Oxidative stress and hypoxia/reoxygenation trigger CD95 (APO-1/Fas) ligand expression in microglial cells

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Abstract Apoptosis plays an important role in neurodegeneration, although the mechanisms and mediators in the brain are largely unknown. Because microglial cells have been suggested to contribute to apoptosis in neurological disorders, we investigated the expression of the death ligand CD95L in this cell type. We found that, compared to classical mediators of microglial activation, the most potent inducer of CD95L was oxidative stress. Exposure of microglial cells to H_2O_2 or paraquat rapidly triggered CD95L mRNA and protein expression, associated with the activation of transcription factor NF-κB. Enhanced expression of CD95L was further found following exposure of cells to hypoxia and subsequent reoxygenation. Our results indicate a potential role of CD95L in oxidative stress-mediated cell death, ischemia/reperfusion and other diseases with a disturbed redox balance.

Key words: Apoptosis; CD95 (APO-1/Fas) ligand; Hypoxia; Nuclear factor-κB; Reactive oxygen intermediate

1. Introduction

CD95 (APO-1/Fas) belongs to the tumor necrosis factor (TNF) receptor superfamily whose members have a wide range of functions related to cell survival, differentiation and regulation of the immune response. It was the first receptor whose role seemed to be largely restricted to induction of apoptosis. Upon crosslinking by agonistic antibodies or its cognate ligand, CD95 mediates apoptosis in sensitive target cells of different origin [1]. The ligand CD95L is a type II transmembrane protein of 40–42 kDa which, after cleavage by a metalloprotease, can also occur as a soluble form. Expression of CD95L has been mainly detected in activated T lymphocytes, although other cell types such as NK cells, monocytes and a number of non-lymphoid cells can also express this cytokine. Recently, it has been demonstrated that CD95L-induced apoptosis involves the activation of death proteases of the caspase family [1]. Ligation of CD95 triggers its association with the cytoplasmic adapter protein FADD through homophilic interaction with the so-called death domain. This event in turn recruits caspase-8 which is assumed to cleave and activate other caspases and death substrates.

The function of the CD95 system has mainly been investigated in the immune system, but it is becoming evident that CD95 also plays a role in other tissues. Naturally occurring mouse strains, which lack expression of either functional CD95 or its ligand, exhibit severe autoimmune disorders, indicating a major role for CD95 in regulation of the immune response and maintenance of self-tolerance. Whereas CD95-mediated apoptosis is mainly controlled by inducible CD95L expression, some cells constitutively express the ligand. High amounts of CD95L are detected, for instance, in Sertoli cells of the testis and epithelial cells of the anterior eye chamber [2,3]. This led to the proposal that CD95 accounts for maintaining the immune privilege of these organs by preventing activated lymphocytes from tissue infiltration. Constitutive expression of CD95L has further been found in tumor cells of different origin, suggesting that similar mechanisms may allow tumor cells to escape the host's immune surveillance [1].

Recent studies provided evidence that CD95 participates in a number of diverse apoptosis settings. Thus, CD95 was suggested to play a role in cell death induced upon viral infection, such as HIV-1-induced T lymphocyte apoptosis and HBV-induced death of hepatocytes [4,5]. In addition, apoptosis evoked by different physiochemical stresses including the exposure of cells to anti-tumor drugs or irradiation, may be at least partially mediated by the CD95 system [6–8]. It has been observed that in these cases cellular stress leads to the upregulation of CD95L expression, which then upon binding to its receptor induces cell death.

Important mediators of different physical and chemical stresses comprise reactive oxygen intermediates (ROIs) including hydroxyl radical, superoxide anion and H_2O_2. Although ROI-mediated damage has been commonly considered as necrosis, recent observations suggest a potential role of apoptosis under these conditions. Thus, excessive formation of ROIs as well as the depletion of cellular antioxidants resulted in apoptosis in different cell types [9–11]. Furthermore, a variety of antioxidant compounds as well as the overexpression of antioxidant enzymes inhibited cell death induced by apoptotic agents [9]. However, although ROIs may be important, they do not exert a common and obligatory role in all forms of apoptosis. CD95-mediated apoptosis, for instance, is not or only weakly diminished by treatment of cells with antioxidants [12].

ROIs have been intimately implicated in the pathogenesis of different neurodegenerative diseases [13–15]. Biochemical and morphological alterations suggested that neuronal damage during cerebral ischemia proceeds, at least partially, by apoptosis [16–18]. Ischemia/reperfusion of brain is usually associated with increased ROI formation and loss of endogenous antioxidants [19]. It is therefore likely that ROIs either directly or indirectly contribute to neuronal damage. An indirect effect may be exerted by ROI-induced novel gene expression. In this respect, ROIs can activate redox-sensitive transcription factors, such as NF-κB and AP-1, which may

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Abbreviations: CD95L, CD95 ligand; EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte/macrophage colony stimulating factor; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; ROI, reactive oxygen intermediate; TNF, tumor necrosis factor

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control the expression of death genes [20]. Supportive for the requirement of de novo gene expression is the observation that inhibitors of RNA and protein synthesis markedly attenuate neuronal damage produced by ischemia/reperfusion [21,22]. The mechanism of ischemic neuronal damage and the genes involved, however, are unknown.

It is well established that microglia, which exert a primary role as brain-resident macrophages, are involved in inflammatory and destructive processes [23]. During ischemia/reperfusion, activation of microglia coincides with the onset of DNA degradation in regions of selective neuronal loss, thus suggesting a possible role in neuronal cell death [24]. It is known that activated microglia can produce several neurotoxins, such as ROIs, nitric oxide, glutamate, cytokines or proteases which could contribute to neuronal cell death [24–26].

Very little information exists about the function of CD95 and its ligand in physiological and pathological processes in the brain. In view of increasing evidence for participation of microglia in neurological disorders, we here investigated the expression and regulation of CD95L in microglial cells. Our experiments revealed that reagents inducing oxidative stress were among the most potent activators of CD95L expression. We further found that hypoxia followed by transient reoxygenation strongly upregulated CD95L expression. These findings suggest that CD95L may potentially contribute to ROI-mediated damage of neuronal or other cell types in diseases characterized by an imbalance of redox homeostasis.

2. Materials and methods

2.1. Cell culture and reagents

N9 mouse microglial cells were grown in RPMI-1640 medium supplemented with 10% FCS and 2 mM glutamine, and passaged by trypsinization. Similar to primary microglia, cells express the phenotypic markers F4/80, Mac-1, FcR, and are able to produce IL-1α, IL-6, and TNF [27,28]. The reagents paraquat, menadione, doxorubicin and H_2O_2 as well as lipopolysaccharide (LPS) were purchased from Sigma (Deisenhofen, Germany). TNF and GM-CSF were obtained from Knoll AG (Ludwigshafen, Germany) and Genzyme (Cambridge, MA, USA), respectively. For hypoxia experiments, cells were subjected to mild controlled hypoxia in an atmosphere of 200 ppm oxygen, a condition that does not inhibit respiration and normal adenylate energy charge [29]. Cells were placed at 37°C in a sealed metal chamber, which was flushed with a water-saturated gas mixture of 0.02% O_2, 5% CO_2 and the balance made up by high purity argon gas. The pH of the medium remained constant throughout the experiments. A Clark-type electrode was placed into a medium-filled culture dish to determine the actual oxygen pressure during the experiments. To reoxygenate hypoxic cultures, cells were transferred into 1/4 volume of medium saturated with 95% O_2 and 5% CO_2. In order to avoid artifacts after treatment, cell culture dishes were quickly removed and the cells were immediately lysed in extraction buffer as described below.

2.2. Detection of NF-κB DNA binding

Cells were plated at 1×10^6/well in six-well plates and treated for 45 min with the indicated concentrations of H_2O_2. Total cell extracts for electrophoretic mobility shift assays (EMSAs) were prepared essentially as described [30]. Equal amounts of the extracts (about 10 μg crude protein) were incubated with the ^{32}P-labeled NF-κB-specific oligonucleotide. After 15 min binding reaction at room temperature, samples were loaded on a 4% non-denaturing polyacrylamide gel. The oligonucleotide with a high-affinity NF-κB binding motif (Promega, Heidelberg, Germany) was labeled using γ-[^{32}P]-ATP (3000 Ci/mmol; Amersham-Buchler, Braunschweig, Germany) and T4 polynucleotide kinase (Boehringer, Mannheim, Germany).

2.3. Detection of CD95L mRNA expression by RT-PCR

Expression of CD95L mRNA was examined by reverse transcription (RT)-PCR. Total cellular RNA was extracted from 1×10^6 N9 cells by the acidic guanidinium thiocyanate phenol-chloroform method. One μg of total RNA was reverse transcribed after heat denaturation (3 min, 60°C) and annealing with 2.5 μM random hexamer primers (Perkin Elmer, Weiterstadt, Germany) in the presence of 50 U MnLV RT (Perkin Elmer), 5 mM $MgCl_2$ and 1 mM of each dNTP in 20 μl for 30 min at 42°C. The reaction was stopped by heat inactivation for 5 min at 95°C. Aliquots of 10 μl of the cDNA were then amplified in a DNA thermocycler (Stratagene, Heidelberg, Germany) with 1.25 U of Ampli-Taq DNA-polymerase (Perkin Elmer), 100 pM of both upstream and downstream CD95L primers and 2 mM $MgCl_2$ in a volume of 50 μl. Each of the PCR cycles consisted of a denaturation step (94°C, 1 min), an annealing step (57°C, 1 min) and an elongation step (72°C, 1 min). For histone H3.3 mRNA expression, which was analyzed as a control for sample loading and integrity, 2 μl cDNA were amplified (1 min 94°C, 1 min 60°C, 1 min 72°C). The PCR products (836-bp mouse CD95L fragment, 522-bp H3 fragment) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Primers used for amplification were mouse CD95L sense primer corresponding to nucleotides 179–200 (5′-CCATGCAGCAGCCCATGAATTA-3′) and antisense primer complementary to nucleotides 1015–993 (5′-GCTTATA-CAAGCCGAAAAAGGTC-3′), and H3 sense (nucleotides 40–59; 5′-ACTGGTGGTAAAGCACCCAG-3′) and antisense primer (nucleotides 562–543; 5′-TGTCCCCTATTTTCCACTCG-3′).

2.4. Western blotting

Cells were seeded on 10-cm dishes at 2×10^6/dish and stimulated with H_2O_2. After the indicated time, cells were washed in ice-cold PBS and lysed for 10 min in 50 mM Tris-HCl pH 7.6 containing 1% NP-40, 300 mM NaCl and protease inhibitors (3 μg/ml leupeptin, 3 μg/ml aprotinin, 2 mM PMSF). Cellular proteins from 1×10^6 cells were loaded in each lane and electrophoretically separated on a 10% polyacrylamide gel in the presence of SDS and reducing conditions. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes by semi-dry electroblotting. Membranes were blocked overnight with 5% non-fat dry milk powder in PBS and then incubated for 1 h with anti-CD95L (0.25 μg/ml; Signal Transduction; Dianova, Hamburg, Germany). Membranes were washed 3 times with PBS/0.05% Tween-20 and incubated with peroxidase-conjugated affinity-purified rabbit anti-mouse IgG for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL reagents (Amersham-Buchler).

2.5. Immunocytochemical detection of CD95L

For indirect immunofluorescence, cells were plated on Permanox plastic chamber slides (Lab-tek, Nunc, Wiesbaden, Germany) at 1×10^4 cells/well and fixed after overnight incubation for 10 min with a cold mixture of methanol and acetone (1:1). After rinsing in PBS, slides were blocked for 30 min with 10% FCS and 1% H_2O_2 in PBS. Incubation with anti-CD95L (2 μg/ml) was performed for 30 min, followed by incubations for 30 min each with biotinylated anti-mouse-IgG and streptavidin-peroxidase. Between each of these steps, slides were intensively washed in PBS/0.05% Tween-20. The specimens were then stained for 5 min with a Tyramide signal amplification kit (TSA NEL 701; DuPont NEN, Boston, MA, USA) and mounted in Elvanol. Fluorescence microscopy was performed with a Zeiss Axiovert microscope. Negative controls included omission of the primary antibody, and the use of isotype-matched irrelevant antibody. In addition, preadsorption experiments with recombinant CD95L were performed.

3. Results and discussion

3.1. The pro-oxidants H_2O_2 and paraquat are strong inducers of CD95L expression

We investigated the inducible expression of CD95L in mouse N9 microglial cells, which exhibit several functional characteristics of primary microglia [27]. In the initial experiments, cells were stimulated with different inducers of microglial activation, before mRNA was isolated, reverse-transcribed and amplified by RT-PCR using CD95L-specific primers. As shown in Fig. 1, CD95L-specific PCR products

were barely detected in unstimulated cells. Treatment of cells with H_2O_2 potently induced CD95L mRNA expression without affecting expression of the histone gene H3.3, which was analyzed as a control for equal mRNA loading. Although to a lesser extent, CD95L mRNA was also inducible by LPS and TNF, which act as potent activators of microglial cells [23]. In contrast, GM-CSF, a frequently used microglial mitogen, was unable to increase CD95L-specific transcripts. A potent activation was further observed following exposure of N9 cells to paraquat, a compound increasing intracellular formation of ROIs by redox cycling. The chemotherapeutic drugs doxorubicin and menadione, however, were almost ineffective in inducing CD95L expression. These results indicate that, in comparison to traditional stimuli of microglial activation such as LPS, reagents generating oxidative stress including H_2O_2 and paraquat are the most potent activators of CD95L expression in microglial cells.

3.2. Time course and dose-dependency of H_2O_2-induced CD95L mRNA expression

In order to investigate the kinetics of CD95L expression, cells were stimulated with 250 μM H_2O_2 over different time intervals, before CD95L mRNA expression was analyzed. As shown in Fig. 2A, the time course of CD95L expression was fast, and significant amounts of CD95L-specific PCR products were already detected 1 h after stimulation. The kinetics of CD95L expression in response to H_2O_2 was transient. Maximal expression was obtained within 2–4 h and thereafter declined. In addition, induction of CD95L mRNA was dose-dependent (Fig. 2B). Strong expression was detected after a 4-h exposure to 250 μM H_2O_2, whereas lower as well as higher concentrations resulted in weaker induction levels.

3.3. CD95L mRNA expression is associated with increased NF-κB DNA binding

The proximal 300-bp sequence upstream of the CD95L gene contains several *cis*-regulatory binding sites for transcription factors [31]. Although only a limited number of studies have yet addressed their functional importance, the region at nucleotides −275 to −264 (GGAAACTTCC) constitutes a putative κB-binding motif. Because transcription factor NF-κB is controlled by redox processes [20], we investigated the activation of NF-κB DNA binding in response to H_2O_2 in microglial cells. As shown in Fig. 2C, stimulation of cells with

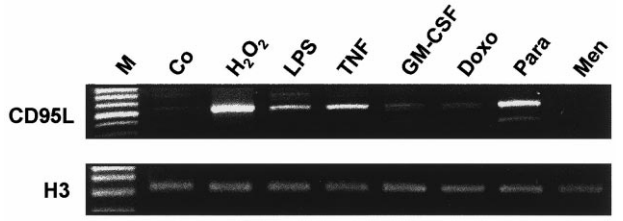

Fig. 1. Induction of CD95L mRNA in microglial N9 cells. Cells were either left untreated (Co) or stimulated with the following reagents: H_2O_2 (500 μM), LPS (100 ng/ml), TNF (50 ng/ml), GM-CSF (50 ng/ml), doxorubicin (Doxo, 1 μg/ml), paraquat (Para, 500 μM) or menadione (Men, 500 μM). After 4 h, RNA was isolated and subjected to RT-PCR. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The products of CD95L and H3 gene amplification migrated at the predicted size of 836 bp and 522 bp, respectively. A DNA size marker (M) is shown on the left.

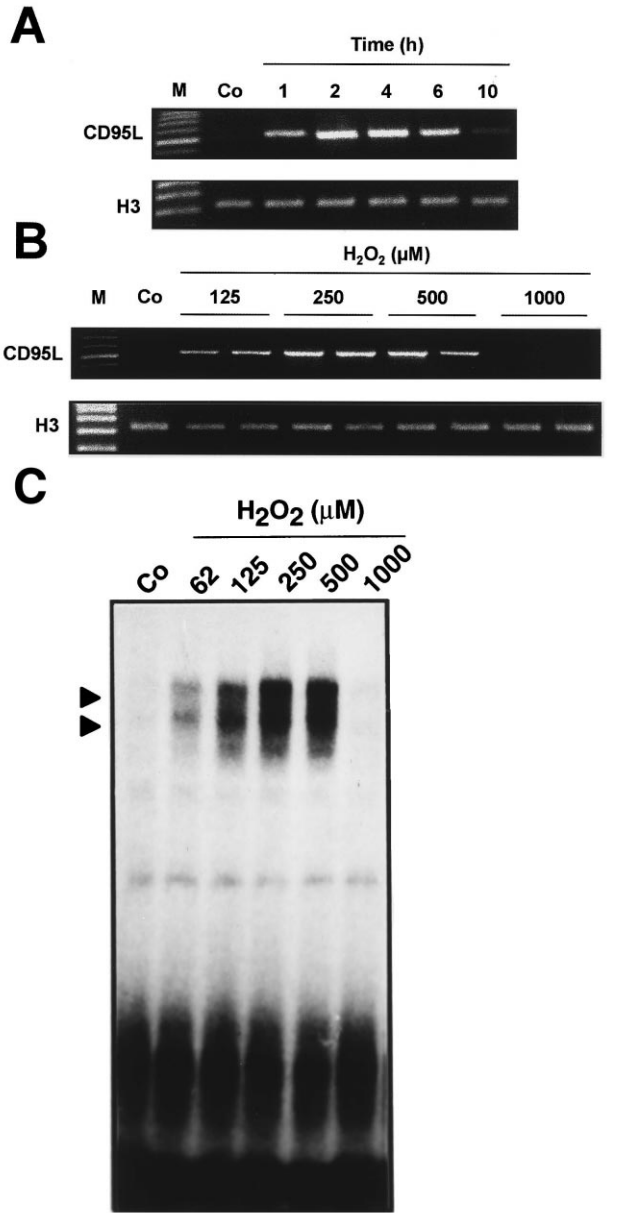

Fig. 2. A: Kinetics of CD95L mRNA expression in response to H_2O_2. Total RNA of cells treated for the indicated times with 500 μM H_2O_2 was reverse-transcribed and analyzed for CD95L- and H3-specific amplification products. B: Dose-response of CD95L mRNA expression. Cells were stimulated for 4 h in duplicates with the indicated concentrations of H_2O_2 and analyzed for the presence of RT-PCR specific amplification products. C: The effect of H_2O_2 on NF-κB activation. After treatment of cells for 45 min with the indicated concentrations of H_2O_2, cell extracts were prepared and analyzed by EMSA using a ^{32}P-labeled NF-κB specific oligonucleotide. The position of the NF-κB-DNA complexes is indicated by arrowheads. The specificity of the NF-κB DNA complex was verified by supershift analyses with NF-κB specific antibodies (data not shown, see [28]).

different concentrations of H_2O_2 induced NF-κB activation in a dose-response similar to CD95L as seen in the previous experiments. Although these experiments do not prove a functional role of NF-κB, together with previous reports [32,33], the results indicate that NF-κB may be of importance in oxidative stress-induced CD95L expression.

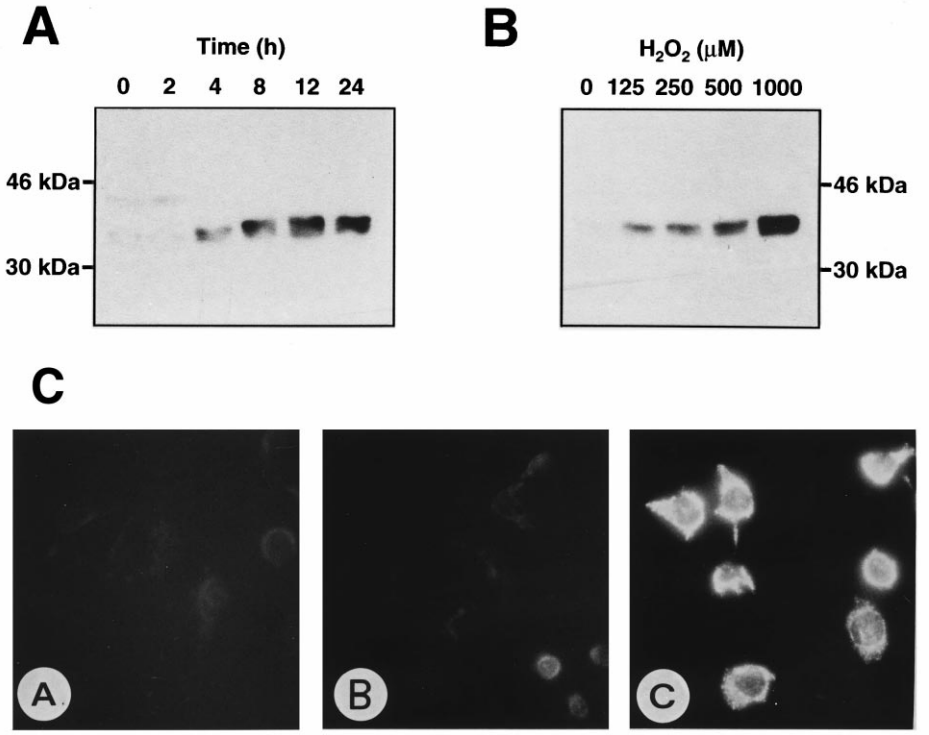

Fig. 3. H_2O_2 increases CD95L protein expression. A: Time kinetics of inducible CD95L expression. N9 cells were incubated with 250 μM H_2O_2. After the indicated time, cell lysates were prepared, electrophoresed on a 10% SDS-PAGE and transferred by Western blotting. CD95L was detected with a CD95L-specific antibody and visualized by enhanced chemiluminescent staining. The Western blot shows a CD95L-specific protein doublet band of 40–42 kDa. B: Dose response of CD95L expression. N9 cells were stimulated with the indicated concentrations of H_2O_2. Cell lysates were prepared after 12 h. C: Immuofluorescent detection of CD95L. Cells were either left untreated (A) or stimulated with 250 μM H_2O_2 (B,C). After 12 h, cells were fixed and incubated with an isotype-matched IgG1 control antibody (B) or with anti-CD95L (A,C), and analyzed by fluorescent microscopy.

3.4. Effects of H_2O_2 on CD95L protein expression

We next investigated whether H_2O_2 also increased the functional expression of CD95L protein. N9 cells were stimulated with 250 μM H_2O_2, and after different time points cell lysates were prepared and subjected to Western blot analysis using CD95L-specific antibodies. CD95L protein, which was not detectable in unstimulated cells, was increasingly expressed following prolonged exposure to H_2O_2 (Fig. 3A). CD95L-specific signals revealed a doublet protein band of approximately 40–42 kDa which corresponded to the published molecular size and probably represented differentially glycosylated forms of CD95L. Fig. 3B demonstrates that CD95L expression was dose-dependent. CD95L protein was most strongly expressed after incubation with 1 mM H_2O_2.

The ability of H_2O_2 to induce CD95L protein expression was verified by immunofluorescence analysis. CD95L-specific signals were almost undetectable in unstimulated cells, whereas stimulation with H_2O_2 produced an intensive cytoplasmic and membrane staining in N9 cells (Fig. 3C). The staining reaction was specific for CD95L, since almost no fluorescent signal was detected, when isotype-matched control antibodies were used (Fig. 3C) or when the antibodies were preabsorbed with recombinant CD95L (data not shown).

3.5. Exposure of microglia to hypoxia/reoxygenation induces CD95L expression

An important pathophysiological correlate of H_2O_2 stimulation is cerebral hypoxia/reperfusion, which results in increased oxidative stress and subsequent neuronal damage caused by apoptosis. It has been documented that microglia may contribute to cell death associated with hypoxic/ischemic injury [23]. To investigate the regulation of CD95L expression in this context, we exposed N9 cells to mild hypoxic conditions, which were not directly cytotoxic and did not inhibit respiration or normal adenylate energy charge [29]. Cells were cultivated in a tight incubation chamber under 200 ppm O_2 over different time intervals, followed by a 1-h incubation under normoxic conditions. As demonstrated in Fig. 4A, this hypoxia/reoxygenation treatment resulted in increased expression of CD95L mRNA. Incubation of cells with prolonged times of hypoxia (up to 8 h) led to elevated CD95L expression. In contrast, transcription of the house-keeping gene H3 was not altered under these conditions. In another experimental setting, cells were incubated under hypoxic atmosphere for 6 or 8 h, followed by different times of reoxygenation. These experiments revealed that a transient reoxygenation period of 1 h was sufficient and optimal to increase CD95L expression, whereas longer normoxic incubations had markedly weaker effects (Fig. 4B). Corresponding to the previous experiment, a prolonged hypoxic incubation of 8 h was superior to activate CD95L expression than a shorter period.

These data demonstrate that hypoxia/reoxygenation potently induces expression of CD95L, which may thus be a potential mediator of microglial-derived cytotoxic activity. Several recent studies have emphasized a causative role of ROIs in apoptosis of neurodegenerative diseases including amyotrophic lateral sclerosis, Parkinson's and Alzheimer's disease [13,14,34]. A critical role of ROI-mediated apoptosis

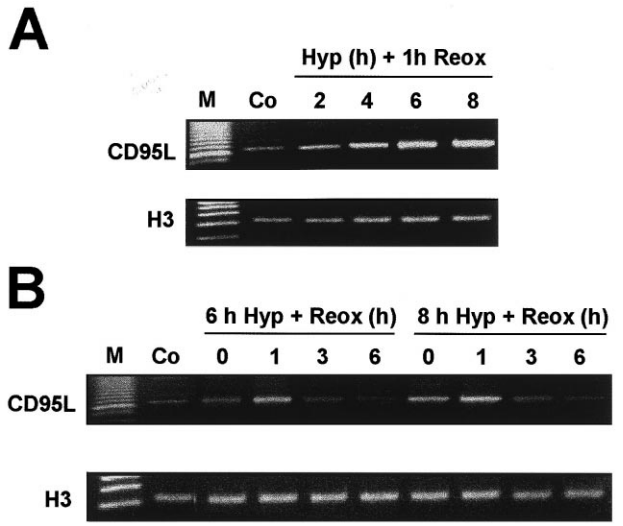

Fig. 4. Effect of hypoxia/reoxygenation on CD95L expression. A: N9 cells were either left under normoxia (Co) or incubated for the indicated time under mild hypoxia (Hyp, 200 ppm O_2) followed by a subsequent 1-h reoxygenation (Reox) period. Total RNA was harvested and amplified by RT-PCR as described in Fig. 1. M denotes a DNA size standard. B: N9 cells were either left untreated or incubated under hypoxia for 6 or 8 h. Following different incubation times of reoxygenation, RNA was isolated and subjected to RT-PCR using CD95L and H3-specific primers.

has been proposed for ischemia/reperfusion injury, where classical morphological alterations of apoptosis can be demonstrated [15]. It has been shown that the apoptosis-inhibitory proteins of the Bcl-2 family as well as pharmacological inhibitors of caspases prevent hypoxic injury [11]. In addition, neurons of Bcl-2 transgenic mice are highly resistant to ischemia [35], suggesting that oxidative damage, although formerly regarded as necrosis, can be attributed to the activation of the apoptotic programme.

Histological studies on transient ischemic insults in brain demonstrated that certain neurons of the hippocampus are particularly susceptible to ischemic apoptosis [16,18]. Cell death of such neurons usually occurred after an interval of 1–2 days following reperfusion. This delay of cell death indicates that apoptosis may be not a direct consequence of hypoxia-mediated effects, but rather requires hypoxia-induced de novo protein synthesis. Consistent with this assumption, a considerable reduction of neuronal cell death in the hippocampus was achieved after injection of the protein synthesis inhibitor cycloheximide [21,22]. Studies investigating the temporal and spatial patterns of apoptosis in hypoxia-mediated injury found that microglial activation coincided with the onset of DNA degradation in regions of selective neuronal loss, suggesting a possible role of microglia in induction of apoptosis [23,24]. Although various small molecules that can trigger apoptosis are known, the mechanisms and identity of death proteins in this process are unclear.

In the present study, we show that oxidative stress induced by H_2O_2 and paraquat is a strong inducer of CD95L expression in N9 microglial cells. Interestingly, pro-oxidant stimuli were significantly stronger in inducing CD95L expression than classical mediators of microglial activation, such as LPS or TNF. Since ischemia/reperfusion represents an important pathophysiological equivalent of oxidative stress, we further investigated the effects of hypoxia/reoxygenation on CD95L expression. Similarly to the pro-oxidants, hypoxia and subsequent reoxygenation were found to be potent CD95L-inducing conditions. The induction of CD95L was more pronounced with increased periods of hypoxia and required brief subsequent reoxygenation. This indicates that gene induction was indeed dependent on pro-oxidative signaling and not mediated by other potential side effects of hypoxia. The activation of CD95L by H_2O_2 corresponded to the increased activation of NF-κB which contains a potential binding site in the CD95L promoter [31]. Although the role of NF-κB in the control of CD95L has not yet been fully established, recent studies using specific inhibitors of the transcription factor pointed out that NF-κB may be involved in CD95L expression in T lymphocytes [32,33]. It is interesting to note that high levels of active NF-κB have been detected in microglia of neurodegenerative diseases [36].

Currently, there exists only a paucity of information about the function of CD95 in the nervous system. Whereas CD95 is weakly expressed in normal brain [37], upregulation of the CD95 receptor has been detected in postischemic brain as well as in brain tissues from Alzheimer's and multiple sclerosis patients [38–41]. Hence, these findings favor a role of the CD95 system in causing or exacerbating neurodegenerative diseases. However, it cannot be excluded that CD95L may, oppositely, exert a protective effect. Since brain is an established immunoprivileged organ, it is possible that, similarly to the eye and testis, CD95L is involved in maintaining the immunoprivilege of the nervous system. A recent study surprisingly demonstrated that mutant mice lacking both TNF receptors are highly susceptible to neuronal damage associated with cerebral ischemia [42]. Thus, TNF obviously plays an undefined protective role in these processes, although previous studies have indicated a neurotoxic effect of TNF under certain conditions. Therefore, until the relative neurotoxic or protective roles of CD95 are not defined, the consequences of CD95L expression have to await further studies.

Hypoxia/reperfusion is an important cause of injury in a variety of tissues other than brain which are highly susceptible to a dysfunctional redox balance. Upregulation of CD95 mRNA has been reported following hypoxia in rat cardiomyocytes [43], suggesting that under this condition CD95-mediated apoptosis may be of functional importance. Our findings that CD95L gene transcription is highly inducible by hypoxia/reperfusion and other forms of oxidative stress may also be relevant for tumor-associated hypoxia. It is conceivable that, in particular, those tumor cells growing in a hypoxic environment may upregulate CD95L expression and thereby establish an immunoprivileged state which allows tumor cells to escape immunosurveillance. Another important aspect may be chemotherapeutic drug-induced apoptosis of tumor cells, which has been proposed to require at least partially induction of CD95L expression [6,7]. Interestingly, many drugs, such as anthraquinones, that undergo redox cycling, continuously produce ROIs and activate NF-κB [44]. Collectively, oxidative signaling leading to CD95L expression may presumably play a role in diverse apoptosis settings involving the CD95 system.

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