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Defined inflammatory states in astrocyte cultures: correlation with susceptibility towards CD95-driven apoptosis

Jeppe Falsig,* Markus Latta† and Marcel Leist*

*H. Lundbeck A/S, Valby, Denmark

†Biochemical Pharmacology, University of Konstanz, Konstanz, Germany

Abstract

A complete cytokine mix (CCM) or its individual components tumour necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β) and interferon-gamma (IFN-γ) were used to switch resting murine astrocytes to reactive states. The transformation process was characterized by differential up-regulation of interleukin-6 (IL-6), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthetase (iNOS) mRNA and protein and a subsequent release of prostaglandin E2, nitric oxide (NO) and IL-6. Both CD95L and anti-CD95 antibodies triggered caspase activation followed by apoptotic death in fully pro-inflammatory astrocytes, whereas resting cells were totally resistant. Two other death-inducing ligands, TNF and TNF-related apoptosis-inducing ligand (TRAIL) did not induce apoptosis in reactive astrocytes. The switch in astrocyte sensitivity was accompanied by up-regulation of caspase-8 and CD95 as well as the capacity to recruit Fas-associated death domain (FADD) to the activated death receptor complex. Neither CD95-mediated death, nor other inflammatory parameters were affected by inhibition of iNOS or COX, respectively. Accordingly, IFN-γ was absolutely essential for up-regulation of iNOS, but not for the switch in apoptosis sensitivity. In contrast, p38 kinase activity was identified as an important controller of both the inflammatory reaction and apoptosis both in astrocytes stimulated with CCM and in glia exposed to TNF and IL-1 only.

Keywords: apoptosis, astrocyte, CD95, inflammation, nitric oxide, p38

Astrocytes may take at least three fundamentally different roles in disorders of the central nervous system. (For reviews see Chen and Swanson 2003; Eddleston and Mucke 1993; Ciccarelli *et al*. 2001; Dong and Benveniste 2001). First, they maintain the metabolic homeostasis within the brain under many conditions of stress, produce neuroprotective substances and are involved in cellular repair processes. Second, moderate insult to brain tissue triggers astrocytes to become 'reactive'. In such a state, they release neurodegenerative and pro-inflammatory mediators, which aggravate the disease process. Reactive astrocytes have been implicated in several neurological diseases with inflammatory components, including HIV-1-associated dementia, Alzheimer's disease, multiple sclerosis and stroke (Minagar *et al*. 2002; Stoll *et al*. 1998). *In vivo* the most commonly used marker for reactive astrocytes is glial fibrillary acidic protein (GFAP), but also increased inducible nitric oxide synthetase (iNOS) and cyclooxygenase-2 (COX-2) expression, as well as p38 activation are commonly observed in such cells (Loihl *et al*. 1999; Maslinska *et al*. 1999; Piao *et al*. 2002). Third, severe brain injury, as observed in mechanical trauma or ischemic core areas of stroke victims, is associated with massive astrocyte death. The factors leading to the death of astrocytes, which are generally resistant towards most known death stimuli, are not well characterized.

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Address correspondence and reprint requests to Jeppe Falsig, Molecular Disease Biology, H. Lundbeck A/S, Ottiliavej 9, 2500 Valby, Denmark E-mail: jfp@lundbeck.com

Abbreviations used: α-CD95, anti-CD95 antibody (JO-2); Ac-DEVD-afc, Ac-Asp-Glu-Val-Asp-7-trifluoromethyl coumarin; AICD, activation-induced cell death; CHX, cycloheximide; CCM, complete cytokine mix; CD95-L, CD95-ligand; COX-2, cyclooxygenase-2; DISC, death-inducing signalling complex; FADD, Fas-associated death domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IFN-γ, interferon gamma; IL-6, interleukin 6; IL-1β, interleukin 1beta; iNOS, inducible nitric oxide synthetase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NMMA, N^G-Methyl-L-Arginine Acetate salt; NO, nitric oxide; NOS, nitric oxide synthetase; p38, p38 MAPK; PBS, phosphate-buffered saline; PBS-G, PBS with 2 g/L glucose; PGE2, prostaglandin E2; Tc, threshold cycle; TNF-α, tumour necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand; zVAD-fmk, *z*-Val-Ala-DL-Asp-fluoromethylketone.

The CD95 (Fas/Apo-1)/CD95L (Fas ligand) system is one of the death receptor systems involved both in normal tissue homeostasis and in pathology (Nagata and Golstein 1995). When CD95L binds to its receptor, Fas-associated death domain protein (FADD) is recruited. This in turn recruits further proteins including the cell death signalling protease procaspase-8. The resulting multimeric receptor complex is termed the 'death-inducing signalling complex' or DISC (Scaffidi *et al*. 1999).

Some controversy still exists regarding the role of the CD95 system in brain, and particularly in astrocytes. During development CD95 expression is seen briefly in a subset of neurones, but in the healthy adult brain no immunoreactivity is seen towards CD95 (Leithauser *et al*. 1993; French *et al*. 1996; Cheema *et al*. 1999). CD95L is expressed in microglia, astrocytes and possibly neurones (Bonetti and Raine 1997; Bechmann *et al*. 2002; Shin *et al*. 2002). In agreement with this, resting astrocytes are generally found to be very resistant to CD95. However, in pathologic situations the expression of CD95/CD95L can be up-regulated in a number of brain cells. For instance, CD95 expression is seen in oligodendrocytes in multiple sclerosis lesions, in both neurones and white matter glia in Alzheimer's disease, in rat astrocytes in a model of anterograde degeneration, and in microglia in mice infused with lipopolysaccharide (LPS) (D'Souza *et al*. 1996; de la Monte *et al*. 1997; Bechmann *et al*. 2000; Terrazzino *et al*. 2002). Like CD95, CD95-ligand (CD95-L) is also up-regulated in various disease states and models (Kohji and Matsumoto 2000; Bechmann *et al*. 2000; Li *et al*. 2000; Terrazzino *et al*. 2002). In stroke, CD95 and CD95-L mRNA are expressed in the ischemic penumbra and a significant reduction of infarct size, i.e. rescue of neurones and astrocytes, is seen in mice deficient for CD95L (*gld*) as well as in *lpr* mice lacking functional CD95 (Matsuyama *et al*. 1994; Martin-Villalba *et al*. 1999, 2001; Raoul *et al*. 1999;). In a rat model of traumatic brain injury, both CD95 and CD95-L are up-regulated in several cell types including cortical astrocytes and caspase-8 is activated followed by cleavage of BID and caspase-9 activation (Franz *et al*. 2002; Beer *et al*. 2000).

The circumstantial *in vivo* evidence showing that reactive astrocytes may die by CD95-mediated apoptosis is partially supported by *in vitro* data. Excellent work on human fetal or adult astrocytes (Choi *et al*. 1999; Saas *et al*. 1999) suggests that generally astrocytes are resistant to CD95 ligation. However, frequent passaging or interferon gamma (IFN-γ) treatment may break this resistance. But there is no unanimous agreement on this finding (Becher *et al*. 1998; Lee *et al*. 2000; Wosik *et al*. 2001), possibly due to slight differences in the experimental set-up used by the different groups. Our intention was therefore to establish a robust and well-characterized experimental rodent model of inflammatory astrocytes using a defined set of stimulatory cytokines and well-established endpoints like iNOS, GFAP and COX-2 induction. On the basis of this system we investigated the switch of astrocytes from total resistance towards CD95-mediated cell death to high sensitivity. Finally we asked the degree to which different inflammatory pathways, prostaglandin production, NO production, and p38 MAP kinase activation, are involved in this switch of astrocyte phenotype.

Materials and methods

Materials

CD95-L supernatant was harvested in serum-free Dulbecco's modified Eagle's medium from N2A cells transfected with a plasmid expressing CD95 ligand as described (Rensing-Ehl *et al*. 1995). Control supernatant was harvested from cells transfected with Neo plasmid. CD95-L and Neo supernatant was concentrated 24 times using a spin-column with a cut-off range of 3 kDa (Centriprep YM-3, Millipore, Glostrup, Denmark). Complete cytokine mix (CCM) contained 10 ng/mL murine tumour necrosis factor alpha (TNF-α), 10 ng/mL murine IL-1β (Sigma-Aldrich, Copenhagen, Denmark), and 5 U/mL recombinant murine IFN-γ (R & D Systems, Abingdon, UK). Other reagents were hamster IgG monoclonal anti-mouse CD95 antibody (JO-2) (BD bioscience, Brøndby, Denmark), W1400-dihydrochloride, soluble human recombinant CD95-L, soluble human recombinant TNF-related apoptosis-inducing ligand (TRAIL), plus enhancer (A.H. Diagnostics, Aarhus, Denmark), zVAD-fmk (z-Val-Ala-DL-Asp-fluoromethylketone) (Bachem, Weil am Rhein, Germany). Basic laboratory chemicals and inhibitors were purchased from Sigma-Aldrich (Copenhagen, Denmark) unless stated otherwise.

Primary astrocyte culture

Pregnant C57bL/6jbom mice were purchased from M & B (Lille skensved, Denmark). All experimental procedures were carried out in accordance with the directives of the Danish National Committee on Animal Research Ethics and the European Communities Council Directive #86/609 for care of laboratory animals.

Primary cortical astrocytes were prepared from 1 to 2-day-old mice according to a slightly modified version of a protocol by David E. Weinstein (Weinstein 1997). In brief, brains from six pups were removed and kept on ice in phosphate-buffered saline (PBS) containing 2 g/L glucose and 0.001% (w/v) phenol red, pH 7.4 (PBS-G). The cortices were dissected out, and hippocampi and meninges were carefully removed before digestion in PBS-G containing 10 mg/mL trypsin TRL (Worthington, Lakewood, USA), 1 mg/mL DNase (Worthington), and 5 mg/mL $MgSO_4$ for 3 min at 37°C. Tissue was washed in PBS-G and triturated in PBS-G with 0.5 mg/mL DNase using sequentially an 18, 20 and a 23-G needle. Cells were filtered through a 70 μm mesh (Falcon), pelleted ($150 \times g$ for 5 min) and re-suspended in PBS-G containing DNase and $MgSO_4$. Cells were carefully layered over a 30% Percoll solution (Amersham Pharmacia biotech, Hørsholm, Denmark) in PBS-G) and centrifuged at $150 \times g$ for 10 min. Cells were recovered from the interface, washed once with 15 mL PBS-G ($100 \times g$ for 5 min) and re-suspended in Dulbecco's modified Eagle's medium (high glucose), 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. This medium was used for growing cell and for all experiments. All medium constituencies were purchased from Invitrogen (Taastrup, Denmark). Cells were

counted in a Trypan blue solution to assess viability, and seeded at a density of 10 000 cells/cm^2 in 185-cm^2 flasks (all dishes used were from NUNC, Roskilde, Denmark). Medium was changed after 3 days, and subsequently twice a week. Cells were trypsinized and re-seeded after 14 days in primary culture and were always used for experiments 6–8 days after re-plating.

Standard cell incubation scheme

CCM model

Cells were pre-incubated with inhibitors for 1 h before addition of cytokine mix (CCM). After 24 h, cytokine production and nitrite production were measured.

CCM/CD95 model

Like CCM model except that after the 24-h incubation period, a CD95 agonist was added to the wells, and the cells were incubated for an additional 24 h before measurement of cell death. For time titration of mRNA or protein expression levels, CCM was added at different times before harvesting cells and supernatant from all wells at the same time.

Enzyme-linked immunoassays

Interleukin 6 (IL-6) and TNF were measured using murine specific OptEIA^{TM} ELISA kits from Pharmingen (Brøndby, Denmark) in MaxiSorp plates from NUNC. Prostaglandin E2 (PGE2) was measured using a competitive immunoassay, correlate-EIA^{TM} kit from Assay designs (Ann Arbor, USA), according to the manufacturer's instruction. The kit has cross-reactivity to PGE1 (70%) and PGE3 (16.3%).

Nitrite measurement

Nitrite was measured by use of Griess reagent. In brief, 50 μL supernatant or $NaNO_2$ standards were mixed with 25 μL *N*-(1-naphthyl)ethylenediamine (0.1% in H_2O) and 25 μL sulfanilamide (1% in 1.2 N HCl) in a 96-well plate. After 3 min, samples were read at (570–690 nm).

Western analysis

The protein levels of COX-2, iNOS, GFAP, and CD95 were assessed using western blot analysis. Astrocytes were washed once in PBS before lysis in ice-cold buffer containing 1% NP-40, 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 4 mM iodo acetamide, 10 mM NaF, 1 mM 4-(2-Aminoethyl)-Benzene sulfonylfluoride HCL (AEBSF), 1 mM Na_3VO_4 and 'Complete mini' protease-inhibitor mix from Roche (1 pill/10 mL buffer). The lysate was transferred to a microfuge tube, and incubated for 15 min on ice before cellular debris was spun down for 10 min at 10 000 × *g*, 4°C. The supernatants were transferred to a fresh tube and stored at −80°C. The protein concentration was determined by the BCA method using a commercial kit from Bie & Berntsen (Rødoure, Denmark). The NuPAGE-kit (4–12% Bis-Tris gel run with MOPS buffer under reducing conditions) from Invitrogen was used for electrophoresis according to manufacturer's instruction. Gels were run for approximately 1 h at 200 V before blotting proteins to an activated PVDF membrane (Immobilon P, Millipore, Glostrup, Denmark) using wet-transfer blot module XCell2 from Invitrogen. Membranes were blocked with 5% milk in Tris-buffered saline/Tween (2.42 g/L Tris base, 8 g/L NaCl, and 0.1% Tween-20 pH 7.6), washed with Tris-buffered saline/Tween and incubated with primary antibody dissolved in 5% milk in Tris-buffered saline/Tween overnight at 4°C. Blots were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (rabbit anti-mouse 1 : 2000, and goat anti-rabbit 1 : 1000 both from DAKO, Glostrup, Denmark) for 1 h at room temperature, and developed using enhanced chemiluminescence or enhanced chemiluminescence+ (Amersham Pharmacia Biotech, Hillerød, Denmark). Primary antibodies used were mouse IgG anti-GFAP clone G-A-5 (Sigma-Aldrich, Vallensbæk strand, Denmark) used at a dilution of 1 : 1000. Rabbit polyclonal anti-mouse COX-2 (1 : 1000), and rabbit polyclonal anti-mouse iNOS (1 : 2000) from Alexis, was purchased through A.H. Diagnostics (Aarhus, Denmark). Rabbit IgG polyclonal anti-mouse CD95 M-20 (Santa Cruz Biotechnology Inc., Santa Cruz, USA) 1 : 200 and mouse anti-β-Actin clone AC-15 (Sigma) 1 : 5000 were also used.

Immunoprecipitation of CD95

Cells (12×10^6/condition) were stimulated for 30 min, washed once in ice-cold PBS before lysis in buffer containing 1% Triton X-100, 150 mM NaCl, 30 mM Tris-HCl pH 7.5, 10% glycerol, and 1 mM Pefabloc. Cells were left to lyse on ice for 15 min before centrifugation at 14 000 × *g* for 15 min at 4°C. The supernatant was transferred to a fresh tube and kept at −80°C. Protein A-sepharose (swollen overnight in PBS) and protein G-sepharose (Sigma), each 50% (v/v) in PBS, were mixed 1 : 1. Thirty microlitres of this mixture was added to 1 mL supernatant and rotated for 2 h at 4°C. The beads were spun down for 1 min at 600 × *g* at 4°C and the supernatant was saved for controls. The beads were washed four times with lysis buffer as above, before addition of 20 μL (2×) Laemmli buffer containing sodium dodecyl sulfate and dithiothreitol. Beads were boiled for 3 min at 95°C and loaded onto a 4–20% Tris-glycine gel. After electrophoresis and transfer of the protein to a PVDF membrane, precipitated protein was immunostained. Antibodies used for western blot analysis were rat anti-CD95 (1 : 500) and mouse anti-FADD (1 : 1000), both from Upstate (Milton Keyes, UK). Secondary antibodies were donkey anti-rat (1 : 20 000) and donkey anti-mouse (1 : 4000) both from Dianova (Hamburg, Germany).

Quantitative PCR

Cells stimulated in 6-well dishes were washed once with PBS and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 μg) was reverse transcribed with TaqMan RT-Reagent (Applied Biosystems, Nærum, Denmark), using random hexamers in a 100-μL reaction on a PTC-200 DNA Engine Thermal Cycler (VWR international, Albertslund, Denmark), using a program of 10 min annealing at 25°C, 30 min reverse transcription at 48°C, 5 min inactivation at 95°C. The cDNA was quantified using the SYBR Green® Quantitative PCR kit (Applied Biosystems, Naerum, Denmark). Each reaction contained 2.5 μL cDNA of the 100 μL RT-product, 300 nM forward and reverse primers, 0.625 U AmpliTaq Gold DNA polymerase, 0.25 U AmpErase UNG, 3 mM $MgCl_2$, and 200 μM of dATP, dGTP, and dCTP, and 400 μM dUTP in a total volume of 25 μL. PCR amplification was run in a 96-well experimental plate format on an iCycler Thermal Cycler equipped with iCycler Optical System (Bio-Rad, Hercules, CA, USA). The program set-up was 10 min at 95°C, 40 cycles of 15 min at 95°C/1 min at 60°C. A melting curve was

obtained to verify the measured signal and the product was run on a 4% agarose gel to verify the presence of only one band. Quantification was performed as follows: using the iCycler data analysis software (Bio-Rad), the threshold cycle (Tc) was determined for each sample. Tc was defined as the cycle at which the level of fluorescence increased significantly above the background levels of fluorescence. The concentration of cDNA was calculated by comparing Tc of samples to Tc of a standard curve. The standard curve was obtained by a serial dilution of cDNA. Each sample was run in two reactions, one with the primer set of interest and one with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set, and all data are displayed as the ratio between the calculated starting concentration of the cDNA of interest and GAPDH. All primers except the housekeeping gene GAPDH were intron spanning in order to distinguish cDNA from genomic DNA. For GAPDH the order of magnitude between the samples and the samples without reverse transcriptase was above 10^6. Primers used were GAPDH sense (Accession no. NM_008084): 5′-TGCACCACCAACTGCTTAG-3′, antisense: 5′-GGATGCAGGGATGATGTTC-3′; CD95 sense (Accession no. AK_002590): 5′-GCTGAGGAGGCGGGTTCGTG-3′, antisense: 5′-CATGGGGCGCAGGTTGGTG-3′; COX-2 sense (Accession no. NM_011198): 5′-GTGTGACTGTACCCGGACTGGATTCTA-3′, antisense: 5′-ACTGTGTTTGGGGTGGGCTTCA-3′; Caspase-8 sense (Accession no. AF_067834): 5′-CAGAAAATAACTTGGAAACCCTAAAATCA-3′, antisense: 5′-GTGGCAACTCTTCCCTTCCTTCAA-3′; IL-6 sense (Accession no. J_03783): 5′-GGAGCCCACCAAGAACGATAGTCA-3′, antisense: 5′-GAAGTAGGGAAGGCCGTGGTT-3′; iNOS sense (Accession no. NM_010927): 5′-TTGCCACGGACGAGACGGATAGG-3′, antisense: 5′-GGGCACATGCAAGGAAGGGAACTC-3′; TNF sense (Accession no. NM_013693): 5′-CTATGGCCCAGACCCTCACACTCA-3′, antisense: 5′-CACTCCAGCTGCTCCTCCACTTG-3′. Primers were designed using DNA-star software package (DNASTAR Inc., Madison, USA) and all primers were blasted using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/).

Immunocytochemistry

Astrocytes were seeded on uncoated glass coverslips and stimulated appropriately. The following procedure was performed at room temperature. The cells were washed once with PBS and fixed in 4% paraformaldehyde for 12 min. After three washes with PBS the coverslips were blocked for 30 min with PBS/0.05% Tween (PBS/Tween) containing 5% fetal calf serum and then incubated for 45 min with mouse IgG anti-GFAP clone G-A-5 (Sigma-Aldrich, Vallensbæk strand, Denmark) diluted 1 : 1000 in blocking buffer. The cells were washed three times in PBS/Tween and incubated with Alexa Fluor 488 goat anti-mouse IgG diluted 1 : 300 in blocking buffer and dye Hoechst-33342 1 μg/mL (both purchased from Molecular Probes, Eugene, OR, USA) for 30 min. The coverslips were washed with PBS/Tween and rinsed in water, before mounting and sealing them on a slide using Dako fluorescence mounting fluid (Dako, Albertslund, Denmark) and nail varnish. Pictures were taken using Zeiss Axiovert S100TV fluorescence microscope equipped with a coolsnap camera, shutter and shutter driver controlled by Metamorph software, Universal Imaging Corporation (Downingtown, PA, USA), through a 40× lens. Apoptotic cells were fixed in 4% paraformaldehyde and stained with H-33342, before being mounted and visualized through a 100× oil immersion lens.

Viability and caspase-3 assays

Lactate dehydrogenase (LDH) release was measured using the cytotoxicity detection kit from Roche (Hvidovre, Denmark) as follows: cell culture supernatant was sampled before lysis of the cells in 0.1% Triton X-100 in medium. An equal amount of cell lysate and supernatant was incubated with the reagent mixture and after 15 min of incubation at room temperature a formazan product was measured spectrophotometrically at 492–690 nm. Data are displayed as the percentage of LDH activity in the supernatant compared to total LDH activity in the well. Caspase-3 activity was measured as the ability to cleave the fluorogenic substrate Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-afc) (Biomol Research Laboratories, Plymouth Meeting, PA, USA). Cells were washed once in PBS, lysed in ice-cold caspase lysis buffer: 25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1 mM Pefabloc for 15 min on ice and centrifuged at 14 000 × *g* to obtain a clear supernatant. Cell lysates (or 10 μL cell lysate from jurkat cells treated with CD95-L, positive control) were mixed with 80 μL assay buffer (50 mM HEPES, 10 mM dithiothreitol, 1% sucrose, 0.1% CHAPS) and 10 μL substrate (500 μM stock Ac-DEVD-afc dissolved in assay buffer). The kinetics of Ac-DEVD-afc cleavage was measured on an Ascent Fluoroskan FL (Labsystems) (Ex 385, Em 510) (Hentze *et al*. 2001).

Statistics

Unless otherwise indicated, all results are displayed as mean ± SD of triplicates. PGE2 and IL-6 data are displayed as means of duplicates ± SD (range). Only data from experiments confirmed at least once are displayed. One-way analysis of variance followed by the Dunnett's test was used for statistical evaluation. (*$p < 0.05$, **$p < 0.01$).

Results

An *in vitro* model of inflammation in astrocytes

In disease processes, inflammatory mediators switch astrocytes from a resting state to an activated, pro-inflammatory state. We designed an experimental system to model this process *in vitro* by stimulating primary murine astrocytes with a cytokine mix (CCM) consisting of IL-1, TNF and IFN-γ. First, time-scale and expression level of COX-2, IL-6 and iNOS were determined by quantitative RT–PCR. The expression of the immediate early gene COX-2 peaked already 2 h after CCM addition. IL-6 and iNOS up-regulation was low during the first 2 h and then steadily increased, peaking after 8 h treatment (Fig. 1a). On the protein level, a similar but delayed profile was observed. A COX-2 protein band was first observed after 4 h (Fig. 1b) and western-blot analysis of iNOS (Fig. 1b) also showed an increase in the protein levels after 4 h.

Up-regulation of GFAP is a commonly used marker for glial activation *in vivo*. After 4 h of CCM treatment a large increase in GFAP expression was observed (Fig. 1b). This correlated with a clear morphological change showing GFAP

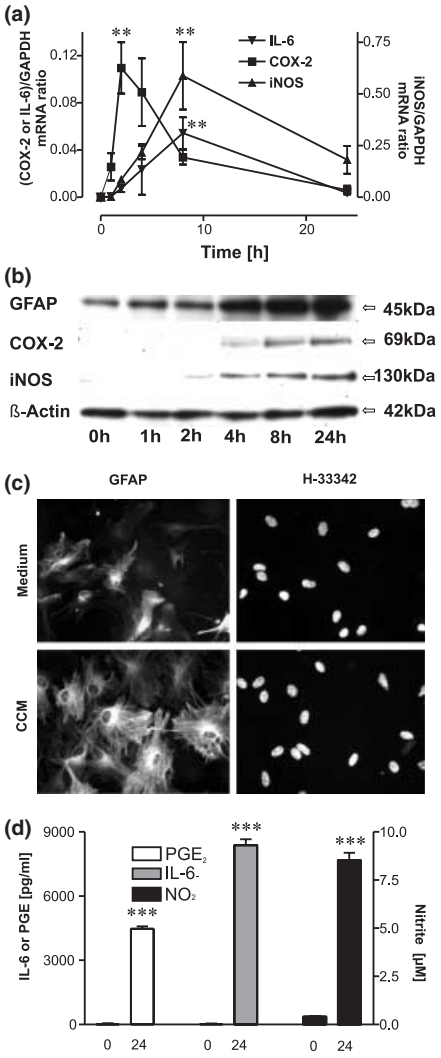

Fig. 1 Induction of a pro-inflammatory state in astrocytes. (a) Cells were stimulated with CCM for the times indicated, total RNA was extracted and inflammatory markers were quantified by real-time RT–PCR. Experiments were performed in triplicates and data are displayed as means ± SD. Statistics was performed for peak concentrations vs. resting cells $^{**}p < 0.01$. (b) Astrocytes were treated with CCM for the times indicated and cell lysates were analysed by western blot analysis for the expression of GFAP, COX-2 and iNOS. β-Actin was used as loading control in all blots. (c) Astrocytes were seeded onto glass coverslips and treated with CCM for 24 h. The cells were fixed in 4% paraformaldehyde and stained using a monoclonal a-GFAP antibody and the nuclear counterstain H-33342. Pictures representative of wells were taken at a magnification of 40×. (d) Astrocytes were treated for 24 h with CCM and NO_2^-, IL-6 and PGE2 released into the supernatant were measured. Data are means ± SD of duplicates (triplicates for NO_2^-). $^{***}p < 0.001$ (unpaired Student's *t*-test).

stress fibers, and suggesting that GFAP is a suitable marker for astroglial activation also in our *in vitro* model (Fig. 1c). The pro-inflammatory activation status induced by CCM in astrocytes was further confirmed by measurement of the secreted product, PGE2, NO and IL-6. All were found in high concentrations in astrocyte-conditioned medium 24 h after stimulation. Thus, the stimulation of astrocytes for 24 h with CCM proved to be a robust and defined way to switch the cells into a pro-inflammatory state.

Lack of a LPS effect on astrocytes

There is conflicting evidence (Chung and Benveniste 1990; Molina-Holgado *et al*. 1995; Sola *et al*. 2002) on the potential contribution of contaminating microglia to the inflammatory response measured in astrocyte cultures. LPS is a powerful microglia stimulator, whereas murine resting astrocytes have been reported to express no (Sola *et al*. 2002) or low levels of the TLR-4 LPS receptor (Bowman *et al*. 2003). In our system, LPS (10 μg/mL) triggered neither NO nor TNF production within a time of up to 72 h (data not shown). LPS responses were readily measurable when microglia cells were seeded onto astrocytes. The cell culture was also examined for microglial markers (stained with lectins and anti-CD11b) but < 1% positively staining cells were observed (data not shown). Although our data do not exclude a contribution of contaminating microglia to the initiation and acceleration of the inflammatory response, they suggest that the inflammatory products measured are all derived from astrocytes.

Contribution of individual cytokines to the inflammatory response

As an important characterization of our experimental inflammatory system the importance of the different components in the CCM was examined. Astrocytes were stimulated for 24 h with a cytokine mix containing from zero to three of the CCM components. Both TNF-α and IL-1β were essential for all the endpoints measured. The combination of these two cytokines was also sufficient for triggering maximal PGE2 or IL-6 production, whereas they only lead to an intermediate NO release (Figs 2a–c). IFN-γ showed a pattern different from TNF-α and IL-1β. IFN-γ was not necessary for the production of PGE2, but was absolutely required for maximal production of NO as measured by nitrite accumulation in the medium (Figs 2a–c).

Pharmacological separation of inflammatory pathways

Next we examined whether the inflammation endpoints chosen truly reflected independently triggered pathways that could be separately correlated with CD95 signalling. The cellular responses were measured in the presence of inhibitors of COX and iNOS. The COX inhibitor indomethacin (or similarly ibuprofen, not shown) prevented the production of PGE2 without affecting levels of nitrite produced during the 24 h CCM incubation (Fig. 3a). IL-6 and NO_2 levels were also not altered by COX inhibition, even after 48 h CCM exposure time (data not shown). Vice versa, W1400, an

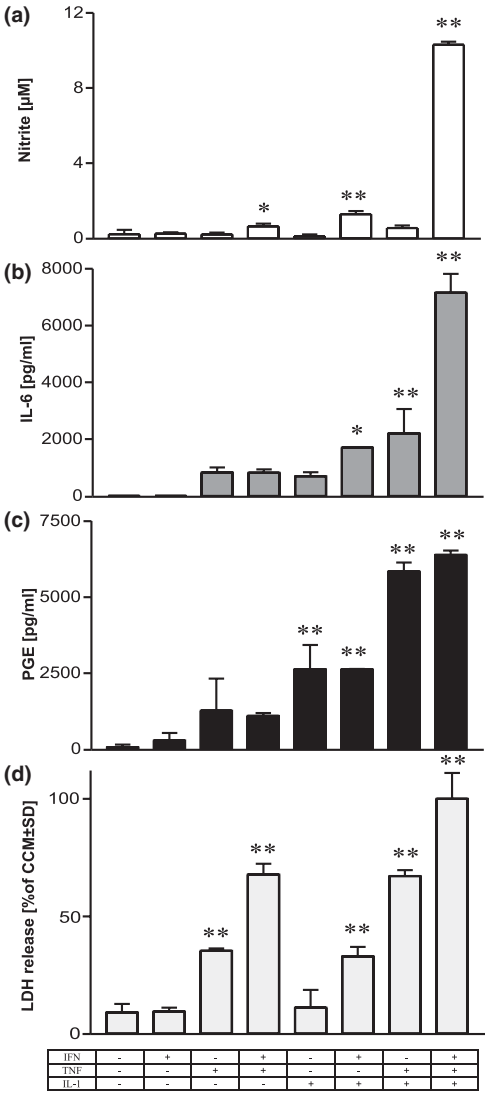

Fig. 2 Contribution of TNF, IL-1 and IFN to the astrocyte inflammatory response. (a–c) Astrocytes were seeded in 96-well dishes and stimulated with different combinations of cytokines constituting CCM. NO_2^-, IL-6 and PGE2 were measured in the supernatant after 24 h. Experiments were performed in triplicate for NO_2^- and duplicates for IL-6 and PGE2 and repeated twice with similar outcome. Data are displayed as means ± SD. $^{**}p < 0.01$. (d) Set-up as in previous experiment except that α-CD95 (1 µg/mL) was added after the 24 h CCM stimulation. LDH was measured 24 h after antibody addition and data are displayed as mean LDH release ± SD $^{**}p < 0.01$.

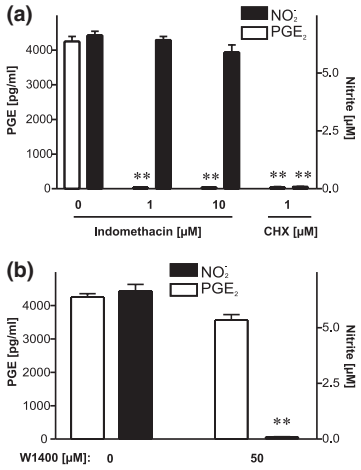

Fig. 3 PGE2 and NO production, two independent inflammatory pathways? Cells were seeded in 96 wells and after 1 h pre-incubation with (a) indomethacin or cycloheximide, or (b) W1400 cells were challenged with CCM for 24 h. Supernatant was used for PGE2 and NO_2^- measurements. Data are means ± SD of duplicates (triplicates for NO_2^-). $^{**}p < 0.01$.

inhibitor of iNOS, totally abolished nitrite production without affecting the levels of PGE2 significantly (Fig. 3b). The general NOS inhibitor N^G-Methyl-L-Arginine Acetate salt (NMMA) showed the same results (data not shown). The protein synthesis inhibitor cycloheximide completely prevented the release of NO, PGE2 and IL-6 (data for IL-6 not shown) without affecting cell viability (Fig. 3a). Having characterized the astrocyte inflammation system pharmacologically, the role of inflammatory activation in apoptosis sensitivity was addressed.

Change in sensitivity of astrocytes towards CD95 depends on inflammatory status

The controversy about the role of CD95 in neural cell death might be due to changed signalling under resting and inflammatory conditions. Resting astrocytes were completely insensitive to CD95-mediated cell death under any stimulation condition. In contrast astrocytes exposed to CCM for 24 h and subsequently treated with CD95-L containing supernatant were killed concentration-dependently (Figs 4a–c). Measurement of LDH release indicated up to a 60% cell loss and these data were fully confirmed by an MTT reduction assay. Similar results were also obtained by morphological analysis and scoring of dead cells after staining with SYTOX. The specificity of the cell death stimulus was tested in parallel experiments, using conditioned cell medium containing recombinant CD95-L, control supernatant, purified rhsCD95-L, TRAIL and CD95 agonist JO-2 antibody as stimuli. Any agonist ligand of CD95 lead to death of CCM-treated astrocytes, whereas resting astrocytes were insensitive (Figs 4b and c). TRAIL, on the other hand, did not affect the viability of astrocytes whether they were pre-treated with CCM or not (Fig. 4c). The specificity of the JO-2 antibody was also confirmed by heat-inactivating the antibody and by using an isotypic antibody (data not shown). We observed that treatment with CCM alone lead to a small decrease in MTT reduction (5–15% depending on the experiment), but only a minor release of LDH (3–5%) (data not shown).

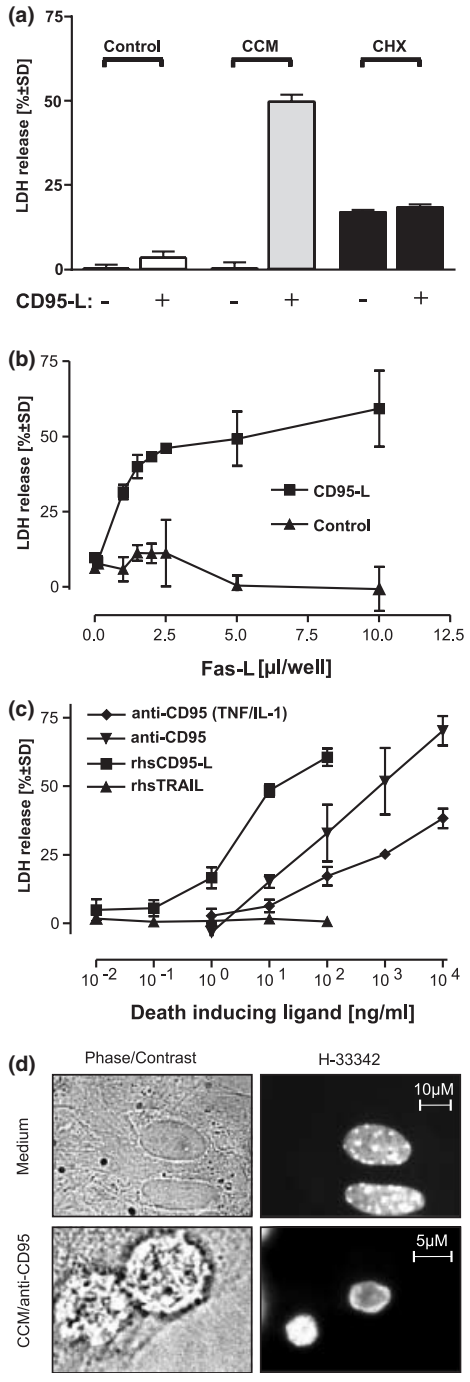

Fig. 4 CD95-mediated death of CCM-treated astrocytes. (a) Supernatant from N2A cells transfected with a CD95-L expressing vector was harvested and concentrated. Supernatant was added for 24 h (2% v/v) to untreated astrocytes or astrocytes pre-treated with either CCM or cycloheximide (CHX) for 24 h (N.B. total exposure time to CCM or CHX was 48 h). (b) Titration of CD95-L/control supernatant in astrocytes pre-treated with CCM for 24 h. CD95-L/control supernatant was harvested from N2A cells transfected with Neo-plasmid (Neo) or with Neo plasmid expressing CD95-L. All data are displayed as mean LDH release ± SD (2 µL compare to 2% v/v as shown in a). (c) Titration of JO-2 α-CD95 antibody, rhsCD95-L (+ 1 µg/mL enhancer) and TRAIL (+ 1 µg/mL enhancer) on astrocytes pre-treated for 24 h with CCM. The JO-2 antibody was also titrated in astrocytes pre-treated for 24 h with 10 ng/mL IL-1β and 10 ng/mL TNF-α. All data are displayed as mean LDH release ± SD. (d) Astrocytes were treated for 24 h with CCM and then exposed to α-CD95 antibody for 24 h. Cells were stained with a nuclear stain (1 µg/mL H-33342) and pictures were taken at a 100× magnification using fluorescence and light microscopy.

Having established a robust cell death measurement, the contribution of the different CCM components towards switching the cell death sensitivity was examined (Fig. 2d). IFN-γ has been reported as the major death sensitivity switch, but brain cells do not express IFN-γ and this cytokine is not found in all brain disease states. We observed here that there was no absolute requirement for IFN-γ in CD95-mediated death (Fig. 2d). Titration of the α-CD95 antibody in astrocytes pre-treated with only TNF-α and IL-1β (Fig. 4c) gave a similar but 30% less severe death profile to that of CCM-treated cells, suggesting that IFN-γ is an aggravating but not a determining factor.

A change in the apoptotic machinery in response to pro-inflammatory conditions

In order to find a rationale why CCM sensitized astrocytes to CD95-L, quantitative PCR was performed in order to examine the expression levels of CD95-receptor, caspase-8, FADD and cellular FLICE-like inhibitory protein (c-FLIP). CD95-receptor expression was significantly increased after 2 h, peaking after 4 h in response to CCM treatment (Fig. 5a). In order to confirm this up-regulation, western blot analysis was performed, showing an increased expression of the receptor (Fig. 5b). The increase in CD95-receptor was prevented by administration of cycloheximide (Fig. 5b). Caspase-8 expression was also increased approximately four-fold (Fig. 5c), whereas FADD and c-FLIP expression were not significantly changed (data not shown).

The expression data were further supported by functional signalling analysis. Cell death signalling via CD95 ligation leads to recruitment of FADD to CD95, to form a so-called DISC. DISC formation (FADD recruitment) was confirmed for astrocytes treated for 24 h with CCM and exposed to anti-CD95 antibody for 30 min (Fig. 5d). DISC formation was absent in astrocytes not treated with CCM or when the antibody was added after cell lysis.

Apoptotic processes in CCM/anti-CD95 antibody-treated astrocytes

In order to examine whether DISC formation led to the activation of caspases, caspase-3 enzymatic activity was measured in astrocyte lysate. In CCM pre-treated astrocytes, CD95L triggered caspase activation within 8 h (Fig. 6a).

Astrocytes were incubated with zVAD-fmk before CD95 ligation, in order to examine the causal role of caspases in

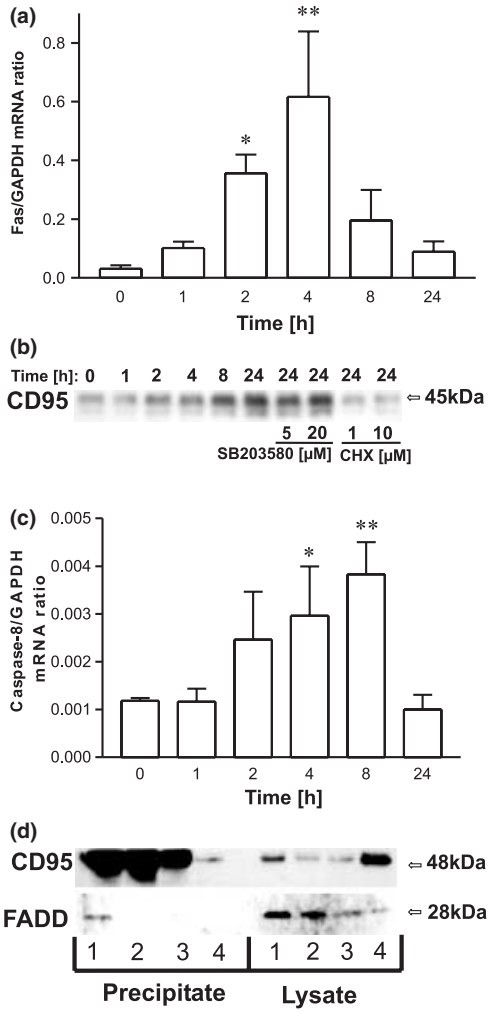

Fig. 5 Change of the CD95 signalling system by CCM. (a) Cells were stimulated with CCM for the times indicated; total RNA was extracted and CD95 mRNA was quantified by real-time RT–PCR. Data are displayed as mean ± SD. (b) Western blot analysis of cell lysates using an anti-CD95 antibody. Lanes 7–10 shows data for cells treated with SB203580 or cycloheximide during 24 h CCM treatment. (c) Cells were stimulated with CCM for the times indicated, total RNA was extracted and Caspase-8 mRNA levels were quantified by real-time RT–PCR. Data are displayed as means ± SD of triplicates. (d) Immunoprecipitation of the CD95 receptor and associated proteins from astrocytes treated as follows: sample 1: CCM 24 h followed by α-CD95 30 min; sample 2: CCM 24 h, α-CD95 added after cell lysis; sample 3: α-CD95 30min, without CCM pre-treatment; sample 4: CCM 24 h, without addition of α-CD95 antibody. Western blot analyses were performed against CD95 and FADD, both in the precipitate and in the supernatant.

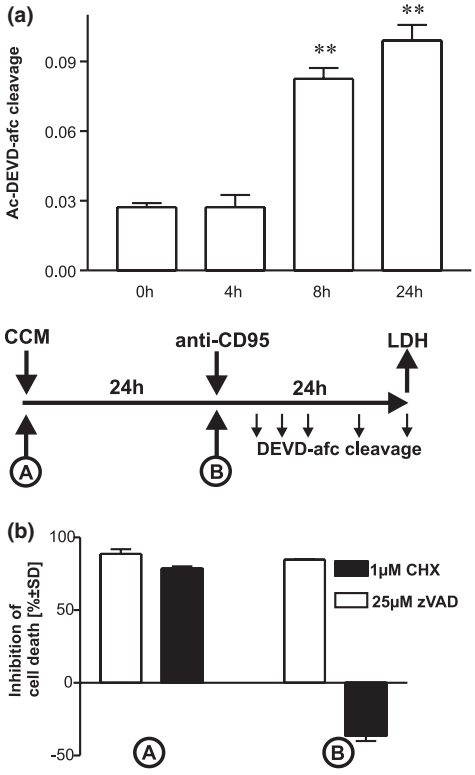

Fig. 6 A caspase-dependent apoptotic cell death induced by anti-CD95 antibody (a) Cells were pre-treated for 24 h with CCM before addition of anti-CD95 antibody. At the indicated times after αCD95 addition, cells were lysed and DEVDase activity determined. Data are displayed as means of Emission (510 nm)/(min × mg protein) ± SD. $^{**}p < 0.01$. (b) The caspase inhibitor, zVAD-fmk (25 μM) or cycloheximide, respectively, were added to astrocytes at the time of CCM stimulation (A), or at the time of α-CD95 antibody addition (B). LDH release was measured and data are displayed as means ± SD of triplicates.

death signalling. The caspase inhibitor z-VAD-FMK blocked astrocyte apoptosis in this system completely at 25 μM and >50% at 1 μM. The protein synthesis inhibitor cycloheximide saved astrocytes only when added at the time of cytokine addition but it enhanced cell death when added together with the JO-2 antibody (Fig. 6b), suggesting a role for protein synthesis in the up-regulation of the death machinery, but not in death execution.

Finally, the mode of cell death was examined morphologically by staining of cultures with the chromatin dyes H-33342 and SYTOX (non-cell-permeable). Most of the SYTOX-positive cells (increasing from 1–24 h) had apoptotically condensed nuclei. On the other hand, 10–20% of the cells with apoptotic chromatin were SYTOX negative. This suggests that the main mode of death was apoptotic or apoptosis-like, with membrane lysis occurring very rapidly after chromatin condensation (Fig. 4d).

The involvement of different inflammatory pathways in CD95 sensitization in the murine astrocyte model

Complete inhibition of COX (as measured by PGE2 ELISA) and iNOS (as measures by nitrite in medium) did not affect cell death caused by CD95 ligation (Figs 7a and b). The

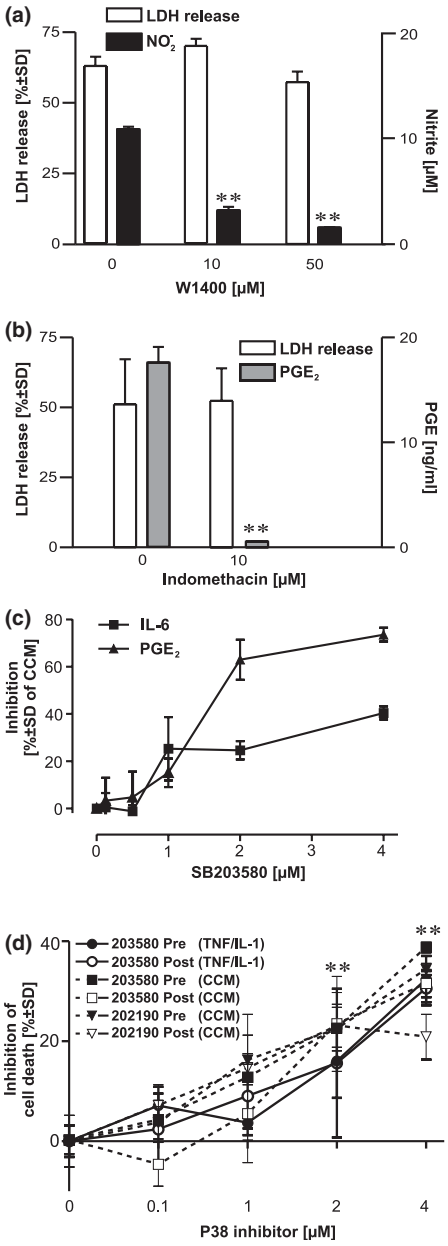

Fig. 7 Modification of CD95 sensitivity by anti-inflammatory agents. (a) W1400, an inhibitor of iNOS, was added prior to CCM addition. After 24 h CCM treatment α-CD95 antibody was added for 24 h. Data are displayed as mean LDH release ± SD of triplicates. (b) COX-2 inhibitor, indomethacin was added prior to CCM addition. After 24 h CCM treatment α-CD95 antibody was added for 24 h. Data are displayed as mean LDH release ± SD of triplicates. (c) Astrocytes were pre-treated for 30 min with different concentrations of p-38 MAPK inhibitor SB203580 before adding 10 ng/mL TNF-α and 10 ng/mL IL-1β for 24 h. Supernatant was harvested and examined for IL-6, NO_2 and PGE2. Data is displayed as percentage inhibition (compared to CCM) ± SD. (d) SB203580 or SB202190 was added together with either CCM or TNF/IL-1 or after 23.5 h (delayed-treatment). After 24 h a-CD95 antibody was added and LDH release was measured 24 h later. Data are displayed as mean LDH release ± SD of triplicates, $^{**}p < 0.01$.

COX inhibitor ibuprofen and the iNOS inhibitor NMMA were also tested with the same result (data not shown). P38 has been linked to CD95-L-induced cell death in other cell types (Chen *et al*. 1998; Davidson *et al*. 2002; Raoul *et al*. 2002). Since p38 mitogen-activated protein kinase (MAPK) has been reported to be involved in cytokine production in astrocytes we explored the role of this inflammatory regulator. In the TNFα/IL-1β model, inhibition of p38 reduced production of PGE2 and IL-6 (Fig. 7c). In astrocytes treated with CCM, PGE2 was reduced up to 70%, as in the previous model, and both IL-6 and nitrite were reduced up to 40% in the presence of SB203580 (data not shown). Having defined the reasonable pharmacological range of action of SB203580, this compound was now used to examine the role of p38 in CD95 signalling.

SB203580 reduced the specific cell death by approximately 40% at 4 μM, both when added at the time of cytokine addition (pre-treatment) and when added together with the CD95 antibody (delayed treatment) (Fig. 7d). To confirm that p38 inhibition can rescue astrocytes, we repeated the experiment with another p38 inhibitor, SB202190 with approximately the same result (SB202190 was a little less efficient in the delayed treatment). SB203580 was also effective in the TNF/IL-1 model, showing the same inhibitory profile as in the CCM model. Since CD95 is up-regulated in the model we wondered if perhaps p38 prevented the enhancement of receptor expression. Inhibition of p38 MAPK did not affect receptor expression in astrocytes (Fig. 5b).

Discussion

In the brain CD95 signalling has been attributed several beneficial features such as elimination of activated T-cells and developmental nerve growth (Bechmann *et al*. 1999; Desbarats *et al*. 2003), as well as negative features such as cell death. (Raoul *et al*. 1999; Lee *et al*. 2000; Choi *et al*. 2002). Strong evidence exists for the contribution of the CD95 system to the massive cell death seen in the penumbral area after an ischemic insult (Martin-Villalba *et al*. 2001), as well as in the cortex after traumatic brain injury (Martin-Villalba *et al*. 1999; Franz *et al*. 2002).

Until now, work on astrocytes and CD95-mediated death has employed human fetal astrocytes with a heterogeneity of described responses, and this is to our knowledge the first murine *in vitro* model allowing characterization of the inflammation-driven switch in death sensitivity.

Both human and rodent astrocytes are generally resistant to CD95-mediated death even though the cells express CD95, and do respond to CD95 ligation by releasing chemokines and cytokines (Lee *et al*. 2000; Choi *et al*. 2002). It is reported in the literature that human astrocytes can become sensitive to CD95-mediated death by either repeated passaging or by treatment with IFN-γ either alone

(Saas *et al*. 1999) or when combined with TNF-α (Malipiero *et al*. 1997; Choi *et al*. 1999). In our model of relatively pure and resting murine astrocytes, we see an almost equal contribution towards the switch in cell death of all the pro-inflammatory cytokines used (IL-1β, TNF-α, and IFN-γ), but only one cytokine, TNF-α, modestly sensitized cells on its own. IFN-γ alone is not sufficient but does contribute when added together with other cytokines. In contrast, TNF plus IL-1, the main mediators found in stroke or Alzheimer's disease plaques, were potent phenotypic switches in the absence of IFN-γ.

Mutations that interfere with the CD95 system confer significant protection for the penumbra tissue in stroke models (Martin-Villalba *et al*. 1999, 2001) Since lymphocytes are hardly found in the penumbral area at earlier time points after ischemia, little IFN-γ may be present in this area (Martin-Villalba *et al*. 2001). Our findings that TNF-α/IL-1β treatment in itself is sufficient to sensitize astrocytes to CD95-mediated death suggests that CD95-mediated astrocyte death is a possibility in many inflammatory situations, including post-ischemic damage where both TNF-α and IL-1β are present.

The markers used here to characterize the inflammatory phenotype, COX-2, iNOS and IL-6, are all induced in reactive astrocytes *in vivo* (Endoh *et al*. 1994; Maeda *et al*. 1994; Hirst *et al*. 1999; Loihl *et al*. 1999; Maslinska *et al*. 1999). Doubts have been raised whether NOS induction is a good activation marker in LPS-treated murine astrocyte cultures (Sola *et al*. 2002; Lehnardt *et al*. 2002). However, astrocytes have the potential to express iNOS in response to cytokine treatment (Simmons and Murphy 1992; Hua *et al*. 2002) exactly as found in our model. The regulation of the expression pattern of these inflammatory mediators is complex, but the involvement of p38 MAPK has been implicated in all cases by the use of the p38 inhibitor SB203580 (Hua *et al*. 2002; Molina-Holgado *et al*. 2000). In fact, activated p38 MAPK is found in astrocytes *in vivo* (Hensley *et al*. 1999; Barone *et al*. 2001; Piao *et al*. 2002) and *in vitro* (Da Silva *et al*. 1997).

Some laboratories have reported that pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ and IL-6) can up-regulate CD95 in human fetal astrocytes (Malipiero *et al*. 1997; Choi *et al*. 1999; Saas *et al*. 1999), whereas others did not see such a regulation (Lee *et al*. 2000; D'Souza *et al*. 1996; Becher *et al*. 1998). Also the matter of cell death is controversial. Three groups observe cell death in astrocytes treated with IFN-γ, or TNF/IFN-γ together with CD95-L (Saas *et al*. 1999; Malipiero *et al*. 1997; Choi *et al*. 1999), whereas others do not see cell death in response to IFN-γ and CD95-L (Wosik *et al*. 2001; Becher *et al*. 1998; Lee *et al*. 2000). Our data show that astrocytes become sensitive to CD95-mediated death depending on the strength of the overall inflammatory stimulation more than on the exact cytokine used. This could explain most of the confusion regarding cell death. Those groups that do not see any cell death only use IFN-γ/CD95-L, a stimulus that does not give significant cell death neither in our cultures nor in the cultures of Choi or Malipiero (Malipiero *et al*. 1997; Choi *et al*. 1999). Saas *et al*. claim 70% cell reduction by pre-treatment with IFN in astrocytes passaged four times (Saas *et al*. 1999). The authors themselves show that astrocytes at passage 8–10 are sensitive to CD95-L without any further treatment, so one could speculate that the repeated passage of their cultures could sensitize astrocytes to CD95-mediated death so that IFN-γ gives a sufficiently strong inflammatory signal.

When cycloheximide was added to CCM-stimulated astrocytes at the time of CD95-L addition, we observed aggravation of cell death. This indicates that protein synthesis-dependent protective processes are ongoing or triggered by CD95 in activated astrocytes. One potential protective pathway has been described in resting human astrocytes, where IL-8 is released in response to CD95-L. IL-8 is able to protect astrocytes against cell death in an autocrine fashion by signalling through the CXCR2 (Saas *et al*. 2002). This mechanism depends on a pertussis toxin sensitive G-protein, but murine astrocytes express neither IL-8 nor CXCR2 (Dorf *et al*. 2000; Saas *et al*. 2002) and we were unable to sensitize astrocytes by using pertussis toxin (data not shown). It was reported that cycloheximide is sufficient to sensitize human astrocytes by preventing IL-8 release, but we found that it did not sensitize murine astrocytes. We conclude that resting murine astrocytes do not need to induce a protective pathway, and are protected possibly due to the absence of certain elements of the apoptotic machinery, or the presence of anti-apoptotic regulators.

The classical inflammatory enzymes NOS and COX have been implicated in apoptosis signalling in other cells, but did not play a role in astrocytes. In contrast, we found that p38 MAPK is involved in the death of astrocytes. It is known from other systems that p38 can be involved in apoptosis. Treatment with p38 MAPK inhibitors protects several cell types including cerebellar granule neurones and motor neurones against CD95-dependent apoptosis (Chen *et al*. 1998; Davidson *et al*. 2002; Hou *et al*. 2002; Raoul *et al*. 2002). A variety of cells are also protected against CD95-independent apoptosis (Bordin and Whitfield 2003). Two different p38 inhibitors used at relevant concentrations inhibited the cell death induced by CCM/α-CD95 by about 40% without affecting CD95 expression. The same degree of inhibition also took place in astrocytes pre-treated with TNF-α/IL-1β. It is unknown at this time by what mechanism p38 MAPK affects cell death but the fact that delayed treatment protects as well as early treatment suggests that p38 is involved, not in the switch in sensitivity, but in the signalling downstream of CD95. Similar processes appear to operate also in other *in vitro* models. SB203580 protects T-cells from

CD95-L-induced apoptosis to the same extent as caspase inhibitors and seemed to work through the same signalling pathway as the caspases (Davidson *et al*. 2002). It has been shown in other cell types, including gliomas, that p38 is phosphorylated in response to CD95 ligation but how that affects cell death is yet unknown (Chen *et al*. 1998; Choi *et al*. 2001).

CD95-L is normally expressed at detectable levels in neurones, microglia and astrocytes, forming part of the so-called immunological barrier of the brain (Bechmann *et al*. 1999; Shin *et al*. 2002). CD95-L expression is up-regulated in reactive microglia by LPS infusion in the hippocampus (Terrazzino *et al*. 2002) in astrocytes and microglia in experimental autoimmune encephalomyelitis and in autopsy specimens after pontosubicular neurone necrosis (Kohji and Matsumoto 2000; van Landeghem *et al*. 2002), in neurones after an ischemic insult (Martin-Villalba *et al*. 1999), and in reactive astrocytes in both a model of anterograde degeneration (Bechmann *et al*. 2000), and in Alzheimer's disease (Ferrer *et al*. 2001). However, we have been unable to detect CD95-L in astrocytes by means of real-time RT–PCR (data not shown), possibly explaining why we observe very little paracrine cell death in our system.

Most immune-privileged tissues show a similar response as inflammatory astrocytes. Certain cell populations from the spleen, thyroid gland, testis, corpus luteum, follicles, kidneys, and pancreas can be sensitized to undergo apoptosis in response to CD95 when pre-treated with various combinations of TNF, IFN and IL-1 (Ortiz-Arduan *et al*. 1996; Harrison *et al*. 1998; Quirk *et al*. 2000; Riccioli *et al*. 2000; Moreau *et al*. 2001; Mezosi *et al*. 2002). We speculate that the sudden change in the sensitivity towards CD95-mediated death could be a general mechanism involved in the termination of an inflammatory response during a 'normal' localized inflammatory episode in an immune-privileged tissue. Such mechanism can also be seen in some other non-immune-privileged sites: when activated, $CD4^+$ and $CD8^+$ T-cells become highly sensitive toward CD95-L-induced apoptosis, while at the same time they express CD95-L. The T-cells auto-regulate their immune response by eliminating themselves by what is termed activation-induced cell death (AICD). A mechanism such as AICD of astrocytes would be beneficial to the CNS by limiting inflammation, but could be detrimental in diseases involving massive insult, such as in stroke or traumatic brain injury. This has major bearing on the designing and application of anti-inflammatory compounds, because the glial immune response may be prolonged by inhibition of a pathway (such as p38 MAPK) involved in AICD.

The exact mechanism surrounding the change in susceptibility towards cell death in astrocytes has not yet been elucidated, but we have shown that as astrocytes become activated, a shift in the expression of the apoptotic machinery is seen. We have also shown that TNF-α and IL-1β treatment is sufficient to make astrocytes undergo apoptosis in response to CD95-L, indicating that this process can be relevant in a number of neurodegenerative diseases where an increased expression of CD95/CD95-L is seen. The p38 MAPK is implicated as an important player that regulates both inflammation and cell death, but precisely how p38 MAPK is involved in apoptosis is yet unknown. Altogether we have characterized a new and robust murine model system allowing the study of inflammation and its involvement in astrocyte apoptosis.

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