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Role of Reactive Oxygen Intermediates in Activation-induced CD95 (APO-1/Fas) Ligand Expression*

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Activation-induced cell death of T lymphocytes requires the inducible expression of CD95 (APO-1/Fas) ligand, which triggers apoptosis in CD95-bearing target cells by an autocrine or paracrine mechanism. Although execution of the CD95 death pathway is largely independent of reactive oxygen intermediates, activation-induced cell death is blocked by a variety of antioxidants. In the present study, we investigated the involvement of redox processes in the regulation of CD95 ligand (CD95L) expression in Jurkat T cells. We show that various antioxidants potently inhibited the transcriptional activation of CD95L following T cell receptor ligation or stimulation of cells with phorbol ester and ionomycin. Conversely, a prooxidant such as hydrogen peroxide alone was able to increase CD95L expression. As detected by Western blot and cytotoxicity assays, functional expression of CD95L protein was likewise diminished by antioxidants. Inhibition of CD95L expression was associated with a decreased DNA binding activity of nuclear factor (NF)-κB, an important redox-controlled transcription factor. Moreover, inhibition of NF-κB activity by a transdominant IκB mutant attenuated CD95L expression. Our data suggest that, although reactive oxygen intermediates do not act as mediators in the execution phase of CD95-mediated apoptosis, they are involved in the transcriptional regulation of CD95L expression.

Apoptosis is an active form of cell death that is fundamental for a number of biological processes and which can be induced by a variety of stimuli and conditions. In the immune system, apoptosis has been recognized as a major element controlling the development of lymphocytes and the extent and duration of an immune response (reviewed in Green and Scott (1)). Activation of T lymphocytes through the T cell receptor-CD3 complex under certain circumstances leads to apoptosis. This process, referred to as activation-induced cell death (AICD),[1] is assumed to play an essential role in the induction of peripheral tolerance and in downsizing of the immune response (1). An important mediator of apoptosis in the immune system is the CD95 (APO-1/Fas) receptor/ligand system (reviewed in Refs. 2–4). CD95 belongs to an increasing family of cell surface receptors with homology to the TNF/nerve growth factor receptors. Its ligand CD95L is a type II transmembrane protein of 40–42 kDa that induces apoptosis in sensitive target cells (5). CD95L can be proteolytically cleaved from the membrane by a metalloprotease and also occur as a soluble cytokine of about 26 kDa (5, 6).

Several studies have revealed that AICD is largely controlled by the CD95 system (7–9). Activation of T cells with anti-T cell receptor/CD3 antibodies or mimicking agents, such as phorbolester and calcium ionophore, rapidly induces CD95L expression. CD95L then triggers cell death in CD95-positive target cells by an autocrine or paracrine mechanism. AICD mediated by CD95L represents an important safeguard in the immune system to control the expansion of activated T lymphocytes. The consequences of a failure of this process are demonstrated by the phenotype of *lpr* and *gld* mice. In these animals, the lack of functional CD95 receptor and ligand leads to the accumulation of previously activated T cells with extensive lymphoadenopathy and splenomegaly (10, 11). Among T lymphocytes, CD95L can be expressed in CD8 and CD4-positive cells (12, 13). Within the CD4 compartment, CD95L exhibits the highest expression in Th1 cells (12–14). In CD8-positive cells, it has been shown that, besides the perforin/granzyme B, CD95L constitutes a second mechanism for T cell-mediated cytotoxicity (12, 15, 16).

CD95L is not only expressed in activated T cells but can be also found in NK cells (17), activated monocytes (18), and a number of nonlymphoid cells (19). High amounts of CD95L are expressed in Sertoli cells of the testis and in epithelial cells of the anterior eye chamber (20, 21). This led to the proposal that the CD95 system accounts for maintaining the immune privilege of these organs by preventing activated lymphocytes from tissue infiltration. Constitutive expression of CD95L has been detected also in tumor cells of different origin, such as melanoma (22), astrocytoma (23), hepatocellular (24), and colon carcinoma (25). This suggested that the expression of CD95L may allow tumor cells to escape the host's immune surveillance and promote tumor progression (26).

Rapid progress has been achieved in the elucidation of the signaling pathway of CD95-mediated apoptosis (reviewed in Fraser and Evan (27)). Upon binding of CD95L or agonistic anti-CD95 antibodies, an adapter molecule, called FADD, is instantly recruited to the so-called death domain of the intra-

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[2] The abbreviations used are: AICD, activation-induced cell death; CD95L, CD95 ligand; GFP, green fluorescent protein; NF, nuclear factor; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; ROI, reactive oxygen intermediate; DTT, dithiothreitol; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); XTT, 2,3-bis[methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide.

cellular part of CD95. This then results in binding and activation of FLICE (Mach) which is a member of a growing family of apoptosis-relevant proteases, called caspases. Further downstream in the death pathway, activation of FLICE presumably triggers the proteolytic processing and activation of other caspases, which then finally cleave various not well defined cellular substrates (28, 29).

Another class of mediators that have been implicated in several forms of cell death are reactive oxygen intermediates (ROIs) (reviewed in Refs. 30–32). ROIs are highly reactive compounds that are generated during normal metabolism, and cells possess many systems to limit their damaging effects (33). The major source of ROIs in most cell types is probably the leakage of electrons from the mitochondrial electron transport, which results in the formation of superoxide anions. Superoxide anions can be converted to hydrogen peroxide by superoxide dismutases. Hydrogen peroxide is then detoxified by glutathione peroxidase and catalase. However, hydrogen peroxide can also generate the highly toxic hydroxyl radical in the iron-dependent Fenton or Haber-Weiss reaction (33).

The notion that ROIs are involved in apoptosis is mainly based on the following lines of evidence. First, excessive formation of ROIs as well as the depletion of cellular antioxidants can result in apoptosis in a number of cellular systems (34, 35). Second, when cells are stimulated to undergo apoptosis, they often hyperproduce ROIs that subsequently causes lipid peroxidation and other noxious consequences of ROI-mediated damage (36). Third, antioxidant compounds as well as the overexpression of antioxidant enzymes inhibit cell death induced by different apoptotic agents (34, 37, 38). A number of data, however, indicate that ROIs may be important but not common and obligatory mediators of all forms of apoptosis. In the CD95 pathway, a role of ROIs is less obvious, since treatment of cells with antioxidants does not or only weakly diminishes cell death (39, 40). Apoptosis induced upon CD95 ligation or by other agents can even proceed in nearly anaerobic conditions where no ROIs are generated (41). It has been demonstrated that an increase in superoxide anions blocks apoptosis triggered through CD95 in melanoma cells (42).

Several studies have shown that AICD is blocked by antioxidant compounds (38, 43, 44). Since AICD is a two-step mechanism, one involving the expression of CD95L and the other the execution of the CD95 death pathway, the possibility arises that ROIs may be selectively implicated in the first activation step of AICD. There is increasing evidence that small amounts of ROIs are not cytotoxic but may rather fulfill a signaling role as second messengers in the control of gene expression. We and others have recently shown that particularly two transcription factors, NF-κB and AP-1, are largely controlled by redox-dependent processes (reviewed in Refs. 45 and 46).

In the present study, we investigated the involvement of ROIs in the activation-induced expression of CD95L. In Jurkat T cells, we show that antioxidants potently inhibited the transcriptional activation of CD95L. Conversely, the sole exposure of cells to hydrogen peroxide was sufficient to up-regulate CD95L. Prevention of CD95L expression by antioxidants was associated with a suppression of the activation of NF-κB, a prooxidant-induced transcription factor. In addition, constitutive inhibition of NF-κB activity by a dominant IκB mutant attenuated inducible CD95L expression. Our data indicate that, although ROIs are not involved in the execution of AICD, activation-induced expression of CD95L in T lymphocytes is redox-controlled. These findings may be also relevant in apoptotic pathways induced by other agents, such as chemotherapeutic drugs, which induce NF-κB activation and subsequent CD95L expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The human T cell line Jurkat and the murine fibrosarcoma line L929 stably transfected with human CD95 cDNA (39) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics. Recombinant human CD95L was expressed in stably transfected 293 cells as a soluble Flag-tagged fusion protein and purified by affinity chromatography.[2] Chimeric receptor decoy proteins consisting of the extracellular part of CD95 or TNF-R1 fused to IgG_1-Fc were kindly provided by Immunex (Seattle). Mouse-anti-human CD95L (clone G247-4) was purchased from Pharmingen (Hamburg, FRG). Anti-CD3 (OKT3) was obtained from the American Type Culture Collection (Rockville, MD). Phorbol 12-myristate 13-acetate (PMA) and the antioxidant compounds clotrimazole, dimethyl sulfoxide (Me_2SO), dithiothreitol (DTT), pyrrolidine dithiocarbamate (PDTC), and rotenone were purchased from Sigma (Deisenhofen, FRG).

Detection of NF-κB DNA Binding—Cells were plated at 2×10^6/well in six-well plates and pretreated for 30 min with the inhibitors, followed by the addition of a combination of PMA (50 ng/ml) and ionomycin (1 μM) for additional 60 min. Total cell extracts were then prepared by resuspending PBS-washed cell pellets in a high salt buffer containing 20 mM Hepes, pH 7.9, 350 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin. Extracts were incubated on ice for 10 min and then cleared by centrifugation. Electrophoretic mobility shift assays were carried out essentially as described previously (47). Equal amounts of the extracts (about 10 μg of crude protein) were incubated with the ^{32}P-labeled NF-κB-specific oligonucleotide and loaded onto a 4% nondenaturing polyacrylamide gel. The oligonucleotide with a high affinity NF-κB binding motif (Promega, Heidelberg, FRG) was labeled using [γ-^{32}P]ATP (3000 Ci/mmol; Amersham-Buchler, Braunschweig, FRG) and T4 polynucleotide kinase (Boehringer Mannheim, FRG) followed by P-10 gelfiltration (Bio-Rad, Munich, FRG) to remove nonincorporated radioactivity.

Detection of CD95L mRNA Expression by Reverse Transcription-PCR—Expression of CD95L mRNA was examined by reverse transcription-PCR essentially as described previously (48). Total cellular RNA was extracted from 1×10^6 Jurkat cells by the acidic guanidinium thiocyanate phenol-chloroform method (49). 1 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, FRG) in the presence of 50 units of MnLV reverse transcription (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, FRG) with 1.25 units 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 (54 °C, 1 min), and an elongation step (72 °C, 1 min). For GAPDH mRNA expression, which was analyzed as a control for sample loading and integrity, 2 μl of cDNA were amplified (1 min 94 °C, 1 min 62 °C, 1 min 72 °C). The PCR products (498-bp human CD95L fragment and 397-bp human GAPDH fragment) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. The quantities of the PCR products were determined by densitometric scanning using the Image Master 1D Prime software (Pharmacia, Freiburg, FRG). Primers used for amplification were human CD95L sense primer corresponding to nucleotides 386–410 (5′-ATGTTTCAGCTCTTCCACCTACAGA-3′) and antisense primer complementary to nucleotides 884–858 (5′-CCAGAGAGAGCTCAGATACGTTGACA-3′), and GAPDH sense (5′-ATGGCACCGTCAAGGCTGAGA-3′) and antisense primer (5′-GGCATGGACTGTGGTCATGAG-3′).

Western Blotting—Jurkat cells were pretreated for 30 min with the antioxidants and then stimulated by incubation in anti-CD3-coated (OKT3, 10 μg/ml) culture dishes. After 24 h, cells were washed in ice-cold PBS and lysed for 10 min in 50 mM Tris-HCl, pH 7.6, containing 1% Nonidet P-40, 300 mM NaCl, and protease inhibitors (3 μg/ml leupeptin, 3 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride). Cellular proteins from 4×10^5 cells were loaded in each lane and electrophoretically separated on a 10% polyacrylamide gel in the presence of SDS and reducing conditions. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes by semidry electroblotting. The loading and transfer of equal amounts of protein was confirmed by staining the nitrocellulose membrane with

[2] M. Vogt and K. Schulze-Osthoff, manuscript in preparation.

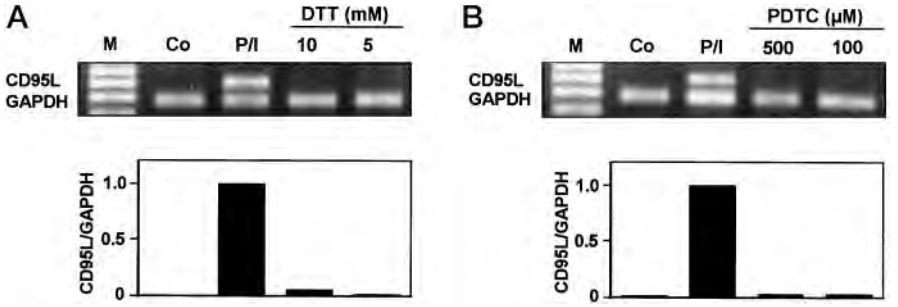

FIG. 1. **Antioxidants inhibit CD95L mRNA expression in response to PMA/ionomycin.** Jurkat cells were preincubated for 30 min with the indicated concentrations of DTT (*A*) and PDTC (*B*) and then either left untreated (*Co*) or stimulated with PMA and ionomycin (*P/I*). After 4 h, RNA was isolated and subjected to reverse transcription-PCR. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining (*upper panel*). The products of CD95L and GAPDH amplification migrated at the predicted size of 498 and 397 bp, respectively. A DNA size marker (*M*) is shown on the *left*. The relative quantities of the CD95L PCR products were determined by densitometric analysis and normalized with the density of the GAPDH profile (*lower panel*). The ratio of the CD95L *versus* the GAPDH product from cells stimulated with P/I in the absence of inhibitors was set to 1.

Ponceau S. Membranes were blocked overnight with 5% non-fat dry milk powder in PBS and then incubated for 1 h with anti-CD95L (1.5 μg/ml). Membranes were washed three 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).

Cytotoxicity Assays—The biological activity of CD95L was measured in supernatants of Jurkat cells pretreated with the various antioxidants and stimulated with anti-CD3 and a combination of PMA and ionomycin. 18 h following stimulation of cells, supernatants were harvested and dialyzed thoroughly to eliminate any side effects of the inhibitors during the bioassays. Dilutions of the supernatants were then incubated with sensitive L929-CD95 target cells (39). Apoptosis was assessed after 24 h by measuring DNA fragmentation to hypodiploid DNA. Briefly, following incubation with CD95L-containing supernatants, L929-CD95 cells were pelleted, washed twice in PBS, and fixed for 30 min in cold acetone-methanol (1:1). Cells were then washed again and incubated on ice for 1 h in PBS containing RNase A (1 mg/ml) and propidium iodide (100 μg/ml). Histograms of DNA were determined by fluorescence-activated cell sorter analysis (FACS-Calibur, Becton Dickinson, Heidelberg, FRG) using the CellQuest program. Cells to the left of the 2N peak containing hypodiploid DNA were considered as apoptotic.

Transfection Experiments—For constitutive and specific inhibition of NF-κB activity, cells were transiently transfected with expression vectors encoding a transdominant IκB mutant and green fluorescent protein (GFP) which was used as marker of transfection. The mutant IκB-α S32/36A, in which the serine phosphorylation sites are substituted by alanine residues, lacks the sequences for signal-induced proteolytic degradation of IκB-α (50). The plasmid pcDNA3-EGFP encoded a codon-optimized GFP suitable for flow cytometry and was kindly provided by W. Wybranietz (University of Tübingen, FRG). Briefly, Jurkat cells were washed twice in Tris-buffered saline, resuspended at 5×10^7 cells/0.2 ml Tris-buffered saline, and cotransfected with 12 μg of pcDNA3-EGFP and 8 μg of either pCMV-IκB-α S32/36A or the parental vector pRcCMV (Invitrogen, Leek, The Netherlands). Transfection was performed by electroporation using a Bio-Rad gene pulser (150 μF, 500 V). After transfection, cells were seeded in a 6-well plate at 1×10^6 cells/well and stimulated with the indicated reagents after 24 h. Eight hours later, cells were analyzed for CD95L expression by flow cytometry. For flow cytometric analyses, transfected GFP-positive cells were gated in the FL-1 channel. Transfection efficiency was between 10 and 18%. CD95L expression in the transfected subpopulation was analyzed in the FL-2 channel following staining with anti-CD95L and phycoerythrin-labeled goat-anti-mouse IgG (Dianova). Dead cells were excluded from the analysis by staining with propidium iodide.

RESULTS

Antioxidants Inhibit Activation-induced CD95L mRNA Expression—In the initial experiments, the involvement of ROIs in activation-induced CD95L expression was examined by reverse transcription-PCR. Jurkat cells were stimulated with the phorbolester PMA and the calcium ionophore ionomycin, and after 4 h mRNA was isolated, reverse-transcribed, and amplified using CD95L-specific primers. As shown in Fig. 1, CD95L-specific PCR products were not detected in unstimulated cells, whereas treatment with PMA and ionomycin resulted in potent CD95L mRNA expression. Pretreatment of cells with the thiol agent DTT almost completely abrogated the induction of CD95L mRNA (Fig. 1*A*). In contrast, expression of GAPDH mRNA, which was measured as a control for equal loading and integrity of the RNA, was not affected by this treatment. Furthermore, pretreatment of Jurkat cells with different concentrations of the dithiocarbamate PDTC, an iron chelator and ROI scavenger, strongly inhibited inducible CD95L expression (Fig. 1*B*).

Since hydroxyl radicals have been proposed to act as signaling molecules (51–53), we next examined the effect of Me_2SO, a cell-permeable hydroxyl radical scavenger. Fig. 2*A* shows that Me_2SO dose-dependently inhibited CD95L expression. Likewise, incubation of cells with phenanthroline, which chelates transition metals and thereby prevents formation of hydroxyl radicals in the Fenton reaction, inhibited induction of CD95L mRNA (data not shown). Chain-breaking antioxidants, such as butylated hydroxyanisole or nordihydroguaiaretic acid, however, were less effective in inhibiting CD95L mRNA expression (data not shown).

We further wished to identify the source of ROIs involved in inducible CD95L expression. An important intracellular ROI source is the mitochondrial respiratory chain, where electrons can be transferred directly from reduced ubiquinone to molecular oxygen (33). It has been shown that blockade of electron entry at proximal elements of the respiratory chain, such as complex I, prevents mitochondrial ROI formation (54). Fig. 2*B* demonstrates that preincubation of Jurkat cells with the mitochondrial complex I inhibitor rotenone inhibited CD95L expression. Another important cellular electron transfer reaction, where ROIs can be formed, includes the cytochrome P450 system. Incubation of cells with the cytochrome P450 inhibitors clotrimazole (Fig. 2*C*) and SKF 525A (data not shown) also strongly inhibited expression of CD95L-specific transcripts.

We further investigated the effect of antioxidants on CD95L expression in response to a more physiological stimulus. To this end, Jurkat cells were pretreated with the various antioxidants and enzyme inhibitors, and then stimulated by incubation in anti-CD3-coated culture plates. Similar to the previous experiments, anti-CD3-induced CD95L expression was strongly inhibited by the enzyme inhibitors clotrimazole and rotenone as well as the thiol DTT (Fig. 3). A marked inhibition of anti-CD3-induced CD95L expression was also observed following pretreatment of cells with Me_2SO and PDTC (Fig. 3). Collectively, these data suggest that activation-induced CD95L expression involves formation of ROIs that may be derived from the respiratory chain and cytochrome P450 system.

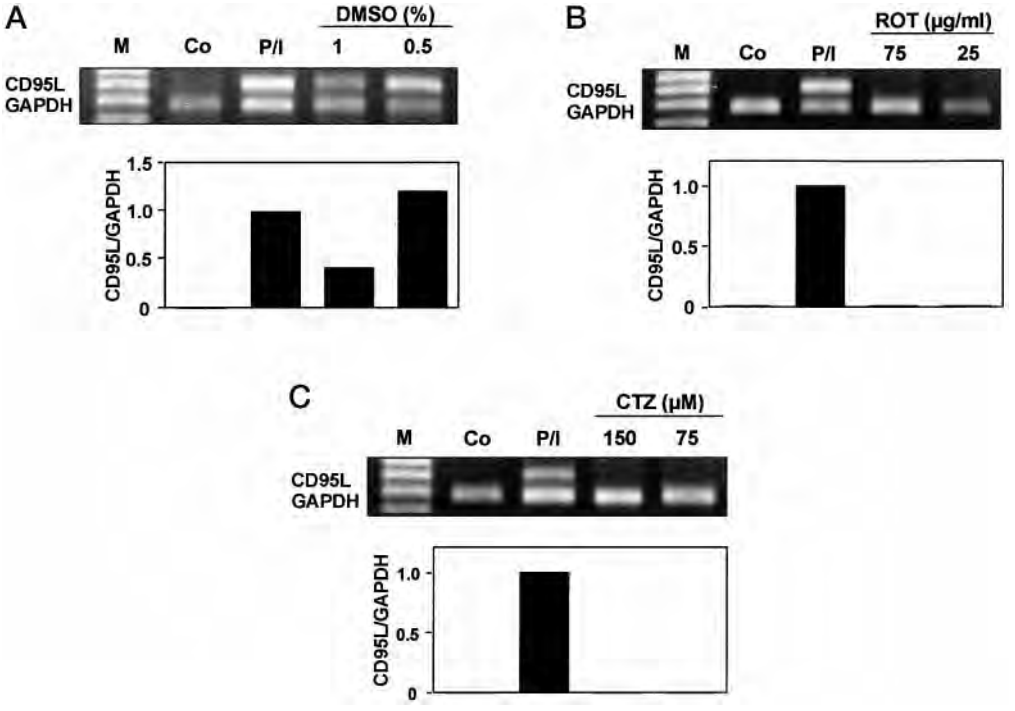

FIG. 2. **Effects of the hydroxyl radical scavenger Me_2S0 (*DMSO*), the mitochondrial inhibitor rotenone and the cytochrome P450 inhibitor clotrimazole on CD95L mRNA expression.** Jurkat cells were pretreated for 30 min with the indicated concentrations of Me_2SO (*A*, *DMSO*), rotenone (*B*, *ROT*) or clotrimazole (*C*, *CTZ*) and then stimulated with a combination of PMA and ionomycin (*P*/*I*). RNA was isolated after 4 h and processed as described in Fig. 1.

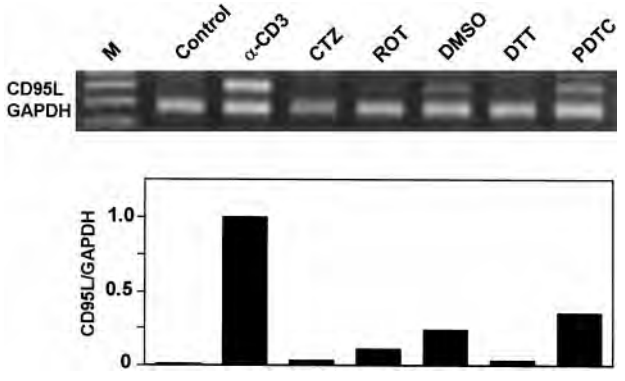

FIG. 3. **Antioxidants inhibit CD95L expression following T cell receptor ligation.** Jurkat cell cultures were preincubated with clotrimazole (*CTZ*, 75 μg/ml), rotenone (*ROT*, 25 μg/ml), Me_2SO (*DMSO*, 1%), DTT (5 mM), PDTC (100 μM), or medium. After 30 min, cells were either left untreated (medium) or stimulated by incubation on anti-CD3-coated culture wells. RNA was harvested after 4 h; the relative amounts of the CD95L PCR product were determined as described in Fig. 1.

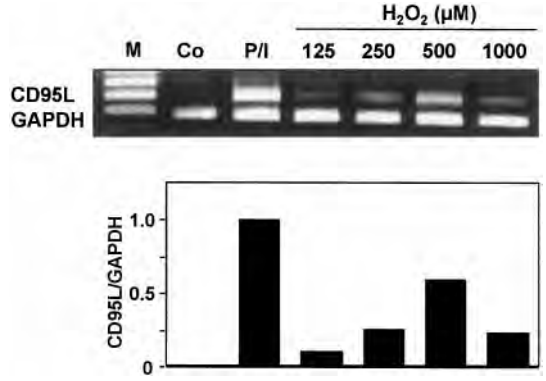

FIG. 4. **Hydrogen peroxide increases CD95L mRNA expression.** Jurkat cells were left untreated (*Co*, control) or stimulated with either PMA/ionomycin (*P*/*I*) or the indicated concentrations of hydrogen peroxide. RNA was harvested after 4 h and subjected to reverse transcription-PCR analysis as described in Fig. 1. *M* denotes a DNA size marker.

Hydrogen Peroxide Induces CD95L Expression in Jurkat T Cells—To further support a role of prooxidant signaling in the induction of CD95L expression, Jurkat cells were incubated with various concentrations of the cell-permeable ROI hydrogen peroxide. Fig. 4 demonstrates that a sole dose of 500 μM hydrogen peroxide was able to significantly increase CD95L transcripts, whereas a higher concentration of 1 mM was less effective. In comparison to stimulation with phorbolester and ionomycin, hydrogen peroxide-induced CD95L expression was less prominent, which may be due to the presence of antioxidants or catalase in the culture medium.

Inhibition of CD95 mRNA Expression by Antioxidants Is Associated with Decreased NF-κB DNA Binding—The proximal 300-bp sequence upstream of the ATG initiation codon of the CD95L gene contains several putative *cis*-regulatory binding sites for transcription factors (55). 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 (consensus sequence RGARNTTCC) (56). Because activation of transcription factor NF-κB is known to be controlled by redox processes, we investigated whether conditions leading to down-regulation of inducible CD95L expression correlated with a decrease of NF-κB activation. To measure NF-κB activation, cells were pretreated with the different inhibitors and then stimulated for 1 h in the presence of PMA and ionomycin. Subsequently, total cell extracts were prepared and analyzed for DNA-binding activity to a ^{32}P-labeled κB-specific oligonucleotide.

As shown in Fig. 5, stimulation of cells with phorbolester and ionomycin induced the appearance of a novel protein-DNA complex that was not detected in untreated cells. A faster migrating, nonspecific DNA complex was not affected by the various treatments and provided an internal control for the

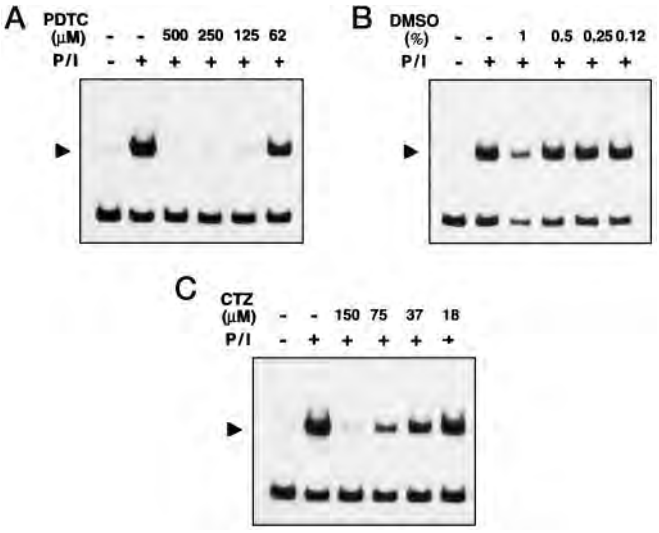

FIG. 5. **The effect of antioxidants on NF-κB activation.** Jurkat cells were pretreated for 30 min with the indicated concentrations of PDTC (*A*), Me_2SO (*B*, *DMSO*) or clotrimazole (*C*, *CTZ*) and then stimulated with PMA/ionomycin (*P/I*). After 1 h, cell extracts were prepared and analyzed by electrophoretic mobility shift assay using a ^{32}P-labeled NF-κB-specific oligonucleotide. The position of the NF-κB protein-DNA complex is indicated by an *arrowhead*.

amount and integrity of the cell extracts. Incubation of cells with PDTC caused a dose-dependent inhibition of the induced NF-κB·DNA complex (Fig. 5*A*). The hydroxyl radical scavenger Me_2SO affected NF-κB activation at a concentration of 1% (Fig. 5*B*). A potent and dose-dependent inhibition was further observed following pretreatment of Jurkat cells with the cytochrome P450 inhibitor clotrimazole (Fig. 5*C*). Although slight differences in the dose-response were found, these results suggest that down-regulation of CD95L expression is associated with the inhibition of NF-κB activation.

Transdominant Inhibition of NF-κB Activity Attenuates CD95L Expression—A key step of NF-κB activation involves the phosphorylation of the inhibitor IκB-α at serine residues 32 and 36, followed by its proteolytic degradation at the proteasome (50). To investigate the role of NF-κB in CD95L expression, we overexpressed a transdominant mutant form of IκB-α which lacks the sequences required for signal-induced degradation. This mutant, IκB-α S32/36A, therefore behaves as a constitutive repressor of NF-κB activity. Following cotransfection of the cDNA encoding mutated IκB and a reporter plasmid for GFP, cells were stimulated with either PMA/ionomycin or different concentrations of hydrogen peroxide. Subsequent flow cytometric analysis revealed that inhibition of NF-κB activity by IκB overexpression strongly attenuated CD95L expression (Fig. 6, *A* and *B*). An inhibitory effect was seen in response to stimulation with both PMA/ionomycin and hydrogen peroxide. These results therefore suggest that NF-κB plays a functional role in the control of CD95L expression.

Effects of Antioxidants on CD95L Protein Expression—In the next experiments, we investigated whether antioxidants inhibit also the functional expression of CD95L protein. Jurkat cells were pretreated with the various antioxidants and then stimulated with anti-CD3 and PMA/ionomycin. After 24 h, cell lysates were prepared and subjected to Western blot analysis using CD95L-specific antibodies. Fig. 7 shows that CD95L was barely detectable in unstimulated cells. As verified with different anti-CD95L antibodies, stimulation of cells led to a significant increase in the expression of CD95L which was mainly detected as the processed 26-kDa form. Incubation of cells with the various antioxidants revealed that their effects on CD95L protein expression largely corresponded to the transcriptional inhibition observed in the previous experiments. A strong down-regulation of CD95L was noted after treatment of cells with DTT (Fig. 7*A*), and also PDTC impaired CD95L expression (Fig. 7*B*). CD95L expression was furthermore suppressed by the enzyme inhibitors rotenone and clotrimazole (Fig. 7, *C* and *D*).

Antioxidants Decrease CD95L-specific Killing Activity in Jurkat Cell Supernatants—The interference of antioxidants with CD95L expression was further confirmed in cytotoxicity assays. Jurkat cells were stimulated in the presence and absence of the various antioxidants. After 24 h, supernatants were harvested, dialyzed and applied as a 1:10 dilution to L929-CD95 cells which were used as CD95L-sensitive target cells. The apoptotic activity in the supernatants was measured by the ability to induce fragmentation to hypodiploid DNA in the target cells. Stimulation of Jurkat cells by anti-CD3 resulted in an about 5-fold enhancement of the cytotoxic activity in the supernatants. The cytotoxicity was dependent on CD95L, since it was almost strongly abolished by neutralizing CD95-Fc but not TNF-RI-Fc decoy constructs (Fig. 8*F*). Pretreatment of Jurkat cells with the antioxidants PDTC, DTT, and Me_2SO dose-dependently inhibited the increase of cytotoxic activity in the supernatants (Fig. 8, *A–C*). Inhibition of expression of CD95L was furthermore observed following incubation of Jurkat cells with various concentrations of rotenone and clotrimazole (Fig. 8, *D* and *E*). The inhibitory effect of the antioxidants on CD95L expression was also verified in cytotoxicity assays using *X*TT as a colorimetric substrate, which measures viability by mitochondrial succinate dehydrogenase activity (data not shown). To exclude that antioxidants did not directly affect CD95-mediated apoptosis on the target cells, we further added the drugs directly to L929-CD95 target cells in the presence of recombinant CD95L. Analogous to previous studies (39, 40), none of the antioxidants interfered with the assay conditions (data not shown). The results therefore demonstrate that antioxidants do not inhibit execution of the CD95 death pathway, but are implicated in activation-induced CD95L expression.

DISCUSSION

Several studies have documented an important role of ROIs in apoptosis (reviewed in Refs. 30–32). Treatment of cells with hydrogen peroxide, ionizing irradiation or cytotoxic drugs, such a quinones that undergo redox cycling and cause ROI formation, induces apoptosis. In these conditions, cell death is caused by the detrimental effects of ROIs which lead to lipid peroxidation, protein denaturation and DNA damage. Intracellular ROI formation has been also implicated in other forms of apoptosis, such as TNF-mediated cytotoxicity or growth factor withdrawal, because antioxidants or the overexpression of antioxidant enzymes prevent cell death in these systems (34, 37, 47, 57).

More recent data suggest that ROIs may be not obligatory for all apoptotic cell death, since at least some forms including CD95-mediated apoptosis are not affected by antioxidants and can even proceed in nearly anaerobic conditions where no ROIs are generated (39–41). Several lines of evidence indicate that small amounts of intracellular ROIs, which are insufficient to induce cellular damage, may play a physiological role as second messengers by regulating gene expression. In particular, activation of important immunoregulatory transcription factors, such as NF-κB and AP-1, is largely controlled by ROIs (reviewed in Schulze-Osthoff *et al.* (46)). Because several forms of cell death require *de novo* protein synthesis, redox-dependent gene expression may therefore constitute another control level where ROIs could affect apoptosis.

Since antioxidants have been reported to inhibit AICD (38, 43, 44), which is dependent on CD95L expression, we hypoth-

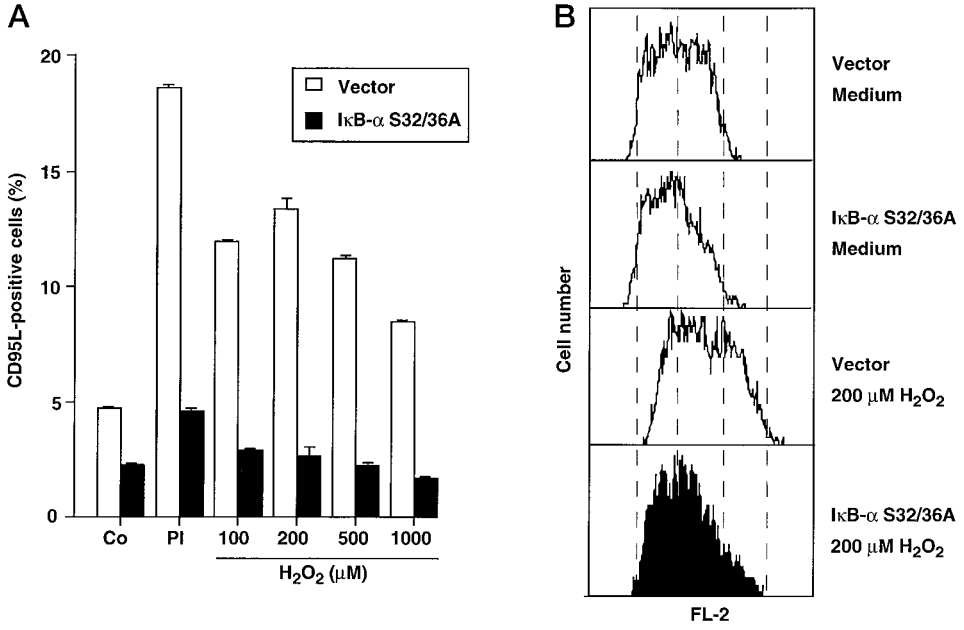

FIG. 6. **Transdominant inhibition of NF-κB activity prevents inducible CD95L expression.** Jurkat cells were cotransfected with a GFP-encoding reporter plasmid together with either the cDNA for a dominant negative IκB mutant (IκB-α S32/36A) or the parental control vector. After 24 h, cells were treated with PMA/ionomycin (*PI*) or the indicated concentrations of hydrogen peroxide for additional 8 h. CD95L expression was analyzed by flow cytometry in the FL-2 channel after gating GFP-positive cells in FL-1. *Panel A* shows CD95L expession in the transfected GFP-positive subpopulation. Representative histograms following treatment of cells with 200 μM H_2O_2 are shown in *panel B*.

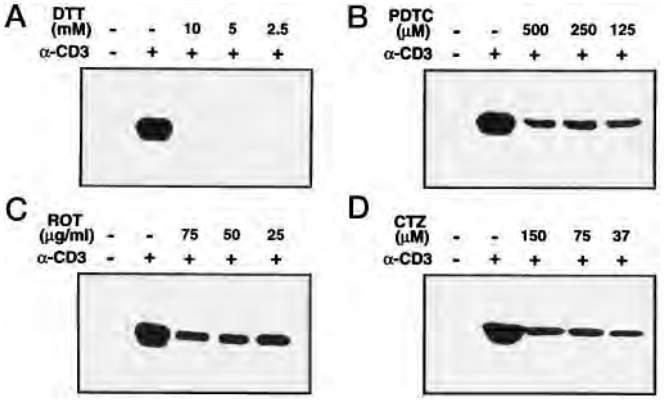

FIG. 7. **The effect of the antioxidants DTT (*A*), PDTC (*B*), the mitochondrial inhibitor rotenone (*C*), and the cytochrome P450 inhibitor clotrimazole (*D*) on CD95L protein expression.** Jurkat cells were preincubated with the indicated concentrations of the antioxidants and then stimulated by incubation on coated anti-CD3. After 24 h, cell lysates were prepared, electrophoresed on a 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred by Western blotting. CD95L was detected with an anti-CD95L-specific antibody and visualized by enhanced chemiluminescent staining. The Western blots show the 26-kDa form of CD95L.

esized that not execution of AICD but rather the activation phase involving CD95L expression may be controlled by redox processes. Our present findings indicate that ROIs are indeed able to control inducible CD95L expression. First, we demonstrated that hydrogen peroxide, which is the most diffusible ROI, itself increases CD95L expression. Secondly, different antioxidants strongly inhibited inducible CD95L expression, as measured in culture supernatants as well as at the protein and mRNA level. The inhibitory effect of antioxidants was observed in response to different stimuli including phorbolester and ionomycin as well as T cell receptor ligation. This suggests that a common downstream component rather than an upstream event in the cascade leading to CD95L expression was affected. Strong inhibitors of CD95L expression were the hydroxyl radical scavenger Me_2SO, the dithiol DTT, and the dithiocarbamate PDTC, which acts as both a sulfhydryl agent and metal chelator. Also phenanthroline, another iron chelator, was effective in preventing CD95L expression. This indicates that formation of hydroxyl radicals in the Fenton reaction, which is inhibited by these compounds, may be of importance in CD95L expression. It is noteworthy that hydroxyl radicals have been also implicated in the inducible expression of a number of other genes including intercellular adhesion molecule-1, interleukin-8, and cyclooxygenase-2 (51–53).

Intracellular ROIs are generated in different cellular compartments and electron transfer reactions (33). An major source are mitochondria where ROIs are formed at the respiratory chain. Mitochondria-derived radicals have been implicated in biological activities of TNF (47, 57). Our finding that rotenone, a mitochondrial complex I inhibitor, strongly abrogated inducible CD95L expression suggests that this pathway may be of major importance. In contrast to mitochondrial inhibitors of ROI formation, drugs that interfere with lipid peroxidation and arachidonic acid metabolism only weakly affected CD95L expression. This indicated that cyclooxygenases and lipoxygenases are presumably not involved in redox regulation of CD95L expression. However, inhibitors of cytochrome P450, such as clotrimazole and SKF 525A, exerted inhibitory effects suggesting that ROI formation by the cytochrome P450 system is involved in CD95L expression. Such a dependence on ROIs derived from cytochrome P450 has recently been proposed in a study measuring CD95L-dependent cytotoxicity in supernatants from activated T cells (44).

The transcriptional elements controlling CD95L expression are rather undefined at present and await further promoter studies. The finding that CD95L expression is sensitive to cyclosporin A argues for the importance of the calcineurin/NF-AT pathway (7, 58, 59). Calcineurin not only activates NF-AT but when in combination with protein kinase C-derived signals can also activate NF-κB (60). Indeed, a putative NF-κB binding site has been identified within the CD95L promoter (55). The sensitivity of CD95L expression to antioxidants makes NF-κB a good candidate for regulating CD95L transcription. We show that antioxidants that inhibited CD95L

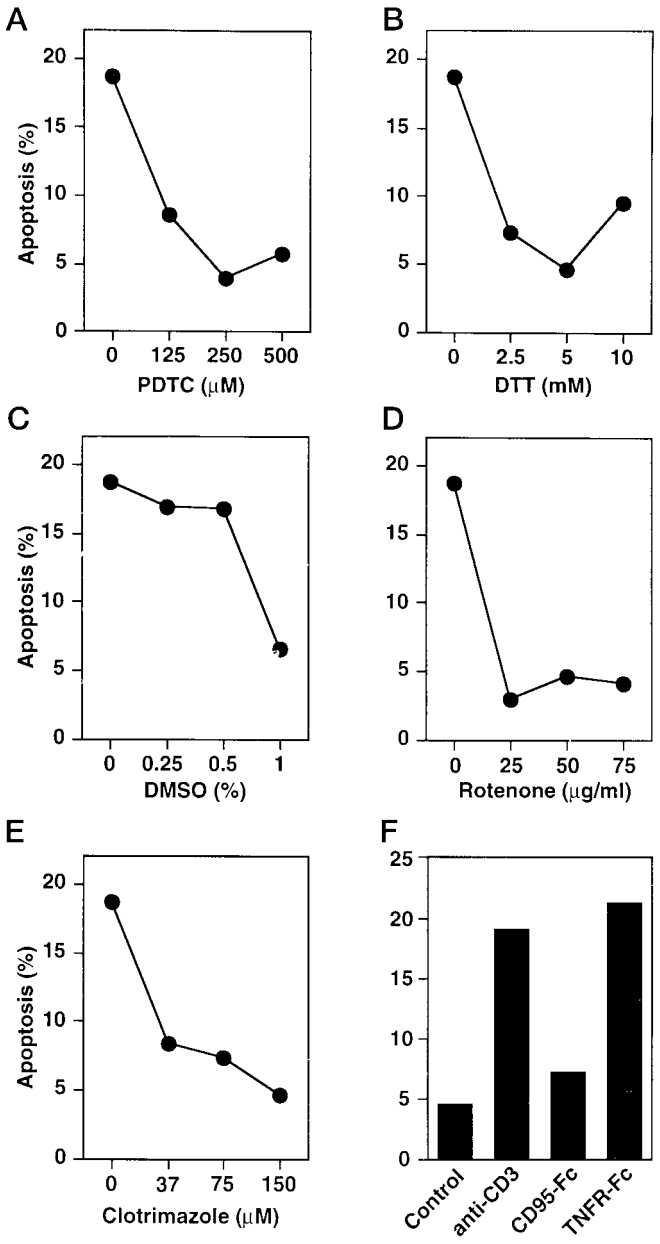

FIG. 8. **The effect of antioxidants on CD95L-dependent killing activity in Jurkat cell supernatants.** Jurkat cell cultures were preincubated for 30 min with the indicated concentrations of *A*, PDTC, *B*, DTT; *C*, Me_2SO (*DMSO*); *D*, rotenone; *E*, or clotrimazole, and then stimulated with anti-CD3. Following 24 h after cell stimulation, supernatants were assessed for killing activity using L929-CD95 target cells. Apoptosis in target cells was measured by analyzing formation of hypodiploid DNA. Target cell death induced by supernatants of unstimulated Jurkat cells was less than 5%. *F*, inhibition of killing activity in Jurkat cell supernatants by a CD95-Fc construct. Target cells were incubated with supernatants from control cells or anti-CD3 stimulated Jurkat cells in the absence or presence of 50 μg/ml CD95-Fc or a Fc-decoy construct consisting of the extracellular part of TNF-RI (*TNFR-Fc*).

were also able to prevent NF-κB activation. Although slight differences in the dose-response on effects on NF-κB activation and CD95L expression were observed, these may be caused by the different incubation periods or the interaction of NF-κB with other transcription factors, such as AP-1 and NF-AT. Support for the notion that NF-κB may be of functional importance in CD95L expression came from transfection experiments. A key step in the process of NF-κB activation is the phosphorylation of its inhibitor IκB at serine residues 32 and 36, followed by the inducible degradation of IκB at the proteasome (45, 50). We demonstrate that a transdominant form of IκB-α, which lacks the sequences required for signal-induced degradation, strongly attenuated CD95L expression. Similarly, a recent study reported that prevention of NF-κB activation by a specific inhibitor of proteasome function was associated with the down-regulation of CD95L expression in T lymphocytes (61). In addition, forskolin, an inhibitor of cyclic AMP, prevented anti-CD3-induced NF-κB activation accompanied by a strong suppression of CD95L synthesis (62). HIV-1 Tat protein is another example where CD95L expression may require oxidant-induced NF-κB activation. Tat, a potent inducer of CD95L, has been reported to induce oxidative stress and subsequent NF-κB activation through the down-regulation of the antioxidant enzyme manganese superoxide dismutase (63, 64).

The involvement of redox processes in CD95L expression, as shown in this study, may be not only restricted to AICD of T lymphocytes. Chemotherapeutic drug-induced cell death of hepatoma and leukemic cells has been proposed to be at least partially mediated through the induction of CD95L expression (48, 65). Interestingly, many of these drugs, such as anthraquinones that undergo redox cycling and continuously produce ROIs, are able to activate NF-κB by conferring oxidative stress (66, 67). Collectively, our data suggest that ROIs, although they are not involved in the execution of AICD, they may be of major importance in the first signaling phase of AICD involving CD95L expression. Oxidative signaling leading to NF-κB activation presumably plays an important role in signal transduction of AICD and also in other apoptotic processes that depend on CD95L expression.

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