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Modification of apoptosis-related genes and CD95 signaling in cytokine-treated astrocytes

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Inflammatory activation of astrocytes with a complete cytokine mix consisting of tumor necrosis factor, interleukin-1 and interferon-gamma renders these otherwise resistant cells highly susceptible to cell death induction via the CD95 pathway. In dying cells, we observed several classical apoptotic features such as chromatin condensation and cytoplasmic blebbing. These events were however quickly followed by a rupture of the cell membrane. For a screen of the transcriptional changes taking place during the transformation from a CD95L-resistant to a CD95L-sensitive cell, we employed a small custom-spotted oligonucleotide microarray. The significantly regulated mRNA species were then further analyzed over a 24 h period by quantitative PCR. We observed a complex pattern of transcriptional regulations showing changes of pro-apoptotic genes (*cd95, caspase-8, bid, bak, caspase-11*), as well as anti-apoptotic genes (*c-flip, iap-1, iap-2/3, bcl-2*). Since inflammatory astrocyte sensitization increased linearly with the time of cytokine-treatment the anti-apoptotic genes never seemed to be able to take over a dominating role in this model. Finally, the response of activated astrocytes to CD95 stimulation was compared with several other death-inducing stimuli. Cells became also more sensitive towards the classical apoptosis inducer staurosporine, but not towards necrotic stimuli such as H_2O_2 and N-Methyl-N′-nitro-N-nitrosoguanidine.

Keywords: Apoptosis / inflammation / CD95 / microarray / Astrocytes

Introduction

Reactive astrocytes are a hallmark of most, if not all neurodegenerative diseases. The term reactive astrocyte refers to hypertrophic astrocytes expressing high amounts of glial fibrillary acid protein (GFAP) [1]. These cells are in a highly activated state producing many factors normally associated with immune cells. One marker that is associated with a certain subset of the reactive astrocytes is CD95 (Fas/Apo-1) [2]. CD95 is a member of the TNF-receptor superfamily. Generally, binding of the CD95 ligand (CD95L) to its receptor induces apoptosis, but some cells are relatively resistant and proliferative or inflammatory responses are triggered instead [3−5].

CD95 mediated apoptosis appears to play a role in several neurodegenerative diseases. Mice deficient for CD95 (*lpr*) or with a defective CD95L (*gld*) are protected in an animal model of stroke [6, 7]. Mice deficient for TNF and IL-1 signaling are also protected in this model [7, 8] and mice defective for both TNF and CD95 signaling, show an even larger protection than mice deficient for only one of the two signaling events [7]. In autopsy cases of people who died from traumatic brain injury (TBI) the activated death inducing signaling complex (DISC), consisting of CD95, FADD, Caspase 8 and 10 can be found in the brain [9]. In a rat experimental model of TBI, CD95 and CD95L were upregulated on neurons, oligodendrocytes and astrocytes and it was found that these cells underwent apoptosis. Furthermore, activated caspase 3, 8 and 9 were found and BID was cleaved in neurons, oligodendrocytes and astrocytes undergoing apoptosis, suggesting that neurons and macroglia can express a complete apoptotic machinery [10−12].

Astrocytes are normally completely resistant to CD95 mediated cell death *in vitro* but it has been suggested that there is a link between inflammation and apoptosis. We have previously established an astrocyte model of inflammation, and found that CD95 was upregulated in murine astrocytes by cytokine treatment. The cells underwent a CD95 mediated

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apoptotic-like cell death with DISC-formation, caspase activation and chromatin condensation. The strength of the inflammatory signal correlated with cell death sensitization [13]. We have now examined the mode and kinetics of the CD95 mediated cell death and used a custom spotted oligonucleotide microarray to examine transcription of apoptosis related genes in the apoptosis-sensitization phase (during cytokine-treatment).

Materials and methods

Materials

Complete cytokine mix (CCM) contained 10 ng/mL murine TNF-α, 10 ng/mL murine IL-1β (Sigma-Aldrich, Copenhagen, Denmark), and 5 U/mL recombinant murine IFN-γ (RD Systems, Abingdon, UK). All laboratory chemicals were purchased from Sigma unless stated otherwise. Hamster IgG monoclonal anti-mouse CD95 antibody (JO-2) was obtained from (BD bioscience, Brøndby, Denmark).

Primary astrocyte cultures

Pregnant C57bl/6jbom mice were purchased from Harlan (Horst, Holland). 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 1−2 day old mice according to a slightly modified version of a protocol by *David E. Weinstein* [14]. In brief, brains from 6 pups were removed and kept on ice in a PBS buffer 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 DMEM (high glucose), 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. This medium was used for growing cells but for all experiments cells were changed into 2% FCS. All medium constituents 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 d, and subsequently twice a week. Cells were trypsinized and re-seeded after 14 d in primary culture and were always used for experiments 6−8 d after re-plating.

Viability assessment

To distinguish between viable, apoptotic and necrotic astrocytes, cells were stained with various combinations of the three fluorescent dyes Hoechst 33342, SYTOX or Calcein-AM by adding the dyes 5 min prior to counting. Hoechst 33342 (1 μg/mL final conc.) is membrane permeant and stains all nuclei, whereas SYTOX (2 μM) is membrane impermeant and thus only stains lysed cells. Calcein-AM is a membrane permeant dye that is loaded in viable cells but not in lysed cells. Cells with condensed nuclei but no SYTOX stain were classified as early apoptotic, cells with condensed nuclei + SYTOX stain as late apoptotic and cells with no condensed nucleus, but SYTOX stain as necrotic. At least 100 cells per field, 3 fields per well were counted using a standard fluorescent microscope.

LDH release was measured using a cytotoxicity detection kit (Roche) 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 minutes of incubation at room temperature the 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. A positive control routinely yielded a LDH release of 95%. This value was set to 100% release and all samples was normalized to the positive control.

Quantitative PCR

Cells stimulated in 10-cm dishes were washed once with PBS and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Purified RNA was treated with DNA-free™, DNase-1 (Ambion, Huntingdon, United Kingdom) according to the manufacturer's protocol. Total 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® PCR Master Mix kit (Applied Biosystems, Nærum, Denmark). Each reaction contained 2.5 μl cDNA of the 100 μL RT-product, 300 nM forward and reverse primers, 12.5 μL master mix and 7 μL water 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 (BIORAD, Hercules, CA). The program set-up was 10 min at 95 °C, 40 cycles of 15′ at 95 °C/L 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's 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 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 (Acc. No.: NM008084): 5′-TGC ACC ACC AAC TGC TTA G-3′, anti-sense: 5′-GGA TGC AGG GAT GAT GTT C-3′. BAK sense (NM_07823): 5′-AGC CGG GAA TGC CTA CGA ACT-3′, anti-sense: 5′-GCC CCA GCT GAT GCC ACT CT-3′. Bcl-2 sense (NM_09741): 5′-GGG TGA ACT GGG GGA GGA TTG T-3′, anti-sense: 5′-TGT GCA GAT GCC GGT TCA GGT-3′. BID sense (NM_07544): 5′-GGC CAG CCG CTC CTT CAA C-3′, anti-sense: 5′-GGG CGA GAT GTC TGG CAA TGT-3′. Caspase 8 sense (AF_67834): 5′-CAG AAA ATA ACT TGG AAA CCC TAA AAT CA-3′, anti-sense: 5′-GTG GCA ACT CTT CCC TTC CTT CAA-3′. Caspase 11 sense (NM_07609): 5′-ACT GAG GTA TGG GGC TAA CTT TGA CA-3′, anti-sense 5′-ATC TCT GAC TCC ATG CCC TCT GCT-3′. CD95 sense (NM_07987): 5′-GCT GAG GAG GCG GGT TCG TG -3′, anti-sense 5′-CAT GGG GCG CAG GTT GGT G-3′. cFLIP sense (NM_09805): 5′-CCG CCG TGC CTT TAC CTC TC-3′, anti-sense 5′-CAC ACT CTG GCA AAA CAT CCG ACT-3′. IAP-1 sense (NM_07464): 5′-CCC CCG GAG ATC AGA GGT CAT T-3′, anti-sense 5′-GGC TTC TGG TCG GTT TTA CTG CTA-3′.IAP-2 sense (NM_07465) (IAP2/3): 5′-GTC TGG CTC GTG CTG GCT TTT ATT AT-3′, anti-sense 5′-TGT CCC CTT GTT TCC AGT TAT CCA-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/).

Transcript analysis by oligonucleotide hybridization analysis

We selected a short list of mouse genes known to be upregulated in astrocyte inflammation. For each of these genes one oligonucleotide (40−50mer) was designed by MWG (MWG, Ebersberg, Germany) using their proprietary Oligo4array software and CodeSeq database, which selects the oligos preferentially from the 3′-region of each coding sequence. Furthermore, each oligomer was scrutinized to meet physicochemical parameters (like melting temperature, self-complementarity, secondary structure etc.) and extensively tested to minimize cross-hybridization to other sequences of the mouse genome in silico. All oligos were synthesized using MWG's HPSF technology followed by MALDI-TOF quality control.

The oligomeres were spotted onto activated glass slides (Pan Epoxy, MWG or CodeLink, Amersham) using a 417 Affymetrix 'ring and pin' spotter. Sample preparation and labeling was carried out as described by Joseph DeRisi (www.microarrays.org/protocols.html), a protocol, derived from (*Hughes* et al. [15]). In brief: 12−15 μg of total RNA were reverse transcribed using random hexamere and dT16 primers and Superscript II reverse transcriptase (Invitrogene), incorporating amino-allyl dUTP into the 1st strand cDNA. After the cDNA synthesis, the remaining RNA was hydrolyzed and after a clean-up step (Microcon-30 spin filters, Millipore), Cy-3 or Cy-5 dye esters respectively were coupled to the cDNA samples. Excessive dye and buffer were removed with QiaQuick PCR purification columns (Quiagen) and the eluates were concentrated with Microcon-30 spin filters. The hybridization mixture contained the Cy-labeled cDNAs in hybridization buffer (50% formamide, 6x SSC, 5x Denhardts, 0.5% SDS and 50 mM sodium phosphate, pH = 8) and was denatured for 5 min before incubation on the slides for 16 h at 42 °C. Washing was carried out in three steps of increasing stringency: 2x SSC, 0.1% SDS followed by 1x SSC, 0.01% SDS and 0.5x SSC (all solutions were preheated to 30 °C). Finally, each slide was spun dry and scanned in a 428 Affymetrix confocal laser scanner at three different intensities (photo multiplier gains).

The microarrays were analyzed using ImaGene 4.2 (BioDiscovery) for spot location, array alignment and background subtraction. Signal intensities for individual spots were adjusted for local background. Microsoft Excel was used for further statistical analysis of the ImaGene output files. E.g. Cy3/Cy5 ratio normalization was carried out by multiplying each ratio value with a scaling factor, which was defined as the ratio of the overall signal intensity of the Cy5 versus Cy3 channel [16]. Each microarray experiment was performed at least twice independently. To further account for bias introduced by dye bleaching or labeling, each experiment was carried out as dye-swap experiment with the resulting ratio value being the arithmetical mean from two slides of opposite labeled sample pairs. Genes with very low signal intensities (less than 5-fold of the background) were excluded from the analysis.

Statistics

All results are displayed as mean ± SD of triplicates. Array data are displayed as means of duplicates (dyeswap) ± SD (= range). Only data from experiments confirmed at least once are displayed. One-way analysis of variance followed by the Tukey-Kramers test was used for statistical evaluation. (*: $P < 0.05$, **: $P < 0.01$).

Results

We have previously shown that astrocytes become sensitive to CD95-driven cell death depending on the inflammatory state of the cell [13]. Now, we further examined the mode of cell death in non-fixed cytokine stimulated astrocytes. The cells were treated according to the stimulation scheme shown in (Fig. 1A) and stained with H-33342, a membrane-permeable chromatin dye and SYTOX, a membrane-impermeable dye. We counted cells at different times after the addition of CD95-ligand and observed an almost linear induction of cell death over 24 hours after ligand addition (Fig. 1B). The mode of cell death appeared to be apoptosis-like [17] since all cells dying displayed condensed chromatin as seen by H-33342 staining. However, the vast majority of the astrocytes with condensed nuclei were also SYTOX positive (Fig. 1B + 2B, D, F). Only a small population of non-lysed cells with condensed chromatin was observed at any given time point (Fig. 1B + 2A, C, E).In contrast, cells were never SYTOX positive, when they had non-condensed nuclei. This suggests that chromatin condensation is very closely followed by rupture of the plasma membrane. Calcein-AM staining of the cells allowed us to study the gross cytoplasmic morphology of our cells. Viable cells were characterized by a strong cytoplasmic stain and a flat polygonal structure with fine irregular projections (Fig. 3C). CCM-stimulated cells showed many long, brightly stained, streaky cytoplasmic structures and pronounced projections (data not shown). When cells were stimulated with CCM/CD95L, dying cells first lost the fine projections and structures, then rounded off, and finally showed prominent cytoplasmic blebs (Figs.

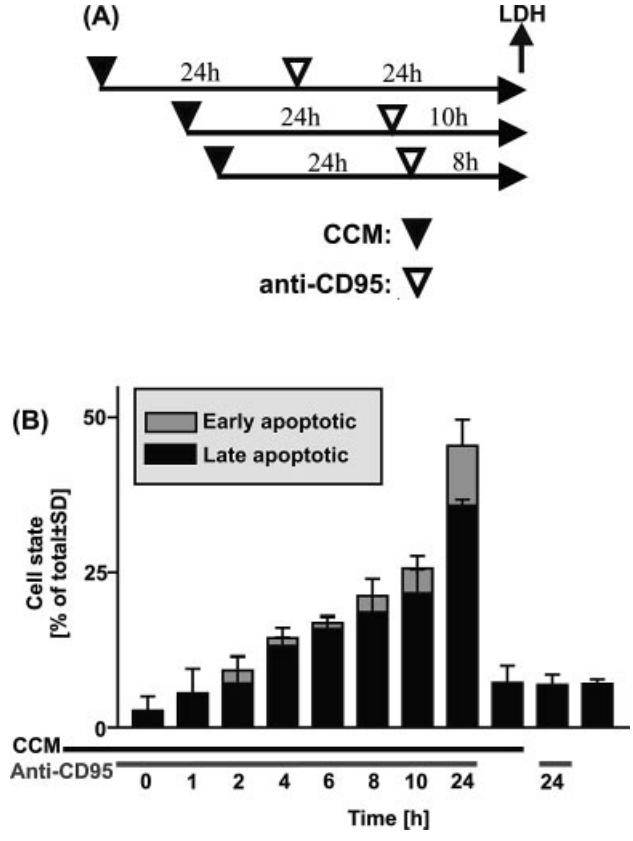

Fig. 1. Cytokine/CD95L-treated astrocytes die in an apoptosis-like fashion.
The α-CD95 antibody was added at various times to wells pre-stimulated for 24 h with CCM (see schematic diagram). To distinguish between viable, early apoptotic and late apoptotic astrocytes, cells were stained with various combinations of the two fluorescent dyes Hoechst 33342 or SYTOX. Cells with condensed or fragmented nucleus were scored as early apoptotic cells if SYTOX negative and as late apoptotic if SYTOX positive. Data are displayed as the means of three wells ± SD.

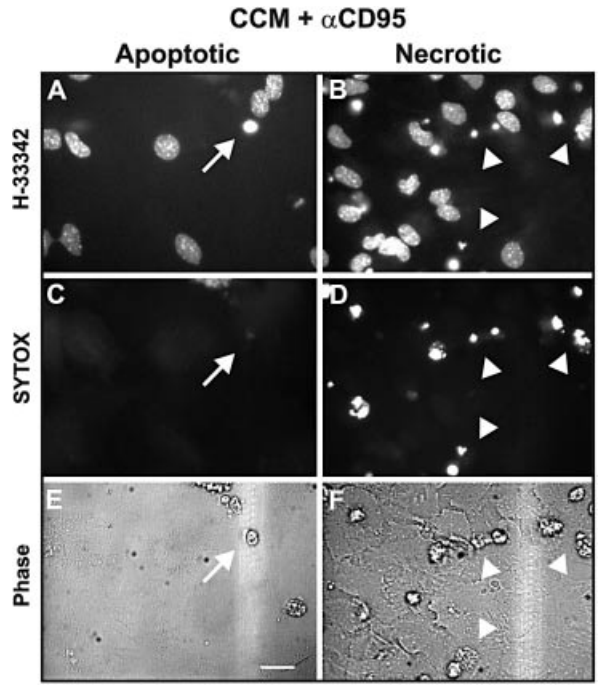

Fig. 2. Nuclear morphology of dying astrocytes.
α-CD95 antibody was added to wells pre-stimulated for 24 h with CCM. After further 24 h cells were stained with SYTOX (2 μM) and H-33342 (1 μg/mL). A, C, E: Images taken from the same field. The arrow indicates a cell looking rounded off in phase contrast (phase), with condensed chromatin, but intact plasma membrane (SYTOX-negative). B, D, F: Images taken from the same field. Examples of late apoptotic cells (here labeled for simplicity as necrotic). Arrowheads marks cells that have an apoptotic nucleus, cytoplasmic blebs and a broken plasma membrane. Scale bar = 20 μM.

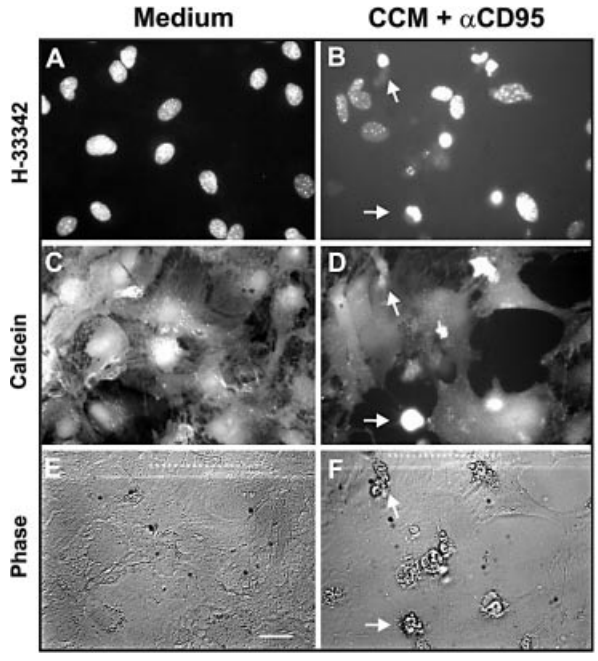

Fig. 3. Morphological examination of dying astrocytes.
α-CD95 antibody was added to wells pre-stimulated for 24 h with CCM. After further 24 h, cell morphology was examined by staining the cells with Calcein-AM (5 μM) and H-33342 (1 μg/mL). Images were taken from the same field. Arrows depict blebbing apoptotic cells. Scale bar = 20 μM.

3B, D, F). The surviving CCM/CD95L treated cells showed morphology similar to CCM-treated cells. The apoptotic-like cell death sequence was rapidly followed by rupture of the outer cell membrane.

To further understand the sensitization process, we examined the gene regulations taking place after 4 h of cytokine treatment. We used a small custom-spotted oligonucleotide microarray to examine a representative set of apoptosis related genes. Besides a strong up-regulation of CD95 as described earlier [13], various other pro-apoptotic genes were regulated (Fig. 4). Two BH3-only proteins, Bid and Bak were upregulated, as was caspase 11. All of the regulations were examined by quantitative PCR and followed over a time course of 24 h. Most of the regulated genes reached peak mRNA levels after 4-8 h of cytokine-treatment, and the expression was decreasing towards baseline at 24 h. However both BID and Caspase 11 remained highly expressed (Fig. 5) over the entire experimental period.

Much to our surprise we also found a strong upregulation of anti-apoptotic genes. Several members of the inhibitors of apoptosis protein-family (IAP) showed increased expression (Birc-1a, 2, 3) and also cFLIP was upregulated. The regulation of cFLIP could not be confirmed with the set of primers we had initially used [13] due to a low efficiency so a new set of primers was designed and a strong signal and upregulation was confirmed (Fig. 6A). Regulations of the IAPs were also confirmed with a primer set for Birc1 and a primer set recognizing a region with equal length and 90% homology in Birc2 and 3 (Fig. 6 B, C).

Using the microarray platform, Bcl-2 was the only gene that was found downregulated. This downregulation was observed immediately after cytokine stimulation, lasting throughout the experiment (Fig. 6D), as measured by PCR. Non-regulated, apoptosis associated genes were e.g. Caspase 1-3, BAD, BAX, BIRC1e, BIRC4 and the important astrocytic apoptosis regulator PEA-15 [18] that displayed a particularly high abundance (data not shown).

In order to test whether the anti-apoptotic genes would predominate later in the cytokine/apoptosis-sensitization phase of our treatment regiment we tried increasing the cytokine pre-stimulation time from 24 h up to 96 h. Such a delayed counter-regulatory response involving pro and anti-apoptotic

Fold induction: > 5 ■:2-5 ■:0.5-2 ■:< 0.50 ■	Fold induction
gene name/product	Ratio CCM / CTR
FAS, CD95	4.1 ± 0.2
c-Flip	2.0 ± 0.2
Caspase 1	1.2 ± 0.2
Caspase 2	0.9 ± 0.2
Caspase 3	1.0 ± 0.8
Caspase 8	0.9 ± 0.0
Caspase 9	0.6 ± 0.6
Caspase 11	7.2 ± 7.3
Bcl2-like	1.3 ± 0.0
Bcl2, B-cell leukemia/lymphoma 2	0.4 ± 0.1
bcl2-related protein a1	0.8 ± 1.0
Bcl2a1a	1.0 ± 0.2
Bim, Bcl2 interacting mediator of cell death	1.9 ± 2.3
Bad, Bcl-associated death promoter	0.9 ± 0.0
Bax, Bcl2-associated X protein	0.8 ± 0.0
Bid, BH3 interacting domain death agonist	2.7 ± 1.3
Bak, N-Bak, Bak1, BCL2-antagonist/killer 1	2.3 ± 0.4
Baculoviral IAP repeat-containing 1a	4.1 ± 4.8
Baculoviral IAP repeat-containing 1e	0.9 ± 0.2
Baculoviral IAP repeat-containing 2	10.0 ± 4.0
Baculoviral IAP repeat-containing 3	3.1 ± 2.9
Baculoviral IAP repeat-containing 4	1.1 ± 0.5
p53, transformation related protein 53	1.6 ± 0.9
Phosphoprotein enriched in astrocytes 15	0.8 ± 0.1

Fig. 4. Regulation of apoptosis related genes by CCM.
Cells were treated for 4 h with CCM. Total RNA was purified and labeled cDNA was prepared. Different competitive cDNA hybridization experiments were done on a small custom array for CCM vs. Control (CTR), Data presented are the average of two dye-swap hybridizations for one cell preparation. All data are presented as means of duplicates ± Range.

genetic changes, has been described for systems, for instance during oncogenic transformation [19]. However, we saw a direct correlation between the time cells had been activated by cytokines and how sensitive they became to undergo apoptosis (Fig. 7). Cytokine treatment itself was slightly toxic to the cells but this toxicity never surpassed 10% even after 5 days of treatment (Fig. 7). The specificity of the JO-2 antibody has previously been confirmed by heat-inactivating the antibody and by using an isotypic antibody [13].

The CD95-activating antibody JO-2, induce cell death of CCM treated astrocytes in a linear, concentration-dependent fashion, whereas control cells were not affect by anti-CD95 treatment (Fig. 8A). Thus the CCM treated astrocytes were >100x more sensitive to CD95 stimulation.To further examine the functional consequences resulting from the complex pattern of gene regulation observed, we tested whether astrocytes changed their sensitivity towards other death inducing stimuli. Astrocytes pre-treated with CCM for 24 h became approximately 4 times (EC_{50} *(control)*: 3.3 μM vs. EC_{50}

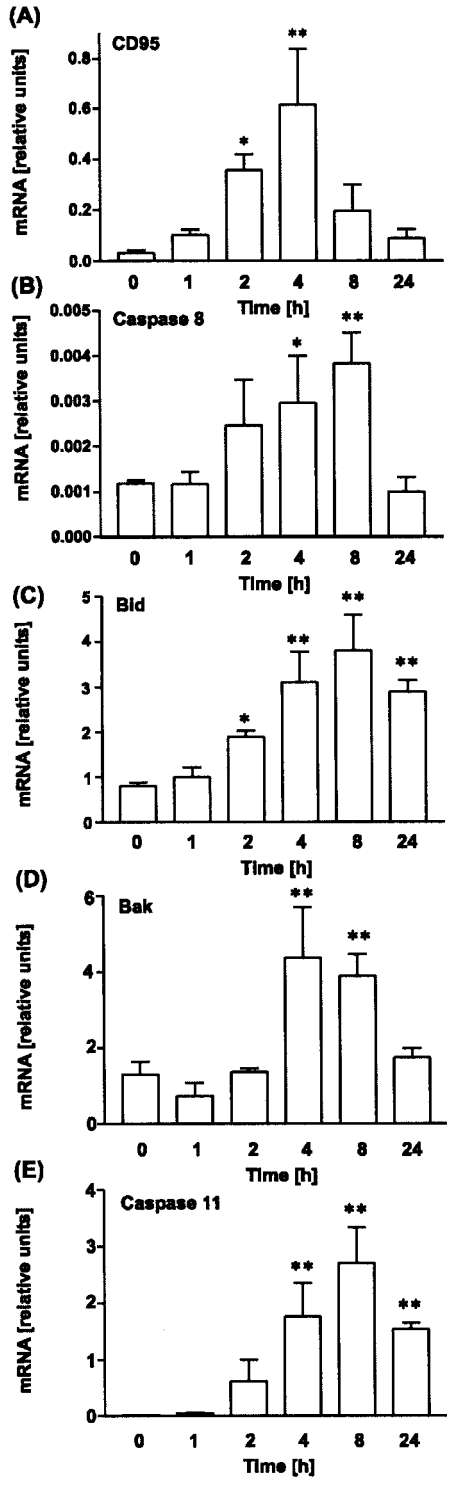

Fig. 5. Change of the pro-apoptotic signaling system by CCM.
Cells were stimulated with CCM for the times indicated; total RNA was extracted and **A.** CD95 **B.** Caspase 8 **C:** BID **D.** Bak **E.** caspase 11 mRNA was quantified by real-time RT-PCR. Data are displayed as means of triplicates ± SD and are displayed as the ratio of gene vs. GAPDH expression.

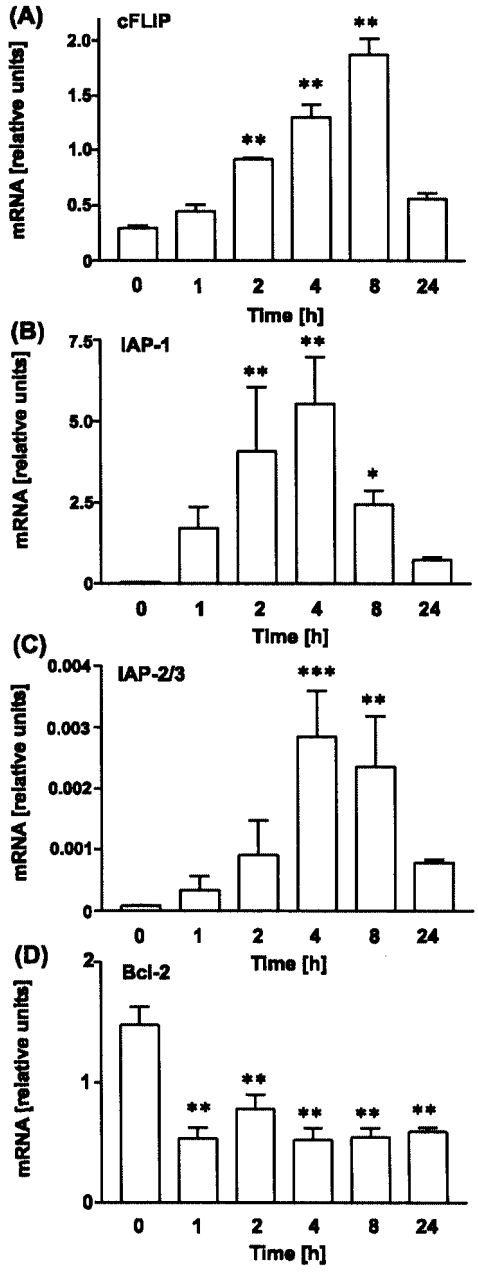

Fig. 6. Change of the anti-apoptotic signaling system by CCM.
Cells were stimulated with CCM for the times indicated; total RNA was extracted and **A.** cFLIP **B.** BIRC-1 **C.** BIRC-2/3 **D.** Bcl-2 mRNA was quantified by real-time RT-PCR. Data are displayed as means of triplicates ± SD and are "ratio of gene vs. GAPDH".

(CCM): 0.8 μM) more sensitive towards staurosporine induced cell death, as measured by the release of LDH (Fig. 8B, Tab. 1). However, this was not a general death sensitization since astrocytes appeared to be slightly protected by

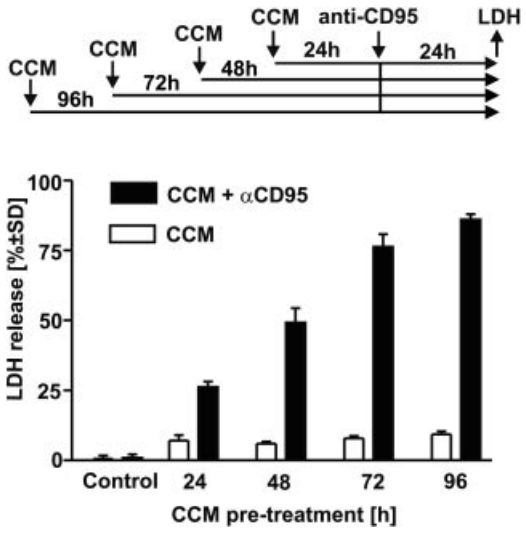

Fig. 7. Time course of cytokine-mediated apoptosis sensitization.
α-CD95 antibody was added to cells pre-stimulated for 0-96 h with CCM (see schematic diagram). After 24 h LDH release was measured. Data are displayed as mean LDH release of triplicates ± SD.

CCM-treatment against the necrosis-inducing stimulus H_2O_2 *(control)*: 92 μM vs. EC_{50} *(CCM)*: 169 μM) (Fig. 8B). A similar tendency of slight protection was also observed with CCM-treated astrocytes when exposed to N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Fig. 8B).

Discussion

In this study we present data showing that reactive astrocytes undergo an apoptotic-like cell death after treatment with CD95L and that the sensitivity increases linearly with the time of cytokine pre-treatment. Furthermore, we show a complete time course of expressions of apoptosis regulating genes, which form a complex pattern as basis for changed cellular sensitivity. We examined the cell death and found that astrocytes die with a slow kinetics after CD95 ligation. Most of the cells had condensed nuclei and perforated cell membranes. This could suggest that the cells undergo an apoptotic-like cell death characterized by quick rupture of the outer membrane following nuclear condensation [17]. An alternative explanation could be that there are two populations of cells, one undergoing an apoptosis-like cell death program at earlier times and a second one undergoing apoptosis at later times. This would be in agreement with the fact that caspase activity was only observed after CD95-ligation at a time when a certain population of cells had already died [13]. The population of cells dying was increased after prolonged cytokine pre-treatment followed by CD95L-treatment. However, cytokine treatment alone was not signif-

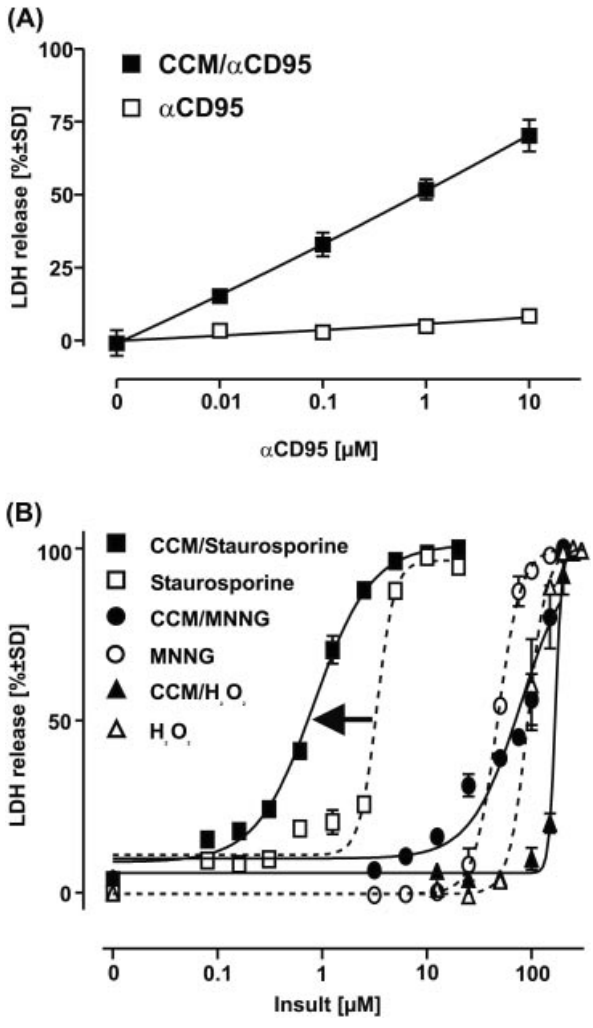

Fig. 8. Cell death sensitivity of reactive astrocytes. **A.** α-CD95 antibody was added to cells pre-stimulated for 24 h with CCM. After 24 h LDH release was measured. **B.** Different death-inducing stimuli (Staurosporine (squares), hydrogenperoxide (triangles) or MNNG (circles)) were added to cells pre-stimulated for 24 h with CCM (solid symbols and lines) and to non-stimulated cells (open symbols and dotted lines) in parallel. After 24 h, LDH release was measured. Data are displayed as mean LDH release of triplicates ± SD.

icantly toxic to the cells. It has been shown in rat astrocytes that a "cytokine-cocktail" similar to ours induced caspase 11 and that this lead to a CD95-independent cell-death on its own [20]. We did see a strong upregulation of caspase 11 (on the transcriptional level), but in our cell system the cell-viability was not significantly affected by the cytokine treatment. This is possibly due to an upregulation of the inhibitors of apoptosis proteins and a resulting, balance of pro and anti-apoptotic factors.

The transcriptional profile of our cells strongly suggests that the balance of Bcl-2 family proteins may play a role in the apoptosis program. Bid, a link between the CD95 pathway and the mitochondria was strongly upregulated as was Bak its interaction partner. Bcl-2 (an antagonist of Bak) was downregulated. This is corroborated by our data showing that astrocytes become more susceptible to staurosporine induced cell death after CCM treatment. Astrocytes are normally more resistant than neurons to this fungal toxin that induces apoptosis via the mitochondrial pathway, but after CCM-treatment astrocytes show approximately the same sensitivity as neurons. However, also at the level of the receptor, strong upregulation of different factors was observed. CD95, caspase 8 and cFLIP were all upregulated. This confirms earlier reports from related models, which found an interaction of inflammatory pathways and the CD95 death receptor system [20−22]. Caspase 8 has previously been shown to be upregulated by PCR [13], but the sensitivity of our array was not sufficient to pick up the regulation most likely due to a low expression of caspase 8 (Fig. 4B).

Cytokine treatment does not appear to render astrocytes sensitive to all types of cell death, but rather switch the death sensitivity towards apoptosis. This is suggested by the fact that sensitivity towards the classical apoptosis inducers CD95 and staurosporine goes up, while the sensitivity towards H_2O_2 and MNNG, two inducers of PARP-1 mediated necrotic cell death [23], goes down. An alternative explanation, at least in the case of H_2O_2 is that cytokine-stimulated astrocytes upregulate genes involved in an anti-oxidant response (manuscript submitted).

In general, CD95/CD95L expression and function seems to be linked very much to inflammation within the brain. CD95-ligation on astrocytes induces release of chemokines or cytokines [5, 21, 24] and cytokine treatment makes the cells sensitive to apoptosis [5, 13, 21, 25, 26]. The role of upregulation of the CD95 system on astrocytes is frequently neglected in the literature on neurodegenerative diseases. We compiled here the extensive evidence on the role of CD95/CD95L within the brain as a discussion background for our findings. Expression of both CD95 and CD95L is increased on astrocytes, in numerous neurodegenerative diseases with inflammatory components (see Tabs. 1 and 2). Many reports show caspase activity and cell death but there are also few reports of caspase activation without the presence of cell death (Creutzfeld-Jakobs disease). There is convincing evidence for a direct involvement of the CD95/CD95L system in acute inflammatory diseases such as TBI and stroke [6, 7, 10−12] but there is also mounting evidence in chronic inflammatory disease such as MS and Alzheimer's disease (see Tab. 1). It is more difficult to assess cell death in a slowly progressing disease, but none the less it would be interesting to see how CD95 inactivation would affect the course of disease in an Alzheimer's model.

In summary, our data confirm the strong link between inflammation and apoptosis in primary astrocytes cultures. Fur-

Tab. 1. Regulation of CD95 in CNS-related diseases.

	Cell-type (CD95 regulation)	Species	Comments	Ref.
Alzheimer's disease	Neurons ↑ , Astrocytes ↑ , White matter glial cells ↑	hu, mu	Caspase 8 activation, neurodegeneration and increased CD95 expression is seen around plaques. Neurons purified from *Lpr* and *gld* mice are protected from $A\beta_{1-42}$ *in vitro* and a blockade of CD95L shedding by inhibition of metalloproteinases exacerbate the $A\beta_{1-42}$ induced damage.	[2, 27–31]
Down syndrome and Pick's disease	Astrocytes ↑ , Neurons ↑ (Pick's not specified)	hu	Frontal/temporal lobe, Cerebellum	[32, 33]
Ischemia/stroke	Neurons ↑ , mRNA ↑	ra, mu, hu	*Lpr* mice have decreased infarct volume (MCAO). Hypothermia decrease infarct, CD95 and Caspase 3 expression in the penumbra (MCAO). DISC formation (4VO). Global cerebral ischemia (cardiac arrest) decreased overall CD95 levels. Human CSF levels of soluble CD95 (sCD95) negatively correlated with infarct volume, IL-1, IL-6, IL-10 and GM-CSF.	[34–39]
Pontosubicular neuron necrosis	Neurons ↑	hu	TUNEL stain positive neurons	[40]
Kainate or Lithium-pilocarpine-induced seizures	Neurons ↑	ra	Co-localises with TUNEL, p53, apoptosis-linked markers and NeuN	[41, 42]
Traumatic brain injury	Astrocytes ↑ , Oligodendrocytes ↑ , Neurons ↑	hu, mu, ra	DISC formation (human), Caspase 3, 8, 9, 10 activation, Bid cleavage and increased CSF levels of sCD95.	[9, 11, 12, 43]
Spinal chord compression	Not specified ↑	ra	CD95 positive glia cells undergo apoptosis and have activated caspase 3, 8.	[44, 45]
MS/EAE	Microglia ↑ , Oligodendrocytes ↑ , Endothelial cells ↑ Astrocytes ↑	hu, mu	CD95 is up during early acute phase of EAE. *Lpr* and *gld* mice show a monophasic course (wt show chronic/relapsing) and lower incidence of disease. Macrophages that have ingested CD95-positive cells are found.	[2, 26, 46–48]
Virus induced demyelination.	Astrocytes ↑	mu	Astrocytes infected with Theiler's murine encephalomyelitis virus can present antigens to CD4+ cells that release IFN. This sensitises astrocytes that die by CD95 driven apoptosis *in vitro* and *in vivo*.	[49]
Bacterial meningitis	Not specified ↑	hu	Increased levels of sCD95 in CSF of patients with bacterial but not viral meningitis or MS.	[50]
Chronic CNS infectious diseases	Not specified ↑	hu	HIV, measles infection and JC-papovavirus infection	[2]
Huntington's disease	Neurons ↓ , Astrocytes ↑	hu		[51]
Parkinson's disease, Diffuse lewy body disease	Neurons (PD) ↓ , Not specified (DLBD) ↑ , Astrocytes ↑	hu	Soluble CD95 is significantly increased in caudate and putamen and correlate with TNF, IL-1 and IL-6	[2, 33, 51, 52]
Creutzfeldt-Jakob disease	Purkinje cells ↑ , Golgi cells ↑	hu	Caspase 3, ERK activation, BAX, Bcl, N-myc upregulated in Purkinje cells. No cell death but loss of dendritic spines.	[53]
Anterograde degeneration	Astrocytes ↑	ra	No Tunel positive cells	[54]
Inflammation	Microglia ↑	mu	After repeated i.p. administration of LPS or treatment with IFN-γ and TNF-α	[55, 56]
ALS/development	Motor neurons	mu, hu	Increased at time of motorneuron pruning during development and in cells from SOD1 mutant mice. In vitro, neurons become sensitive to CD95 ligation and cell death can be blocked by caspase 8, p38 SAPK or nNOS inhibition.	[57–59]
Others	Not specified ↑	hu	Pick's disease, progressive supranuclear palsy, multiple system atrophy	[33]

Tab. 2. Regulation of CD95L in CNS-related diseases.

	Cell-type (CD95L regulation)	Species	Comments	Ref.
Alzheimer's disease	Neurons ↑ , Astrocytes ↑	hu, mu	Surrounding plaques	[27, 30]
Ischemia/stroke	Neurons ↑ , Not specified ↑ , Microglia ↑	mu, ra	*Lpr, gld,* and TNF k.o. mice have decreased infarct volume. Oxidative stress upregulate CD95L *in vitro*.	[6, 7, 36, 39, 60, 61]
Pontosubicular neuron necrosis	Astrocytes ↑ , Microglia ↑	hu	[40]	
Traumatic brain injury	Astrocytes ↑ , Neurons ↑ , Microglia ↑ , CSF levels ↑	ra, mu, hu	[9, 10]	
Spinal chord compression	Not specified ↑ microglia ↑ , astrocytes ↑	ra	Oligodendrocytes, astrocytes and microglia appear to be CD95 sensitive	[44, 45]
MS/EAE	Microglia ↑ astrocytes ↑ oligodendrocytes ↑	hu, mu	In MS or during early acute phase of EAE, co-localised with TUNEL- and CD95-positive glial cells.	[2, 46−48]
Virus induced demyelination.	Astrocytes ↑	mu	Astrocytes infected with Theiler's murine encephalomyelitis virus upregulate CD95L.	[49]
Huntington's disease	Neurons ↓ , Astrocytes ↑	hu	Reactive astrocytes	[51]
Parkinson's disease	Neurons ↓ , Astrocytes ↑	hu	Reactive astrocytes	[51]
Creutzfeldt-Jakob disease	Purkinje cells ↑ , reactive astrocytes ↑	hu	Also in reactive astrocytes in Olivopontocerebellar atrophy ↑	[53]
Anterograde degeneration	Astrocytes ↑	ra	No TUNEL positive cells	[54]
HIV encephalitis	Whole brain ↑	hu	Only in HIV patients with encephalitis, on astrocytes infected *in vitro*.	[62]
Inflammation	Microglia ↑	mu	After repeated i.p. administration of LPS	[55]
Others	Motor neurons ↑	mu	During development	[59]

thermore, we present evidence suggesting that astrocytes respond to cytokine treatment by upregulating pro- and anti-apoptotic genes. In the case of CD95 stimulation, the changed expression clearly tips the death-life balance towards apoptotic-like deaths.

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