal kinase; MAP = mitogen-activated protein; MAPK = mitogen-activated protein kinase; NF-kB = nuclear factor kB; PI = propidium iodide; RIP = receptor interacting protein; TdT = terminal deoxynucleotidyl transferase; TNF = tumour necrosis factor; TRADD = TNF-R1-associated death domain protein; TRAF2 = TNF receptor associated factor-2; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling

Introduction

Tumour necrosis factor (TNF) elicits a wide spectrum of cellular responses by interaction with two distinct cell surface receptors: TNF-Rp55 (TNFR1) and TNF-Rp75 (TNFR2) (Vandanabeele et al., 1995). The outcomes of TNF interaction with p55 receptor depends on selective activation of different signal transduction pathways: the caspase cascade, nuclear factor-kB (NF-kB) family of transcription factors and the mitogen-activated protein kinases (MAPKs), including both the c-jun NH2-terminal protein kinase (JNK) and p38 kinases (Liu et al., 1996). Different signalling pathways are initiated by recruiting different types of adaptor proteins to the cytoplasmic portion of TNF-Rp55. Activation of TNF-Rp55 results in recruitment of the TNF-R1-associated death domain protein (TRADD) (Hsu et al., 1995). Subsequently, TRADD interacts with TNF receptor associated factor-2 (TRA2) or with Fas-associated death domain protein (FADD) (Hsu et al., 1996b). TRAF2 mediates NF-kB activation whereas FADD interacts with procaspase-8, a direct activator of the apoptotic protease cascade (Boldin et al., 1996). Another death domain protein is the serine/threonine kinase receptor interacting protein (RIP). RIP is recruited to the TNF-Rp55 signalling complex by TRADD.
and participates in NF-kB activation (Hsu et al., 1996a). Both signal transducers RIP and TRAF2 also mediate JNK activation (Malinin et al., 1997).

JNK activity is implicated in cell death pathways stimulated by exogenous stress factors and TNF (Tournier et al., 2000; Tobiume et al., 2001). Recently, it was shown that NF-kB protects cells from apoptosis by inhibiting JNK (De Smaele et al., 2001; Tang et al., 2001). The exact mechanism and location of JNK activity in biochemical pathways leading to TNF-induced cell death is not known. Mitochondrial depolarization measured by the change in mitochondrial membrane potential and cytochrome c release seems to be important in many death mechanisms (Kluck et al., 1997).

Multiple sclerosis is an inflammatory demyelinating disease of the CNS (Brosnan and Raine, 1996; Lassmann, 2002). Depletion of oligodendrocytes is a recognized feature of multiple sclerosis lesion (Raine, 1994; Lucchinetti C et al., 1999). Several immune effector mechanisms, including TNF and lymphotoxin-α (Selmaj et al., 1991b; Selmaj and Raine, 1998), have been shown to induce oligodendrocyte death in vitro. In situ (Bonetti and Raine, 1997) and in vitro (D’Souza et al., 1996) studies suggested that the mechanism of oligodendrocyte death does not involve classical apoptotic features.

The objective of this study was to define the role of JNK in the mechanism responsible for mature adult human oligodendrocyte (hOL) death in response to TNF-Rp55 ligation and to determine which of the JNK isoforms is primarily involved in oligodendrocyte death. Although all three JNK isoforms—JNK-1, JNK-2 and JNK-3—are expressed in the brain (Martin et al., 1996; Mielke and Herdegen, 2000), JNK-3 was predominantly localized in the CNS tissue (Davis, 2000). Another goal was to determine whether JNK activation occurs in TNF-induced oligodendrocyte death prior or after mitochondrial dysfunction. Human adult oligodendrocytes were chosen because the importance of JNK for cell death in these glial cells has not been fully evaluated and they represent the primary cell target during demyelination in multiple sclerosis. Our data suggest that mature adult hOL death induced with TNF does require prolonged JNK-3 activation, which is a critical intermediate for mitochondria dysfunction.

Target cell populations
Cells of a murine fibroblast cell line, WEHI 1640, were prepared and maintained following ATCC (American Type Culture Collection) protocols. A MO3.13 rhabdomyosarcoma and oligodendrocyte fusion cell line was used as a positive control for p38 activation.

Oligodendrocytes were prepared from human adult brain resected during neurosurgical procedures as described previously (Jurewicz et al., 1998). Briefly, the tissue was trypsinized, rubbed through mesh and spun down on a 30% Percoll gradient. The dissociated cells were then suspended in minimal essential medium (MEM) (GIBCO, Gaithersburg, MD, USA) with 5% foetal calf serum (FCS) (GIBCO), streptomycin (50 μg/ml) and penicillin (50 U/ml), and then cultured for 48 h in culture flasks. This step separated adherent cells such as microglia from non-adherent cells such as oligodendrocytes. The non-adherent oligodendrocyte fraction was plated onto poly-L-lysine-coated wells of 96-well microtitre plates at 5 x 10^4 cells/well and cultured for 2 weeks. The purity of these cells, as assessed by staining with antibody against galactocerebroside (Sigma) was > 90% as shown previously by Jurewicz et al., 1998. The microglia were cultured in flasks for 1.5 weeks. A few days before the experiment, they were collected, spun down and plated onto culture plates. The purity of microglia cells was assessed by staining with antibody against Mac-1 (CD11b) (Pharmingen) and was >90%. For each experiment, the cells were used at the same time point after removal from the brain. The cells were then cultured with TNF at a concentration range of 0-2000 U/ml for up to 96 h.

Flow cytometric analysis of cell death
Before being detached from the poly-L-lysine coated wells, cells were stained simultaneously with FITC-conjugated annexin V to detect exposure of phosphatidylinerine on the cell surface and PI to detect permeable dead cells. The staining was measured by flow cytometry (Becton-Dickinson, San Jose, CA, USA). Briefly, cells were washed in phosphate-buffered saline (PBS) before 10 μl of FITC-conjugated annexin V and PI (5 μg/ml) in calcium-containing buffer were added according to the manufacturer’s protocols. After cell detachment, we assessed the FITC intensity shift for FITC-conjugated annexin V and PI2 intensity shift for PI staining. As in previous reports (Vermes et al., 1995), the apoptotic cells were defined as those showing annexin V staining before the appearance of staining for PI.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
We also assessed cell death pathway using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-
labelling (TUNEL) technique (Boehringer-Mannheim, Mannheim, Germany). Briefly, air-dried cells cultured on slide chambers were fixed with a freshly prepared 4% paraformaldehyde solution for 30 min at room temperature and DNA strand breaks were stained with FITC-labelled dUTP by terminal deoxynucleotidyl transferase (TdT) according to the manufacturer’s protocol. Cells were assessed using a fluorescent microscope. Approximately 500 cells with clearly defined nuclei were examined in each sample. The apoptotic cells were counted by identifying TUNEL-positive nuclei.

**Western blotting**

Expression of JNK types as well as phosphorylated form of JNK was detected by western blotting. Proteins were separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA, USA). The membranes were blocked with 5% dried milk in Tris-buffered saline (TBS) overnight, followed by incubation for 1–2 h with antibodies (Santa Cruz Biotechnology) specifically recognizing JNK-1, JNK-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), JNK-3 (Upstate Biotechnology, Dundee, Scotland), a phosphorylated form of JNK (Santa Cruz Biotechnology), p38 kinase, a phosphorylated form of p38 kinase, ERK1/2 kinase and a phosphorylated form of ERK1/2 kinase (Santa Cruz Biotechnology). Blots were washed in TBS and incubated with peroxidase-coupled secondary antibodies. Proteins were detected by using an Amersham enhanced chemiluminescence system, Amersham, Little Chalfont, UK).

**Immunoprecipitation**

Lysates of 5 × 10⁶ cells were introduced to immunoprecipitation with three different antibodies: rabbit anti-JNK-1 (Santa Cruz Biotechnology); mouse anti-JNK-2 antibody (Santa Cruz Biotechnology); and rabbit anti-JNK-3 (Upstate Biotechnology). The primary antibodies were conjugated to protein A-agarose (Sigma, Poznan, Poland) (anti-JNK1 antibody and anti-JNK-3 antibody) or to protein G-agarose (Sigma Poland) (anti-JNK-2 antibody) for 2 h at 4°C. The immunoprecipitation step was continued with the cell lysates for another 2 h at 4°C. The immunoprecipitates were used for c-jun phosphorylation and autoradiography as described below.

**JNK activation assay**

The immunoprecipitates of the cell lysates were washed three times with kinase buffer (25 mM Tris-HCl pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na₂VO₄, 10 mM MgCl₂) and used for c-jun phosphorylation with [³²P]ATP in kinase buffer. Briefly, the immunoprecipitated JNK was incubated in 10 μl of kinase buffer with 1 μg of c-jun (Santa Cruz Biotechnology) and 1 μCi [³²P]ATP (Amersham, Little Chalfont, Buckinghamshire, UK) at 37°C for 0.5 h. The phosphorylated c-jun was subjected to 8% SDS–PAGE and JNK activation was assessed by autoradiography of the gel and quantified using a Phosphoimager (Molecular Dynamics, Kent, UK). The assay (immunoprecipitation and autoradiography) was performed on unstimulated hOLs and TNF stimulated for 10, 30 and 120 min.

**Mitochondrial function assay**

Changes in the mitochondrial membrane potential were assessed by ApoAlert Mitochondrial Membrane Sensor Kit (Clontech, Palo Alto, CA, USA) and fluorescence-activated cell sorter (FACS) analysis, according to the manufacturer’s protocol. The kit includes Mitosensor, a cationic dye which aggregates mitochondria and exhibits red fluorescence. However, in apoptotic cells that have lost the ability to maintain normal mitochondrial membrane permeability, the cationic dye cannot accumulate in mitochondria. It therefore remains in a monomeric form in the cytoplasm and exhibits green fluorescence. Cells were rinsed gently with serum-free medium before Mitosensor was added and the cells were incubated at 37°C in a 5% CO₂ atmosphere for 20 min and subjected to FACS analysis.

**Cell transfection**

5 × 10⁶ oligodendrocytes, 5 × 10⁶ microglial cells and 5 × 10⁶ WEHI cells were transfected with pcDNA3.1 vector alone or pcDNA3.1 vector containing a dominant-negative MKK4 (DN-MKK4) insert (SEK1) kindly provided by Dr Rony Seger (Weizmann Institute, Rehovot, Israel) or a constitutively active MEKK1 insert kindly provided by Dr Michael Kracht (Institute of Molecular Pharmacology, Medical School Hanover, Hanover, Germany) or green fluorescent protein (GFP) insert (Promega, Madison, WI, USA). The cells were incubated with 2 μg of pcDNA3.1 vectors and 1 μl Lipofectine (Life Technologies, Inc., Gaithersburg, MD, USA) according to previously described protocols (Skov et al., 1997). The cells were grown for 48 h before further experiments were undertaken. The level of cell transfection was checked by pcDNA3.1 (Invitrogen, Groningen, The Netherlands) containing an GFP insert, thus enabling assessment of living cells by fluorescence microscopy. The efficacy of cell transfection was also confirmed by flow cytometry of cells transfected with pcDNA3.1-GFP (Fig. 1).

**Statistical analysis**

All data were derived from three to eight experiments. Mean values are presented for TUNEL and quantitative phosphorylation. FACS analysis data were derived from counting
at least 10,000 events in each experiment. The means were compared using Student’s t-test. The significance was defined at $P < 0.05$.

**Results**

**TNF induces human adult oligodendrocyte death**

TNF-induced hOL death was dose-dependent (see Fig. 2A). Increasing TNF concentration induced increased death of hOLs as shown by the number of PI-positive cells. For most subsequent experiments, TNF was used at a concentration of 1000 U/ml, the concentration at which cell death reached a maximum. The mechanism of cell death was defined as apoptosis by demonstrating annexin-V cell surface staining preceding PI cell staining (Fig. 2B). Annexin-V staining was detected 24 h after TNF addition, whereas PI staining was observed in oligodendrocytes after 72 h. As previously reported (Selmaj *et al.*, 1991b), oligodendrocyte apoptosis was a delayed process compared with other TNF-sensitive cell types such as WEHI. The apoptotic mechanism of oligodendrocyte death was confirmed by use of the TUNEL technique (Fig. 2C). hOLs exposed to TNF for 72 h and labelled with TdT fluorescein-dUTP showed 90% positive nuclei staining, whereas control hOLs did not show dUTP-FITC staining ($P < 0.001$) (Fig. 2C). Microglia did not show apoptotic features in response to TNF at a concentration of 1000 U/ml up to 96 h of incubation (data not shown).

**TNF-induced hOL death correlates with JNK-3 activation**

It has been postulated that JNK is activated through TNF-Rp55 ligation after TNF stimulation and that this may lead to

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**Fig. 1** Efficiency of cell transfection with pcDNA3.1-GFP. (A) hOLs, microglia and WEHI cells were transfected with pcDNA3.1-GFP as described in Material and methods, and the cells were analysed by flow cytometry. The graphs present fluorescence intensity on the horizontal axis and the relative cell number on the vertical axis. GFP-positive cells (open peaks) are demonstrated by the fluorescence shift in FL1 compared with a control (non-transfected cells, shadow peaks). Data are representative for four identical experiments. (B) hOLs were transfected with pcDNA3.1-GFP as described in Material and methods, and analysed by fluorescent microscope ($\times 100$). The pictures show a representative hOLs culture of green fluorescent protein labelling along with the parallel phase-contrast picture. The inserts represent a higher magnification ($\times 400$) of individual cells showing the cytoplasmatic presence of GFP. (C) hOLs were stained with anti-galactocerebroside antibody and analysed by flow cytometry. The graph presents fluorescence intensity on the horizontal axis and the relative cell number on the vertical axis. Galactocerebroside-positive cells (open peak) are demonstrated by the fluorescence shift in FL1 compared with the control (isotype control antibody staining, shadow peak).
cell death (Tournier et al., 2000; Tobiume et al., 2001). We have found that JNK was activated in hOLs and microglial cells in response to TNF, as measured by autography of c-jun phosphorylation and induction of a phosphorylated JNK form. Autoradiography demonstrated the biphasic activation pattern of JNK in hOLs, with a strong increase at 10 min and 2 h after TNF stimulation (Fig. 3A). The second increase in JNK activation was prolonged and continued until the last time point of assessment at 24 h post exposure. In microglial cells, JNK activation was much lower than in hOLs and

![Diagram](https://academic.oup.com/brain/article-abstract/126/6/1358/330615)

**Fig. 2** TNF induces hOLs apoptosis. (A) TNF-dose dependent hOL death determined as a percentage of PI-positive cells. Cell death was assessed after 72 h of TNF stimulation. (B) Time-dependent FACS analysis of annexin-V-FITC conjugated and PI staining of hOLs. The graphs present fluorescence intensity on the horizontal axis and relative cell number on the vertical axis. The positive cells for annexin V-FITC and PI are demonstrated by the fluorescence shift in FL1 and FL2 compared with a control (no TNF stimulation). Annexin-FITC staining appeared after 24 h and PI after 72 h of TNF stimulation, indicating apoptotic pathway of cell death. (C) TdT dUTP-FITC-positive cell nuclei (TUNEL technique) in hOLs treated with TNF after 72 h of exposure. The pictures show representative hOLs culture of TdT dUTP-FITC-positive cells along with the parallel phase-contrast picture (×100). Please note the morphologic features of TNF-induced hOLs injury. The inserts represent a higher magnification (×400) of individual cells showing the TdT dUTP-FITC nuclear staining. Both panels show representative data for six separate experiments.
showed early activation only at 10 min and with no secondary increase (Fig. 3A). In WEHI cells, JNK activation was negligible. Immunoprecipitation with antibodies recognizing JNK-1, JNK-2 and JNK-3 was performed prior to the kinase assay in order to assess the contribution of each of the JNK isoforms to prolonged JNK activation in hOLs. After TNF stimulation, the highest c-jun phosphorylation was detected in the JNK-3 immunoprecipitate. This indicated that this isoform of JNK is responsible for prolonged JNK activation (Fig. 3A). Autoradiography findings of prolonged JNK activation in hOLs was confirmed by detection of the phosphorylated form of JNK on western blots at corresponding time points to autography data (Fig. 3B). In microglial cells, the TNF-induced phosphorylated form of JNK was seen only at 10 min. In WEHI cells, there was no enhancement of JNK phosphorylation, indicating that JNK was not activated by TNF (Fig. 3A and B).

**JNK inhibition diminished TNF-induced hOL death**

JNK inhibition in hOLs correlated with diminished TNF-induced death of these cells. hOLs transfection with the DN-MKK4 construct, a mitogen-activated protein kinase upstream of JNK activation, led to significant reduction of JNK activation (Fig. 3A) and a reduction of oligodendrocyte death induced with TNF (Fig. 4A), whereas DN-MKK4 did not influence TNF-induced apoptosis of WEHI cells (Fig. 4C). Since M KK4 may potentially also activate p38 kinase, we measured p38 phosphorylation in hOLs exposed to TNF and found no increase in p38 phosphorylation in response to TNF (Fig. 3B) whereas, in control MO3.13 cells, phosphorylation of p38 was achieved easily (data not shown). In addition, inhibition of p38 with a p38 inhibitor, SB203580, at a concentration of 10 μM for 2 h prior to TNF stimulation did not affect TNF-induced hOL death (Fig. 4A). Inhibition of ERK with PD98059 did not prevent TNF-induced hOL death (Fig. 4A).

**MEKK1 over-expression enhances TNF-induced hOL death**

To further confirm JNK involvement in TNF-induced death of hOLs, we induced sustained JNK activation in hOLs with the construction of constitutive active MEKK1, the upstream kinase to JNK and MKK4 in the MAP kinases cascade (Fig. 3A). The MEKK1 transfection of hOLs slightly increased JNK phosphorylation (Fig. 3C) and, in hOLs treated with TNF, constant JNK-3 activation was induced without transient reduction at 30 min after TNF stimulation, which occurred in non-transfected hOLs (Fig. 3A). Whereas over-expression of constitutive active mutant of MEKK1 alone did not induce hOL death (Fig. 4B), the MEKK1 transfection accelerated hOL death after TNF stimulation (Fig. 5). The uptake of PI in TNF treated hOLs transfected with MEKK1 appeared at 24 h instead of at 72 h as in non-transfected hOLs (Fig. 5). TNF-induced death of WEHI cells was not influenced by transfection with MEKK1 (Fig. 4C). In addition, microglial cells that were resistant to TNF-induced death were not affected by over-expression of MEKK1 (data not shown).

**TNF-induced hOL death involves mitochondrial dysfunction**

Mitochondrial depolarization has been proved to be a major checkpoint in cell death machinery. To assess mitochondrial dysfunction in TNF-induced hOL death, we measured changes in mitochondrial membrane permeability in TNF stimulated cells. Increased mitochondrial membrane permeability was detected as early as 4 h, reaching the maximal level after 15 h post TNF stimulation. The control microglial cells, which were resistant to TNF-induced death, did not show any change in mitochondrial membrane permeability (Fig. 6).

**JNK activation precedes mitochondrial dysfunction**

To assess whether JNK activation during TNF-induced hOL death occurs before or after mitochondrial dysfunction, we measured the mitochondrial membrane permeability in cells transfectected with DN-MKK4 as well as in cell transfected with MEKK1. We found that inhibition of JNK activation with DN-MKK4 led to significant diminishing of mitochondrial membrane disruption (Fig. 6), which correlated with diminished hOL death. To strengthen the observation that JNK activation occurs before mitochondrial dysfunction, we measured mitochondrial membrane permeability in cells

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**Fig. 3** (A) Autoradiography of phosphorylated c-jun by JNK immunoprecipitated from hOLs, microglia and WEHI cells as described in Material and methods. JNK-3 showed biphasic, strong and prolonged stimulation with two increases (10 min and 2-24 h) of activation in hOLs, but not in microglia or WEHI cells. Data are representative for three independent experiments. MKK4-DN transfection, but not control empty vector pcDNA3.1, suppressed JNK-3. MEKK1 transfection induced constant activation of JNK3 in TNF-treated hOLs. Substrate phosphorylation was quantified by Phosphoimager and increased stimulation is indicated. (B) Western blot of hOLs, microglia and WEHI cells with antibodies specific for JNK-1, JNK-2, JNK-3, a phosphorylated form of JNK (pJNK), p38, a phosphorylated form of p38 kinase, ERK1/2, and a phosphorylated form of ERK. TNF stimulation of hOLs induced early (10 min) and sustained (2 and 24 h) phosphorylation of JNK. JNK protein levels remained unchanged and p38 and ERK1/2 were not phosphorylated in response to TNF. (C) Western blot of hOLs, microglial and WEHI cells with antibodies specific for pJNK. TNF stimulation of hOLs induced early (10 min) and sustained (2 and 24 h) phosphorylation of JNK. MKK4-DN transfection inhibited TNF-induced JNK phosphorylation and MEKK1 transfection induced increased JNK phosphorylation.
transfected with MEKK1. We found that MEKK1 transfection accelerated the appearance of changes in mitochondrial membrane permeability, which were observed 2 h after TNF stimulation whereas, in non-transfected cells, mitochondrial membrane permeability was detected only 4 h post TNF exposure (data not shown).

Discussion
In this report, we defined intracellular transduction pathways involved in TNF-induced death in primary adult human oligodendrocytes, the putative cell target in multiple sclerosis. We present evidence that TNF-induced death of adult hOLs is dependent on JNK-3 activation, which is critically involved in the induction of mitochondrial depolarization.

During demyelination, considerable oligodendrocyte death occurs in the CNS. These glial cells are highly susceptible to injury and inflammation. The majority of hOLs express both TNF receptors (Wilt et al., 1995). The sensitivity of hOLs is underscored by the effects of TNFα and LTα (lymphotoxin-α) which cause apoptosis at concentrations that do not affect other CNS cell types (Selmaj et al., 1991b). During inflammatory lesions, it is conceivable that hOLs become more reactive to TNF family cytokines released at the site of injury. Support for this notion comes from the detection of increased TNF expression in inflammatory lesions of CNS, including multiple sclerosis (Selmaj et al., 1991a). TNF can induce both apoptotic and necrotic cell death (Vercammen et al., 1998). The differences between apoptosis and other mechanisms of cell death are sometimes ambiguous. Recent results suggest that some apoptotic features, e.g. phosphatidylserine externalization, might occur in other mechanisms of cell deaths (Vermes et al., 1995). TNF-induced cell death can be mediated by different intracellular pathways and which one is employed depends on many factors including cell type and stage of maturation. Hisahara et al. (2001) showed that TNF-induced apoptosis in embryonic mouse mixed glial cell cultures enriched for oligodendrocytes is inhibited by a p35-like inhibitor, Z-Asp-CH-DCB and partially by Ac-DEVD-CHO, the inhibitor of most downstream caspase-3 (Xue and Horovitz, 1995). More recently, the same authors showed that oligodendrocytes derived from caspase-11 deficient mice are partially resistant to TNF-induced apoptosis (Hisahara et al., 2001). However, Andrews et al. (1998) reported that general caspase inhibitor, ZVAD FMK, partially suppressed apoptotic changes elicited by the combined activity of interferon-γ (IFN-γ) and TNF only in CG4-positive oligodendrocyte progenitor cells, but not in differentiated oligodendrocytes. Consistent with these findings, nerve growth factor (NGF) interacting with the p75 receptor of the TNF family induced caspase-3-dependent apoptosis of rat post-natal oligodendrocytes (Casaccia-Bonnefil et al., 1996), but not of human adult mature oligodendrocytes (Ladiwala et al., 1998). These results demonstrate the significant dependence of intracellular transduction pathways involved in TNF-induced death of oligodendrocytes on the developmental stage of cells. Therefore, we decided to use primary mature oligodendrocytes isolated from adult brain instead of embryonic derived oligodendrocytes to test the most relevant cells to the cell target of the immune attack in multiple sclerosis.

Several reports indicated that the JNK transduction pathway is implicated in the control of cell survival mechanism (Chen et al., 1996; Guo et al., 1997). The JNK pathway is activated in response to environmental stress and by the engagement of several classes of cell surface receptors, including TNF receptors (Derijard et al., 1995). Targets for the JNK signal transduction pathway include the transcription factors c-jun, Elk-1 and ATF-2 (Gupta et al., 1995). Verheij et al. (1996) have shown that apoptosis of U937 and endothelial cells induced by C-2 ceramide and sphingomyelinase (intermediates of TNF transduction signalling) was inhibited by a dominant-negative c-jun mutant lacking the N-terminus (TAM-67), as well as by a dominant-negative kinase-inactive SEK-1 construct. Consistent with these finding was the observation that apoptosis signal-regulating kinase 1 (ASK-1), an activating kinase of the JNK pathway, was required for TNF-induced cell death (Ichijo et al., 1997; Tobiume et al., 2001). Similarly, MEKK, the upstream kinase of the JNK, induced apoptosis upon ectopic expression (Johnson et al., 1996). Also apoptosis in cells deprived of NGF is mediated by the JNK pathway (Xia et al., 1995). More recently, it has been shown that apoptosis of T cells was significantly impaired in JNK-1 and JNK-2 deficient mice (Dong et al., 1998; Sabapathy et al., 1999) and neuronal apoptosis in mice lacking JNK-3 (Yang et al., 1997a). Yang et al. (1997c) described a novel signalling protein, Daxx, which mediates Fas-induced apoptosis in L929 cells. Daxx-induced apoptosis involved the JNK pathway, in contrast to FADD-induced apoptosis in the same cells. The possibility of JNK involvement in TNF-induced oligodendrocyte death was first suggested by Ladiwala et al. (1998). They observed a two-fold increase in JNK activation in oligodendrocytes at one time point after stimulation with TNF.

![Fig. 4](https://academic.oup.com/brain/article-abstract/126/6/1358/330615/1366) M KK4-DN transfection suppressed TNF-induced hOL death. (A) TNF-induced hOL death was inhibited with MKK4-DN, but not with control pcDNA3.1, p38 kinase inhibitor (SB203580) and ERK inhibitor (PD98059), as shown by annexin V-FITC and PI staining. (B) DN-MKK4, pcDNA3.1-MEKK1 and kinase inhibitors did not affect oligodendrocyte survival. (C) TNF-induced WEHI cells apoptosis was not inhibited by MKK4-DN, SB203580 and PD98059. (D) DN-MKK4, pcDNA3.1-MEKK1 and kinase inhibitors did not affect WEHI survival. The graphs present fluorescence intensity on the horizontal axis and the relative cell number on the vertical axis. The positive cells (TNF-stimulated: A and C; or stimulated with inhibitors alone: B and D) (open peaks) for annexin-V-FITC and PI are demonstrated by the fluorescence shift in FI1 and FL2 compared with the control (non-stimulated cells, shadow peaks). The results show representative data from live experiments.
Our results clearly showed that JNK is activated after TNF treatment in mature adult hOLs. TNF induced biphasic and prolonged JNK activation within hOLs, but not in TNF-resistant microglial cells. Interestingly in WEHI cells, which were sensitive to TNF-induced death, we did not detect JNK activation, indicating substantial differences in the mechanisms operating in TNF-induced death between different cell populations. By using a dominant-negative construct of a kinase upstream of JNK, MKK4/SEK1, we provide strong evidence that MKK4-JNK activity is involved in TNF-induced death of hOLs. MKK4 may also activate p38 kinase (Guo et al., 1997). However, we did not observe any increase in p38 activity in hOLs in response to TNF. Therefore, it seems unlikely that p38 can be involved in hOL death in response to TNF. In addition, the p38 inhibitor did not prevent TNF-induced apoptosis of hOLs. Three isoforms of JNK have

Fig. 5 Transfection of hOLs with pcDNA3.1-MEKK1 accelerated TNF-induced hOL death. The percentage of dead cells was assessed by counting PI positive cells by flow cytometry (see Material and methods).

Fig. 6 MKK4-DN transfection inhibits TNF-induced mitochondrial membrane permeability in hOLs. The mitochondrial membrane disruption appeared after 4 h and lasted for 15 h of TNF stimulation. It was inhibited by MKK4-DN, but not by control pcDNA3.1. The graphs present fluorescence intensity on the horizontal axis and the relative cell number on the vertical axis. The positive cells (TNF-stimulated) (open peaks) are demonstrated by the fluorescence shift in FL1 compared with the control (non-stimulated cells) (shadow peaks). Data are representative for four identical experiments.
been identified and JNK-3 was associated with neuronal death within the CNS (Davis, 2000). By assessing the JNK activation in cellular protein fraction precipitated with antibodies specific for JNK-1, JNK-2 and JNK-3, we have shown that JNK-3 is responsible for prolonged JNK activation in TNF-induced hOL death. This might indicate a more general JNK-3 function in mediating cell death within the CNS. We have strengthened the conclusion that TNF-induced hOL death depends on JNK activation by demonstrating that over-expression of a constitutive active mutant of MEKK1 in hOLs accelerates the cell death. The mechanism of MEKK1 enhancement of TNF-induced hOL death may be related to the observed constant JNK-3 activation in MEKK1 transfected hOLs treated with TNF. The failure to induce hOL death with MEKK1 over-expression without TNF stimulation might suggest that the additional signal generated by TNF-Rp55 ligation parallel to MEKK1/MKK4 may occur. The existence of a JNK activation pathway independent of M KK4 was suggested by the results of experiments with cells with a disrupted M KK4 gene. Although M KK4-deficient cells were defective in JNK activation, some JNK activation was detected (Yang et al., 1997b). The molecular cloning of members of the MAP kinase kinase group revealed that M KK7 can function as activator of JNK (Tournei et al., 1997). We attempted to identify the presence of M KK7 in hOLs exposed to TNF by reverse transcriptase–polymerase chain reaction (RT–PCR), but were not able to detect mRNA for M KK7 (data not shown). This indicated that other pathways independent of M KK7 and co-operating with the M KK4 may contribute to the JNK-mediated TNF-induced hOL death. A recent finding of a JIP (JNK-interacting protein) family of scaffold proteins, which facilitate the efficient activation of JNK by MEKK1 (Akechi et al., 2001), is of interest here.

The mechanisms involved in JNK-induced cell death are presently unclear. Tournei et al. (2000) have shown that protection against stress-induced apoptosis in primary murine embryonic fibroblasts by JNK gene disruption was associated with a defect in the release of cytochrome c. Consistent with suggestions that JNK is involved in mitochondrial dysfunction is an observation by Kharabanda et al. (2000) on JNK translocation to mitochondria and the phosphorylation of anti-apoptotic Bcl-xL protein in human U937 cells exposed to ionizing radiation. In addition, Daxx-induced apoptosis, which is mediated by JNK, is sensitive to bcl-2 activation (Yang et al., 1997c). Our results have shown that JNK involvement in TNF-induced death of adult hOLs depends on mitochondrial dysfunction by demonstrating that M KK4-DN suppressed mitochondrial membrane permeability. Consistent with this conclusion are recent results that show that down-regulation of JNK by NF-kB, involving induction of Gadd45B, inhibits mitochondrial depolarization in mouse embryo fibroblasts (De Smaele et al., 2001).

In conclusion, we have shown that TNF-induced death of adult hOLs depends on prolonged JNK-3 activation prior to mitochondrial dysfunction. These findings might have significant importance in designing new molecules to prevent oligodendrocyte demise in inflammatory and demyelinating diseases such as multiple sclerosis.

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