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NF-κB-independent actions of sulfasalazine dissociate the CD95L- and Apo2L/TRAIL-dependent death signaling pathways in human malignant glioma cells

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Abstract

Death receptor-mediated apoptosis of human malignant glioma cells triggered by CD95 ligand (CD95L) or Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) share several features, including processing of multiple caspases and mitochondrial cytochrome *c* release. We here report that CD95L-induced cell death is inhibited by sulfasalazine (SS) in all of four human glioma cell lines, both in the absence and presence of cycloheximide (CHX). Coexposure to CD95L and SS prevents the CD95L-evoked processing of caspases 2, 3, 8 and 9, the release of cytochrome *c* from mitochondria, and the loss of BCL-x_L protein. This places the protective effect of SS proximal to most known events triggered by the CD95-dependent signaling pathway in glioma cells. CD95L promotes the accumulation of nuclear factor kappa B (NF-κB) in the nucleus and induces the DNA-binding activity of NF-κB assessed by electrophoretic mobility shift assay. The total levels of p50, p65 and IκBα remain unchanged, but the levels of phosphorylated IκBα and of nuclear p65 increase, in response to CD95L. IκBα phosphorylation as well as nuclear NF-κB translocation and DNA binding are blocked by SS. However, unlike SS, dominant-negative IκBα (IκBdn) does not block apoptosis, suggesting that SS inhibits CD95L-mediated apoptosis in an NF-κB-independent manner. In contrast to CD95L, the cytotoxic effects of Apo2L/TRAIL are enhanced by SS, and SS facilitates Apo2L/TRAIL-evoked caspase processing, cytochrome *c* release, and nuclear translocation of p65. These effects of SS are nullified in the presence of CHX, suggesting that the effects of SS and CHX are redundant or that enhanced apoptosis mediated by SS requires protein synthesis. IκBdn fails to modulate Apo2L/TRAIL-induced apoptosis. Similar effects of SS on CD95L- and Apo2L/TRAIL-induced apoptosis are observed in MCF-7 breast and HCT116 colon carcinoma cells. Interestingly, HCT cells lacking p21 (80S14$^{p21-/-}$) are only slightly protected by SS from CD95L-induced apoptosis, but sensitized to Apo2L/TRAIL-induced apoptosis, indicating a link between the actions of SS and p21. Thus, SS modulates the death cascades triggered by CD95L and Apo2L/TRAIL in opposite directions in an NF-κB-independent manner, and SS may be a promising agent for the augmentation of Apo2L/TRAIL-based cancer therapies.
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Keywords: CD95L; Apo2L; TRAIL; sulfasalazine; NF-κB

Abbreviations: Apo2L/TRAIL, Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand; ATP, adenosinetriphosphate; CD95L, CD95 ligand; CHX, cycloheximide; DISC, death-inducing signaling complex; DR, death receptor; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FADD, Fas-associated death domain; FLIP, FLICE-inhibitory proteins; IκB, inhibitory kappa B; IκBdn, dominant negative IκB; IL, interleukin; NF-κB, nuclear factor kappa B; P-IκB, phosphorylated IκB; ROI, reactive oxygen intermediates; SS, sulfasalazine; TNF, tumor necrosis factor; TRAIL-R1–4, TRAIL receptor 1–4

Introduction

CD95 ligand (CD95L) and Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) are members of a family of cytokines that are cytotoxic to certain cancer cells.[1] These cytokines interact with cell surface death receptors (DRs) to trigger a killing cascade that depends critically on the activation of caspases and on mitochondrial cytochrome *c* release and the subsequent activation of caspase-9 in many cell lines. Activation of these DRs results in the proteolytic degradation of multiple cellular substrates.[2,3] Ligation of CD95 or of the agonistic Apo2L/TRAIL receptors DR4/TRAIL-R1 and DR5/TRAIL-R2 induces receptor oligomerization and formation of a death-inducing signaling complex (DISC) that consists of oligomerized cytoplasmic receptor domains, the adaptor molecule Fas-associated death domain (FADD) and caspase-8. DISC formation occurs both in CD95L- and in Apo2L/TRAIL-induced cell death, and no essential differences downstream of the DISC between both signaling pathways have been identified.[1-3]

Sulfasalazine (SS) was synthesized to combine an antibiotic and an anti-inflammatory reagent (sulfapyridine and 5-aminosalicylic acid).[4] Upon oral administration, 30% of the drug is absorbed in the bowel in unaltered form, whereas 70% is degraded by colonic bacteria and azo-reduction into sulfapyridine and 5-aminosalicylic acid.[5] Its main clinical applications include chronic inflammatory bowel diseases and rheumatoid arthritis. The mechanisms underlying the therapeutic actions of SS have remained a matter of debate, but include inhibition of leukocyte motility, interleukin (IL)-2

synthesis and lymphocyte proliferation, or IL-1 production by monocytes.[6-9] SS may also act as a scavenger of toxic reactive oxygen intermediates (ROI)[10,11] and inhibit the lipoxygenase-dependent formation of leukotrienes and hydroxyperoxyeicosanoids.[12-14] SS has also been suggested to specifically inhibit nuclear factor kappa B (NF-κB.[15])

NF-κB is a multisubunit transcription factor that can rapidly activate the transcription of various proteins (reviewed by Siebenlist *et al.*[16] and Baeuerle and Baltimore[17]). NF-κB dimers are commonly composed of the RelA (p65) and NF-κB1 (p50) or NF-κB2 (p52) subunits. NF-κB is sequestered in an inactive cytoplasmic complex by binding to inhibitory kappa B (IκB). For the activation of NF-κB, IκB has to be phosphorylated and consecutively ubiquinated and degraded by the proteasome pathway. Free NF-κB then translocates to the nucleus, binds to the promoter regions of target genes and activates transcription.[18,19] During DR-mediated apoptosis, be it triggered by tumor necrosis factor (TNF)-α,[20] CD95L[21,22] or Apo2L/TRAIL,[23], NF-κB has commonly been considered to mediate a survival pathway.

We here report a novel effect of SS, that is, the differential modulation of DR-mediated apoptosis in glioma cells. These data have important implications for the further development of death ligand-based cancer therapies.

Results

SS protects human malignant glioma cells from CD95L-induced apoptosis upstream of caspase activation and cytochrome *c* release

Different glioma cell lines were treated with CD95L in the absence or presence of SS. The experiments were performed in the absence or presence of an inhibitor of protein synthesis, cycloheximide (CHX), since inhibition of protein synthesis greatly enhances CD95-mediated apoptosis in glioma cells.[27] There was a distinct prevention of CD95L-induced apoptosis by SS in all cell lines, both in the absence and presence of CHX (Figure 1). CD95L-induced apoptosis of glioma cells depends critically on caspase activation and mitochondrial cytochrome *c* release[26,28]. Exposure to SS prevented the CD95L-induced cleavage of caspases 2, 3, 8 and 9 into the active cleavage products, both in the absence and presence of CHX (Figure 2a). Further, SS abrogated CD95L-induced Acetyl-Asp-Glu-Val-Asp-chloromethylcoumarin (Ac-DEVD-amc) cleaving activity in LN-18 and LN-229 cells, both in the absence and presence of CHX (Figure 2b), consistent with the cytotoxicity data (Figure 1).

The release of cytochrome *c* from mitochondria was also blocked by SS. The loss of BCL-x_L protein mediated by CD95L[29] was prevented by SS in the absence of CHX, and attenuated in the presence of CHX. The loss of p21, which accompanies apoptosis induced by CD95L plus CHX[29] was prevented by SS, but SS alone did not increase the protein levels of p21 (Figure 2a). Further, immunoblot analysis confirmed that the levels of BCL-2 and XIAP proteins, which prevent or directly inhibit caspase activity during CD95L-induced apoptosis,[26,30] were not increased in SS-treated cells (data not shown). The levels of FLICE-inhibitory proteins (FLIP) were also not modulated by SS, but a cleavage

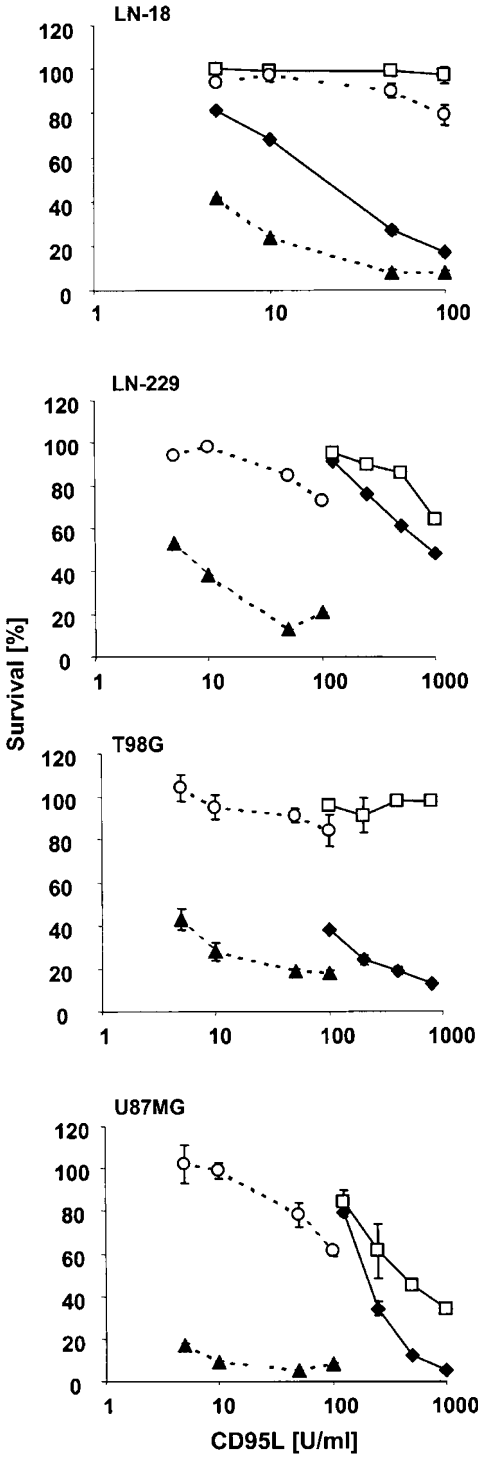
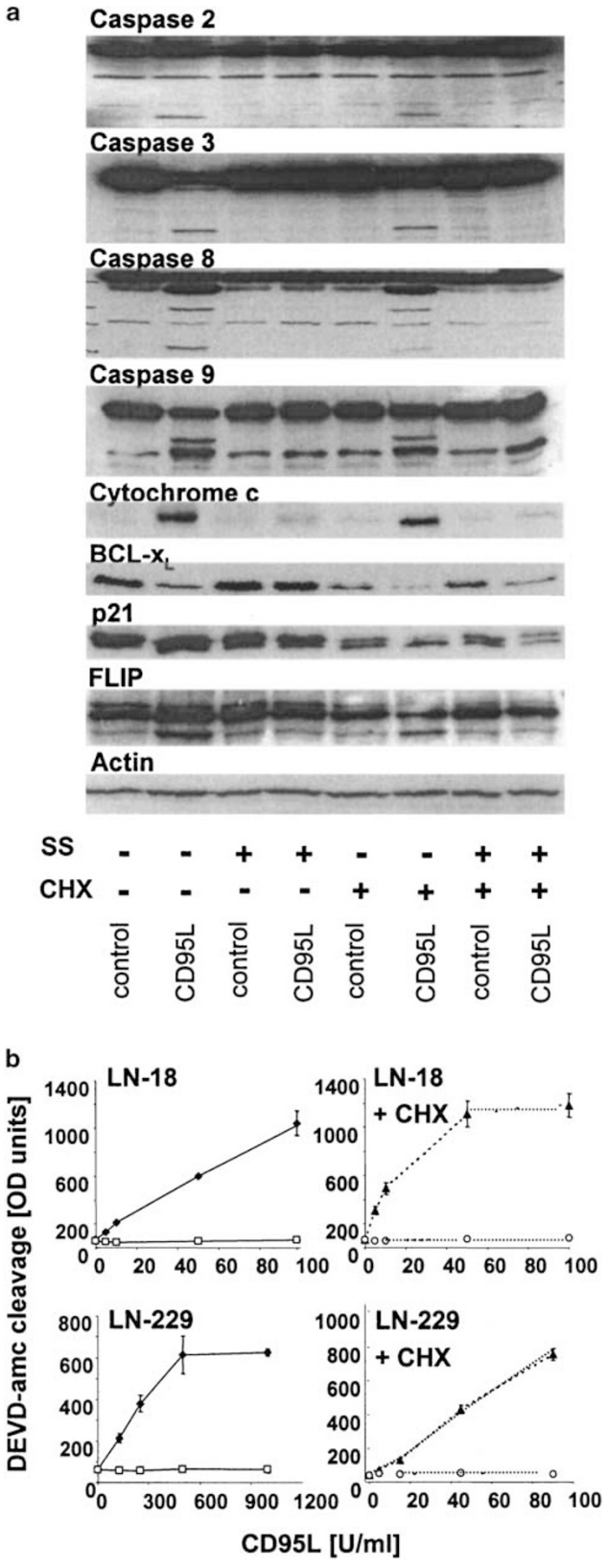


Figure 1 SS prevents CD95L-induced cell death in the absence or presence of CHX. The cells were seeded in 96-well plates (10^4/well), allowed to attach for 24 h, pretreated with 2 mM SS for 1 h (open symbols) or medium alone (filled symbols) and then treated with CD95L in the absence (straight lines) or presence (dashed lines) of CHX (10 μg/ml) (and in the continued absence or presence of SS). Survival was assessed at 16 h by crystal violet assay. Data are expressed as mean percentages of survival and s.e.m. (n=3) relative to untreated cultures or CHX only treated cultures



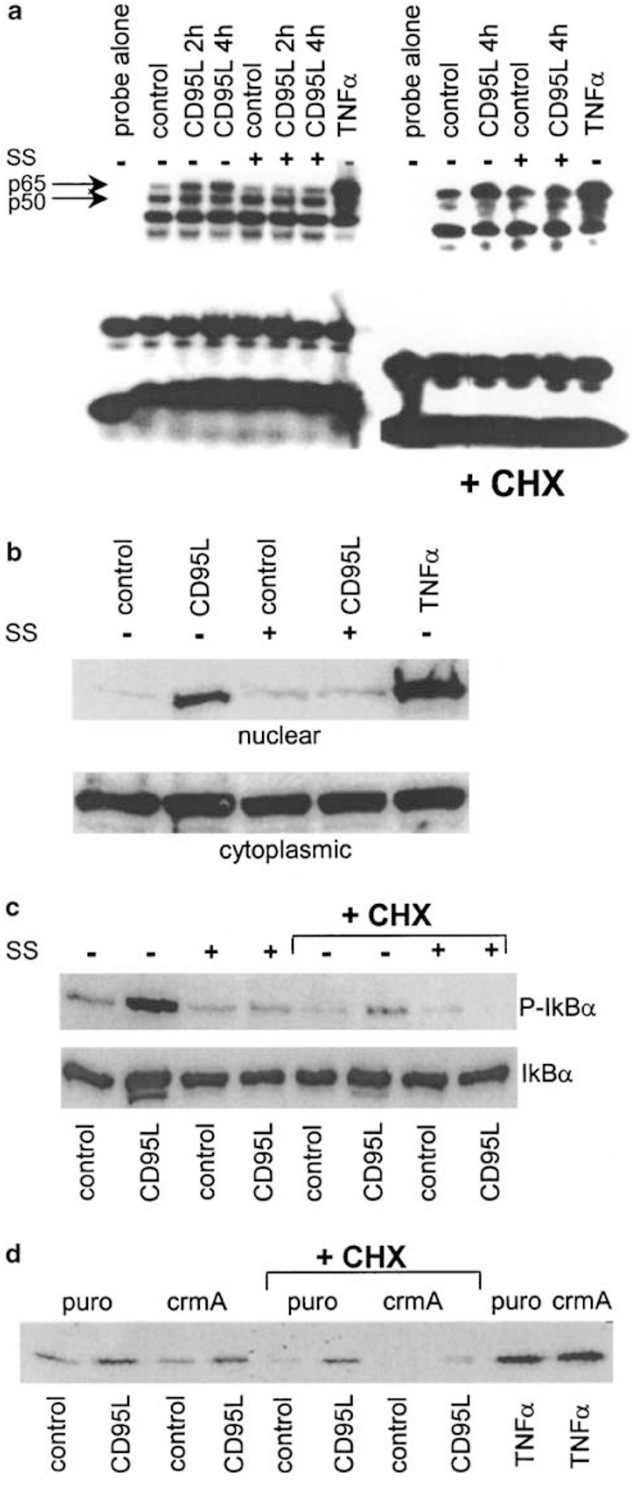
product, consistent with a 43 kDa fragment of FLIP,[31] was detected in the absence, but not in the presence, of SS (Figure 2a). Since CD95 expression at the cell surface is one important predictor of sensitivity to CD95-mediated apoptosis,[32] we monitored whether SS modulated the expression of CD95 or CD95L at the cell surface. LN-18 cells were treated with 2 mM SS or culture medium for 1, 6 or 12 h, and the expression of CD95 or CD95L was assessed by flow cytometry. There was no significant change in CD95 or CD95L expression at the cell surface (data not shown), suggesting that SS-induced CD95 or CD95L expression is not the mechanism underlying SS-mediated prevention of CD95L-induced apoptosis.

SS inhibits CD95L-induced IκB phosphorylation and nuclear translocation and DNA binding of p65 NF-κB

Some effects of SS have been attributed to the inhibition of NF-κB activity.[15] Electrophoretic mobility shift assay (EMSA) revealed a distinct increase in one of the proteins that bound to the NF-κB consensus sequence in response to CD95L, both in the absence or presence of CHX, or TNF-α (positive control) (Figure 3a). Competition assays using an unlabeled consensus sequence for NF-κB or an unspecific consensus sequence for AP-1 showed that the upper two lanes (marked with arrows) bound specifically to the NF-κB consensus sequence (not shown). Supershift assays with antibodies to p65 and p50 NF-κB identified the two lanes as the p65 and p50 subunits of NF-κB. The increase in specific binding was nullified by SS. Immunoblot analysis from nuclear and cytoplasmic extracts accordingly confirmed the nuclear translocation of p65 NF-κB during CD95L-induced apoptosis and its inhibition by SS (Figure 3b). In contrast, the cytoplasmic levels of p65 were unaltered. IκBα binds NF-κB and abrogates its DNA-binding activity. Before NF-κB is activated, IκBα becomes phosphorylated and is then degraded by the proteasome pathway. NF-κB then translocates to the nucleus and binds DNA. IκBα phosphorylation was induced by CD95L with or without coexposure to CHX, and was inhibited by SS, with no change in total IκBα levels (Figure 3c). To determine whether the activation of the NF-κB pathway during CD95L-induced apoptosis requires the caspase cascade, LN-18 cells expressing the viral caspase inhibitor crm-A[26] were examined in parallel. These cells are

Figure 2 SS inhibits CD95L-evoked caspase processing, cytochrome *c* release, loss of Bcl-x_L, and caspase-3-like activity. (**a**) LN-18 cells were pre- (1 h) and cotreated with SS (2 mM) or not, and then treated for 4 h with CD95L (20 U/ml) in the absence of CHX, or with 5 U/ml in the presence of CHX (10 μg/ml). Control cells were untreated or treated with CHX alone. Soluble protein lysates were subjected to SDS-PAGE and immunoblot analysis for caspases 2, 3, 8 and 9, Bcl-x_L, p21, FLIP or ß-actin. Cytosolic cytochrome *c* was extracted as described and analyzed by SDS-PAGE and immunoblot. (**b**) LN-18 or LN-229 cells were seeded in 96-well plates (10^4/well), allowed to attach for 24 h, pre- (1 h) and cotreated with SS (2 mM, open symbols), or not (closed symbols) and then treated for 4 h with CD95L in the absence (straight lines) or presence (dashed lines) of CHX (10 μg/ml). Caspase-3-like enzymatic activity was assessed by DEVD-amc cleavage. Data are expressed as mean optical densities and s.e.m. (n=3)

resistant to CD95L, because the apical caspase-8 is no more activated. Both puro control cells and crm-A-expressing cells showed phosphorylation of IκBα in response to CD95L or TNF-α (Figure 3d), indicating that activation of the NF-κB pathway does not depend on caspase activation and is not sufficient to signal cell death.



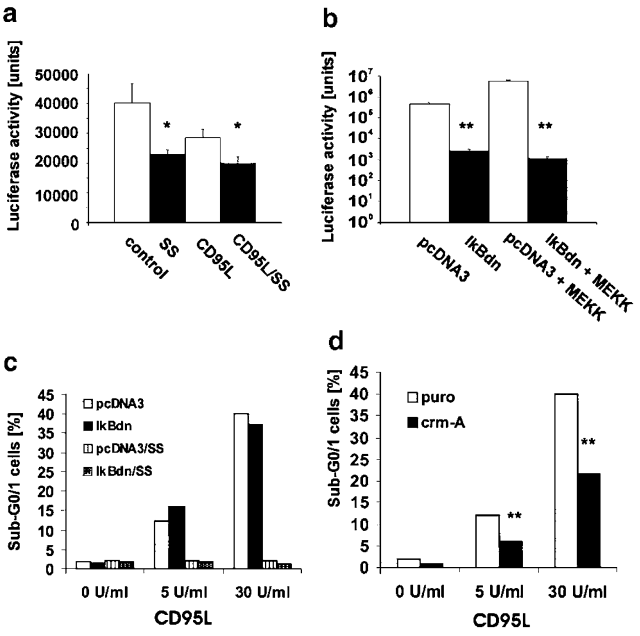


Figure 4 Abrogation of NF-κB activity does not modulate CD95L induced cell death. **a,b**. LN-18 cells were transfected with the NF-κB luciferase reporter gene (0.15 μg/well). At 23 h, the cells were pretreated with 2 mM SS or culture medium for 1 h and then treated with CD95L (20 U/ml) for 4 h (**a**). Alternatively, the cells were co-transfected with pcDNA3 or IκBdn (0.075 μg/well) and MEKK (0.015 μg/well) (**b**). NF-κB transcriptional activity was assessed by luciferase activity. Data in (**a** and **b**) are expressed as mean optical densities and s.e.m. ($^*P<0.05$, $^{**}P<0.01$, *t*-test). (**c**) LN-18 cells were cotransfected with plasmids encoding IκBdn, or pcDNA3 as a control, and GFP at a ratio 2 : 1 and treated with CD95L with or without SS as indicated. The sub-$G_{0/1}$ peak of GFP-positive cells, representing transfected, but dead, cells, was quantified by flow cytometry. (**d**) Cells were transfected as in (**c**) with a plasmid encoding crm-A (black bars) or a puro control plasmid (open bars) and the sub-$G_{0/1}$ peak of GFP-positive cells was assessed by flow cytometry. Data in (**c** and **d**) are representative experiments performed three times with similar results

NF-κB inhibition by IκBdn fails to modulate CD95L-induced cell death

SS inhibited NF-κB luciferase activity in a reporter assay in the absence or presence of CD95L (Figure 4a). Transient transfection with a plasmid encoding dominant-negative IκB (IκBdn), a constitutively active form of IκBα that blocks the activation of NF-κB, markedly inhibited NF-κB luciferase activity too (Figure 4b). IκBdn also abrogated the strong increase in NF-κB activity mediated by transient expression of

Figure 3 SS inhibits CD95L-induced IκBα phosphorylation and nuclear translocation and DNA binding of p65 NF-κB. (**a**) LN-18 cells were untreated or treated for the indicated times with CD95L (20 U/ml) in the absence of CHX (left), or with 5 U/ml in the presence of CHX (10 μg/ml) (right). The cells were pre- and cotreated with SS, or not, as in Figure 1. As a positive control, the cells were treated with TNF-α (10 ng/ml) for 4 h. Nuclear extracts were prepared and EMSA were performed. The arrows indicate the p50 and the p65 subunits of NF-κB as determined by supershift assays. (**b**) Nuclear and cytoplasmic extracts were prepared from CD95L-treated or untreated LN-18 cells (4 h) with or without pretreatment with SS. TNF-α was used as a positive control. Soluble proteins were subjected to SDS-PAGE and immunoblot analysis for p65. (**c,d**). Parental LN-18 (**c**) or LN-18 puro or crm-A (**d**) cells were treated as in (**b**) in the absence or in the presence of CHX (10 μg/ml). Soluble protein lysates were subjected to SDS-PAGE and immunoblot analysis for IκBα or P-IκBα.

mitogen-activated protein/ERK kinase kinase (MEKK), used as a positive control. NF-κB activity induced by MEKK was also strongly reduced by the IκBα-kinase inhibitor, Bay 11-7082 (data not shown). To assess the functional relevance for NF-κB in CD95L-induced apoptosis, the cells were cotransfected with IκBdn and GFP at a 2 : 1 ratio and then treated with CD95L with or without SS. Cell death was assessed by flow cytometry, detected as the sub-$G_{0/1}$ fraction representing apoptotic cells, specifically in the GFP-positive (transfected) cells (Figure 4c). There was no difference in sensitivity to CD95L between LN-18 cells transfected with IκBdn and the control plasmid, and SS rescued both pcDNA3 control cells and cells transfected with IκBdn from CD95L-induced cell death (Figure 4c). In contrast to IκBdn, transient crm-A transfection, used as a positive control, attenuated apoptosis (Figure 4d). Similar to IκBdn, Bay 11-7082 did not modulate CD95L-induced apoptosis (data not shown), indicating that the effects of CD95L on the NF-κB system (Figure 3) are epiphenomenal to cell death, and that SS modulates CD95L-mediated apoptosis in an NF-κB-independent manner.

SS sensitizes human glioma cells to Apo2L/TRAIL-induced cell death in the absence of CHX

Apo2L/TRAIL is a CD95L-homologous death ligand, which has gained specific interest for cancer therapy because of good efficacy in the apparent absence of toxicity *in vivo*.[1,33] Surprisingly, there was a distinct sensitization to Apo2L/TRAIL-induced cell death by SS, using nonhepatocytotoxic Apo2L/TRAIL.0 (Figure 5). Similar results were obtained with His-tagged Apo2L/TRAIL (data not shown). Coexposure to CHX, which sensitizes for Apo2L/TRAIL-induced apoptosis as well as for CD95L-induced apoptosis,[34] nullified or reduced (LN-229) the sensitizing effects of SS. In contrast to the glioma cells, nontumorigenic cells such as postmitotic rat cerebellar granule neurons (day 7) or the astrocytic cell line SV40-FHAS were not sensitive to Apo2L/TRAIL, and SS failed to sensitize these cells for Apo2L/TRAIL-induced apoptosis (data not shown). Exposure to SS resulted in increased expression levels of TRAIL-R2 in LN-229 cells in the absence, but not in the presence, of CHX (Figure 6a). This increase in TRAIL-R2 expression was also observed in LN-229 cells expressing dominant-negative p53, indicating that SS increased TRAIL-R2 expression in a p53-independent manner (data not shown). Further, $p53^{V135A}$-transfected cells were similarly sensitized by SS to Apo2L/TRAIL-induced apoptosis as hygro control cells (data not shown). Expression of the other TRAIL receptors (TRAIL-R1, TRAIL-R3, TRAIL-R4) was not regulated by SS in the absence or presence of CHX in LN-229 cells (data not shown).

Similar to CD95L-induced apoptosis, caspases were cleaved and activated during Apo2L/TRAIL-induced glioma cell death, and cytochrome *c* was released from mitochondria.[25] Cleavage of caspases 2, 3 and 8 and mitochondrial efflux of cytochrome *c* induced in LN-229 cells by low concentrations of Apo2L/TRAIL were strongly enhanced by SS (Figure 6b). The levels of p21 were not modulated by SS or Apo2L/TRAIL, but FLIP cleavage was enhanced in cells cotreated with Apo2L/TRAIL and SS. Similarly DEVD-amc-cleaving caspase activity was markedly enhanced in Apo2L/TRAIL-treated cells coexposed to SS (Figure 6c).

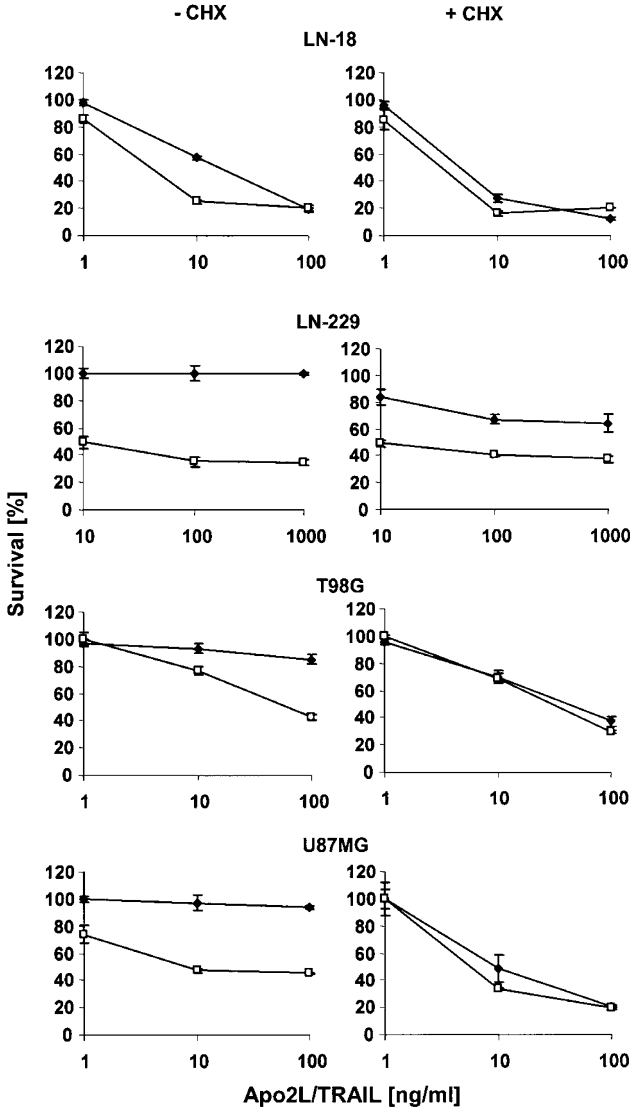


Figure 5 SS sensitizes glioma cells to Apo2L/TRAIL-induced apoptosis in the absence of CHX. The glioma cells were seeded in 96-well plates (10^4/well), allowed to attach for 24 h, pretreated with culture medium alone (filled symbols) or SS (2 mM) (open symbols) for 1 h and treated with Apo2L/TRAIL.0 in the absence (left) or presence (right) of CHX (10 μg/ml) (and in the continued absence or presence of SS). Survival was assessed at 16 h by crystal violet assay. Data are expressed as mean percentages of survival and s.e.m. (n=3) relative to untreated cultures or CHX only treated cultures.

SS-mediated sensitization to Apo2L/TRAIL-induced apoptosis is NF-κB-independent

Using similar approaches as shown above for CD95L-induced apoptosis (Figures 3 and 4), we examined the role of NF-κB for the effects of SS on Apo2L/TRAIL-induced apoptosis. The p65 subunit of NF-κB translocated to the nucleus only upon

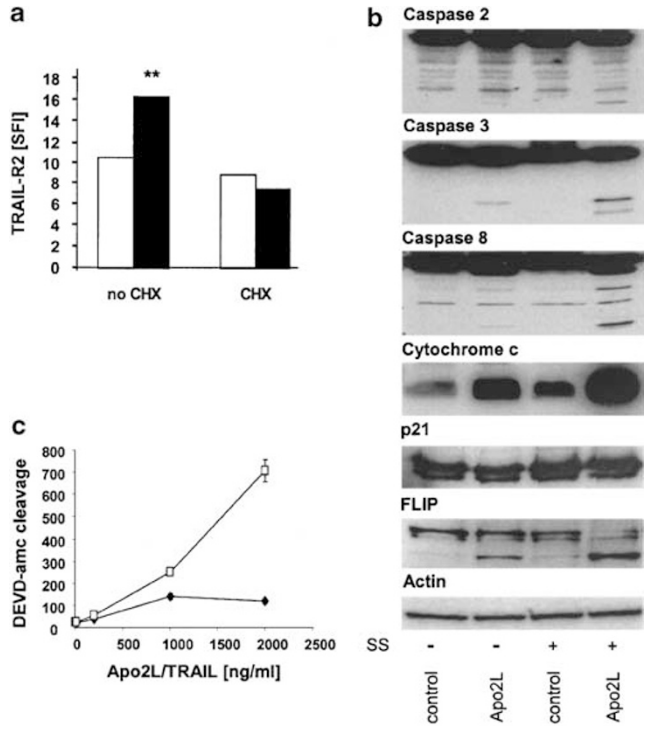
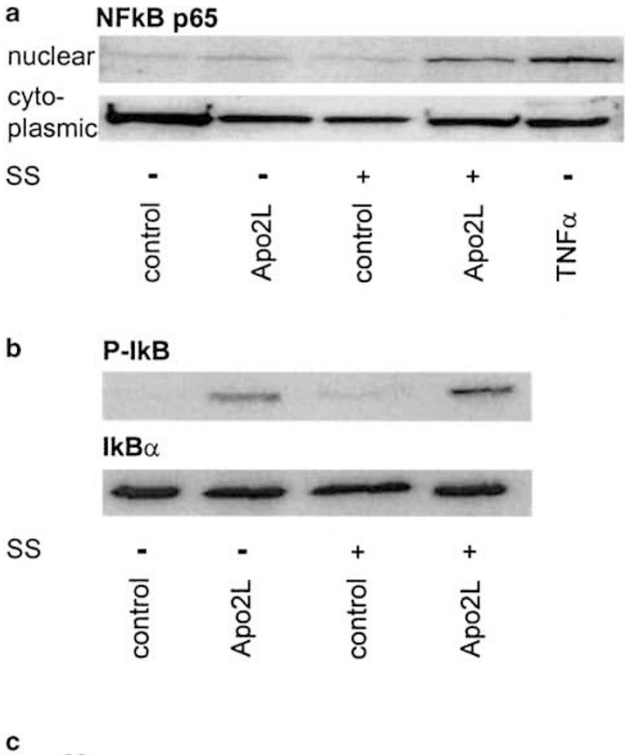
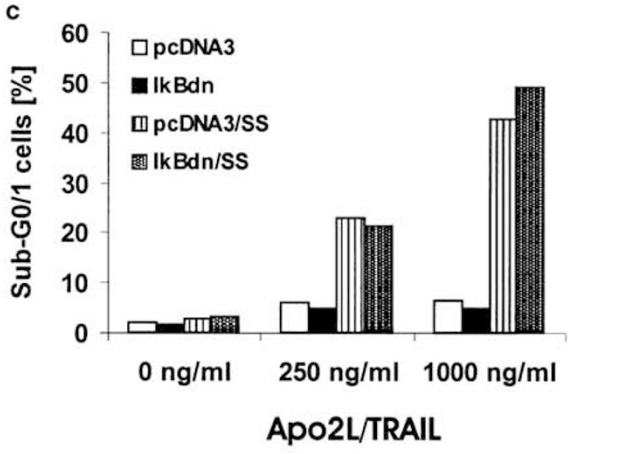


Figure 6 SS enhances TRAIL-R2 expression and Apo2L/TRAIL-induced cell death upstream of caspase activation. (**a**) LN-229 cells were treated with culture medium (open bars) or SS (2 mM, filled bars) for 7 h in the absence or presence of CHX. TRAIL-R2 expression was analyzed by flow cytometry. Data are representative of experiments performed three times with similar results. (**b**) LN-229 cells were pre- and cotreated, or not, with SS as in Figure 1, and treated for 4 h with Apo2L/TRAIL.His (1000 ng/ml). Soluble protein lysates were subjected to SDS-PAGE and immunoblot analysis for caspases 2, 3, 8, cytosolic cytochrome c, p21, FLIP and ß-actin. (**c**) LN-229 cells were seeded in 96-well plates (10^4/well), allowed to attach for 24 h, pretreated with SS (open symbols) or culture medium (closed symbols) and then treated for 4 h with Apo2L/TRAIL.His. Caspase-3-like enzymatic activity was assessed by DEVD-amc cleavage. Data are expressed as mean optical densities and s.e.m.

cotreatment with Apo2L/TRAIL and SS, but not in response to Apo2L/TRAIL alone (Figure 7a). IκBα phosphorylation was induced by Apo2L/TRAIL, and this was enhanced by SS, with total levels of IκBα again unaltered (Figure 7b). To establish a possible link between p65 NF-κB nuclear translocation and enhanced cell death in the presence of SS, experiments similar to those for CD95L, shown in Figure 4, were performed for Apo2L/TRAIL. Inhibition of NF-κB activity by IκBdn determined by a reporter assay was as prominent in LN-229 cells (not shown) as in LN-18 cells (Figure 4b). Similar to the





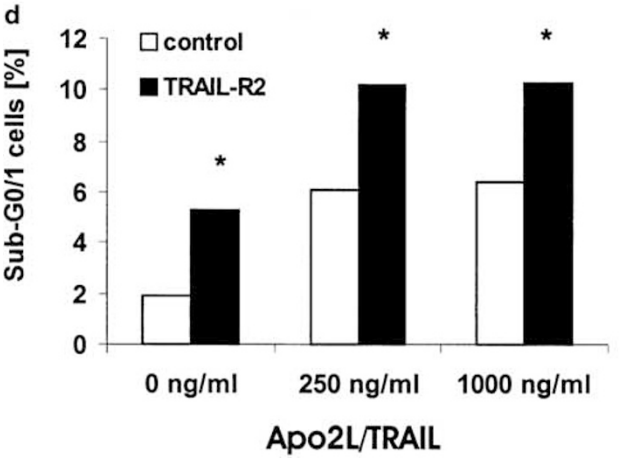


Figure 7 NF-κB-independent sensitization to Apo2L/TRAIL-induced cell death by SS. (**a,b**). LN-229 cells were pre and cotreated, or not, with SS and treated for 4 h with Apo2L/TRAIL.His (1000 ng/ml). Nuclear or cytoplasmic extracts were subjected to immunoblot analysis for p65 NF-κB (**a**). Whole-cell lysates were analyzed for P-IκBα and IκBα (**b**). (**c**) LN-229 cells were cotransfected with plasmids encoding IκBdn, or pcDNA3 as a control, and GFP at a 2 : 1 ratio. After 24 h, cells were treated with Apo2L/TRAIL.His with or without SS (2 mM) as indicated. The sub-$G_{0/1}$ peak of GFP-positive cells was quantified by flow cytometry. (**d**) The cells were transfected as in (**c**) with a plasmid encoding TRAIL-R2 (black bars) or a control plasmid (open bars) and the sub-$G_{0/1}$ peak of GFP-positive cells was assessed by flow cytometry. Data in (**c**) and (**d**) are representative experiments performed three times with similar results

lack of effects on CD95L-induced apoptosis (Figure 4c), IκBdn did not modulate Apo2L/TRAIL-induced apoptosis, whereas SS greatly enhanced cell death in control-transfected and in IκBdn-transfected cells (Figure 7c). In contrast to IκBdn, transient transfection with TRAIL-R2 conferred enhanced baseline cell death and sensitized for Apo2L/TRAIL (Figure 7d).

Given the opposing effects of SS on cell death induced by CD95L and Apo2L/TRAIL, we also examined its effects on cell death induced by other prototype cytotoxic agents, including cytotoxic concentrations of the inhibitor of RNA synthesis, actinomycin D; the inhibitor of protein synthesis, CHX; the broad-spectrum kinase inhibitor, staurosporine; and the free radical generator, H_2O_2. SS was an antagonist to cell death induced by all of these agents (Table 1).

Differential effects of SS on DR-mediated apoptosis in nonglial cell lines

To examine the influence of SS on CD95L and Apo2L/TRAIL-induced cell death in nonglial cancer cell lines, we performed similar experiments with MCF-7 breast and HCT116 colon carcinoma cells (Table 2). In MCF-7 cells, CD95L-induced cell death in the presence of CHX was inhibited, whereas Apo2L/TRAIL-induced cell death was enhanced by SS in the absence and presence of CHX. In HCT116 cells, SS inhibited CD95L-induced cell death, whereas Apo2L/TRAIL-induced cell death was unaffected. Given the putative role of p21 in inhibiting DR-mediated apoptosis,[29] we compared parental HCT116 with isogenic cells lacking p21 (80S14$^{p21-/-}$). Interestingly, in contrast to parental cells, the p21$^{-/-}$ cells were less protected by SS from CD95L-induced cell death, whereas these cells became sensitized to Apo2L/TRAIL in the absence of CHX.

Table 1 SS inhibits cell death induced by actinomycin D, CHX, staurosporine and H_2O_2[a]

	Control	SS (2 mM)
Actinomycin D (10 μM)	50±2	96±5**
CHX (100 μg/ml)	47±4	84±10*
Staurosporine (1 μM)	22±1	69±3**
H_2O_2 (50 μM)	24±2	91±7**

[a]LN-18 cells were seeded in 96-well plates (10^4/well) allowed to attach for 24 h, pre- (1 h) and cotreated with SS (2 mM), or not, and exposed to the agents for 24 h. Survival was assessed by crystal violet assay. Data are expressed as mean percentages of survival and SEM relative to untreated cultures or SS only treated cultures (n=3, *P<0.05, **P<0.01, t-test, effect of SS). Similar results were obtained for LN-229 cells (data not shown)

Modulation of TNF-α-induced cell death in human glioma cell lines by SS

Finally, the glioma cell lines were treated with TNF-α in the absence or presence of SS. The experiments were performed in the absence or presence of CHX. There was a distinct sensitization to TNF-α-induced apoptosis by SS in LN-18 cells in the absence of CHX, whereas there was no significant effect in the other cell lines (Figure 8a). In the presence of CHX, SS failed to sensitize the glioma cells for cell death either, and there was even a trend to inhibition of TNF-α-induced cell death in LN-18 cells (Figure 8a). Further, the cell surface expression of TNF receptor (TNFR) 1 was not significantly modulated by SS (1 and 7 h) in LN-18 cells (Figure 8b).

Discussion

The present study identified SS, a drug used clinically in the treatment of chronic inflammatory bowel diseases or rheumatoid arthritis, as a potent modulator of apoptosis in glioma cells. Unexpectedly, CD95L-mediated apoptosis was

Table 2 Modulation by SS of CD95L- or Apo2L/TRAIL-induced cell death in breast and colon carcinoma cell lines[a]

	Survival (%)			
SS	−	+	−	+
CHX	−	−	+	+
MCF-7				
CD95L (600 U/ml, 200 U/ml+CHX)	61±3.6	62±5.6	58±1.0	80±5.8*
Apo2L (250 ng/ml, 100 ng/ml+CHX)	94±2.6	54±2**	52±4	36±1.7*
HCT116 (p21$^{+/+}$)				
CD95L (100 U/ml, 0.8 U/ml+CHX)	17±0.6	55±6**	57±3.3	85±7.3**
Apo2L (40 ng/ml, 10 ng/ml+CHX)	52±8.8	56±5.2	28±1.2	35±5.4
HCT116 80S14p21$^{-/-}$				
CD95L (100 U/ml, 0.8 U/ml+CHX)	11±0.6	20.6±3*	37±5	42±2.5
Apo2L (40 ng/ml, 10 ng/ml+CHX)	89±7.4	54±3*	26±2	25±0.7

[a]MCF-7 or HCT116 cells were seeded in 96-well plates (10^4/well) allowed to attach for 24 h, pre- (1 h) and cotreated with SS (2 mM for MCF-7, 0.5 mM for HCT116 cells) and CHX (10 μg/ml), or not, and exposed to CD95L or Apo2L/TRAIL for 16 h. Survival was assessed by crystal violet assay. Data are expressed as mean percentages of survival and s.e.m. relative to untreated cultures or SS only treated cultures with or without CHX (n=3, *P<0.05, **P<0.01, t-test, effect of SS)

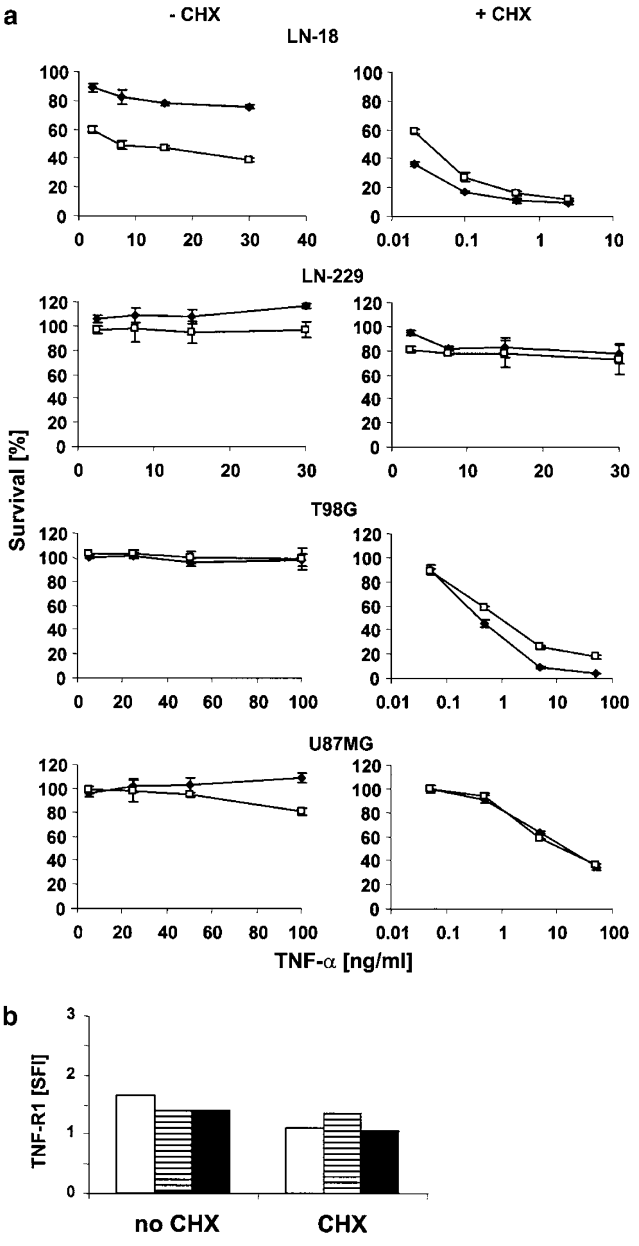


Figure 8 Effects of SS on TNF-α-induced cell death in the absence or presence of CHX. (**a**) The cells were seeded in 96-well plates (10^4/well), allowed to attach for 24 h, pretreated with 2 mM SS for 1 h (open symbols) or medium alone (filled symbols) and then treated with TNF-α in the absence (left) or presence (right) of CHX (10 μg/ml). Survival was assessed at 16 h by crystal violet assay. Data are expressed as mean percentages of survival and s.e.m. (n=3) relative to untreated cultures or CHX only treated cultures. (**b**) LN-18 cells were treated with culture medium (open bars) or SS (2 mM) for 1 h (striped bars) or 7 h (black bars) in the absence or presence of CHX. TNF-R1 expression was analyzed by flow cytometry

inhibited (Figures 1 and 2), whereas Apo2L/TRAIL-induced apoptosis was potentiated (Figures 5 and 6), by SS. The inhibition of cell death by SS induced by various other cytotoxic agents of different modes of action (Table 1) is consistent with a general cytoprotective effect of SS in human malignant glioma cells, whereas the death-promoting effects of SS on Apo2L/TRAIL-induced apoptosis were the exceptional observation in this study. Importantly, the data summarized in Table 2 indicate that our observations are not restricted to glial cancer cells, but may have broader implications for the treatment of cancer.

SS-mediated cytoprotection did not require new protein synthesis since the effect was not nullified by an inhibitor of protein synthesis, CHX (Figure 1). In contrast, the sensitization to Apo2L/TRAIL-induced apoptosis was distinctly stronger (LN-229) or became apparent (LN-18, T98G, U87MG) only in the absence of CHX (Figure 5). These observations indicate that SS and CHX act on the same intracellular target or that the sensitizing effect of SS depends on new protein synthesis. A p53-independent upregulation of TRAIL-R2 detected in the absence, but not presence of CHX, was identified as a candidate mechanism mediating the sensitizing effect of SS (Figure 6).

Since SS has been attributed, among others, NF-κB inhibitory activity,[15] we sought to link a differential activation of the NF-κB pathway to the opposing effects of SS on CD95L- and Apo2L/TRAIL-induced apoptosis. NF-κB is a transcription factor commonly attributed a role in cell survival pathways. The most prominent example is TNF-α-induced cell death where NF-κB inhibits cell death, unless protein synthesis is blocked by inhibitors of RNA or protein synthesis.[20] The role of NF-κB in CD95L-induced cell death is less clear. While NF-κB may mediate enhanced expression of CD95[35,36] and CD95L[37,38] in certain paradigms of cell death, the modulation of cell death induced by exogenous CD95L by NF-κB has not been well defined.[39] Proteolytic cleavage of NF-κB in response to CD95L has been reported in T cells,[40] but this was not observed in a quantitative manner in the glioma cells examined here (Figures 3 and 7). NF-κB may inhibit CD95L-induced cell death via upregulation of cFLIP expression,[22] but there was no modulation of basal cFLIP expression by SS either (Figure 2).

During Apo2L/TRAIL-induced apoptosis, TRAIL receptors 1, 2 and 4 have been proposed to mediate NF-κB activation that inhibits cell death in melanoma cells[41] whereas inhibition of NF-κB augments Apo2L/TRAIL-induced apoptosis in colon carcinoma cells.[42,43] One study reporting the augmentation of Apo2L/TRAIL-induced apoptosis of Jurkat- and colon carcinoma cells by SS[23] concluded that the inhibition of NF-κB by SS was the mechanism of augmentation of Apo2L/TRAIL-induced cell death. Further, the anti-inflammatory agent, sulindac, was proposed to augment Apo2L/TRAIL-induced cell death via the inhibition of NF-κB-dependent Bcl-x_L expression.[43]

We find that cytotoxic concentrations of CD95L induce IκBα phosphorylation (Figure 3c), NF-κB nuclear translocation (Figure 3b) and NF-κB DNA binding as assessed by EMSA (Figure 3a). For unknown reasons, CD95L did not enhance NF-κB-related luciferase activity of NF-κB (Figure 4a). Anyhow, the activation of NF-κB played no decisive role in cell death since transient expression of IκBdn greatly reduced NF-κB activity (Figure 4b), but did not affect cell death (Figure 4c). We assume that the activation of NF-κB upon stimulation of CD95 or the TRAIL receptors has other functions than the modulation of cell death which may be relevant for cells surviving this challenge, for example, to mediate the

CD95L- or TRAIL-induced production of ILs or even proliferation.[44-48] Prevention of such DR-mediated effects by SS might well mediate some of the anti-inflammatory properties of SS.

The molecular mechanisms mediating the protective effects of SS await clarification. The protection from H_2O_2-induced cell death suggests an antioxidant effect of SS, and SS has indeed been attributed oxygen free radical-scavenging activity.[49,50] However, CD95L-induced apoptosis of human glioma cells appears not to be mediated by ROIs[51] (data not shown). Further, SS inhibits lipoxygenases,[12–14] and the 5-lipoxygenase inhibitor nordihydroguaretic acid (NDGA) has been shown to inhibit CD95L-induced cell death in glioma cells.[26,51] Interestingly, NDGA differentially modulates apoptosis induced by CD95L or Apo2L/TRAIL just as does SS (data not shown). In contrast, boswellic acids, another class of lipoxygenase inhibitors proposed to interfere with edema formation in human glioma patients,[52] do not inhibit CD95L-induced apoptosis.[53]

Our data support the notion that the differential effect of SS on CD95L- and Apo2L/TRAIL-induced cell death is mediated upstream in the signaling cascade, for example at the level of DISC formation. In this context, it is also still unknown why ligation of CD95 on normal hepatocytes leads to cell death whereas ligation of Apo2L/TRAIL receptors does not.[54] Altogether these observations raise the possibility of hitherto unknown adaptor molecules, which interact with CD95 or Apo2L/TRAIL receptors and are possibly regulated differentially by SS.

In conclusion, the differential effects of SS on death ligand-induced apoptosis reported here provide a valuable tool to dissociate the pathways mediating CD95L- *versus* Apo2L/TRAIL-induced apoptosis. In view of the current interest in Apo2L/TRAIL as an anticancer agent,[55,56] specifically the potentiation by SS of Apo2L/TRAIL-induced apoptosis may define a novel pathway to potentiate Apo2L/TRAIL-based cancer therapy. This is particularly relevant for gliomas, since SS does not sensitize postmitotic rat neurons or the astrocytic cell line SV40-FHAS for Apo2L/TRAIL-induced apoptosis (data not shown), suggesting that this effect may be specific for tumor cells in the brain.

Materials and Methods

Reagents

Actinomycin D, staurosporine, etoposide, hydrogen peroxide, CHX, luciferin, coenzyme A, Adenosinetriphosphate (ATP), dithiothreitol (DTT), SS, propidium iodide and ribonuclease A were purchased from Sigma (St. Louis, MO, USA). Acetyl-Asp-Glu-Val-Asp-chloromethylcoumarin Ac-DEVD-amc was obtained from Biomol (Plymouth Meeting, PA, USA). TNF-α and FuGene transfection reagent were from Roche (Mannheim, Germany). Accutase was obtained from PAA Laboratories (Wien, Austria). CD95L was obtained from the supernatant of CD95L-transfected N2A murine neuroblastoma cells.[24] The activity of the supernatant was defined as 1/1 : X, where X corresponds to the dilution of supernatant that kills 50% of LN-18 cells within 24 h in a 100 μl volume microtiter assay. His-tagged Apo2L/TRAIL.His and nontagged Apo2L/TRAIL.0 were kindly provided by A. Ashkenazi (Genentech South, San Francisco, CA, USA). TNFR1 antibody was kindly provided by Dr. G Jung (Tübingen, Germany). Goat polyclonal anticaspase 2, rabbit polyclonal anti-p21, mouse monoclonal anti-NF-κB p65 and rabbit polyclonal anti-NF-κB p50 antibodies were obtained from Santa Cruz Biotechnology (Santacruz, CA, USA), mouse monoclonal anticaspase-3 antibody from Transduction Laboratories (Lexington, KY, USA), mouse monoclonal anticytochrome *c* and anti-BCL-x_L antibodies from PharMingen (San Diego, CA, USA), mouse monoclonal anti-IκB antibody from Alexis (San Diego, CA, USA), rabbit polyclonal anti-phosphorylated IκB (P-IκB) antibody from Cell Signaling (Frankfurt, Germany). Caspase-8 antibody C15 (mouse monoclonal) was kindly provided by P.H. Krammer (Heidelberg, Germany), caspase-9 antibody (mouse monoclonal, clone 2-22) was kindly provided by YA Lazebnik (Cold Spring Harbor, NY, USA), Apo2L/TRAIL receptor antibodies were kindly provided by H Walczak (Heidelberg, Germany), mouse anti-FLIP antibody (NF6) was kindly provided by C Scaffidi (Bethesda, MD, USA). The HRP-coupled secondary anti-goat, anti-rabbit and anti-mouse antibodies were from Santa Cruz Biotechnology. A plasmid encoding IκBdn was kindly provided by Dr. P Daniel (Berlin, Germany). The NF-κB-luc *cis*-reporter gene plasmid was from Stratagene (Amsterdam, Netherlands, 219077).

Cell culture

LN-18, LN-229, U87MG and T98G human malignant glioma cells were kindly provided by Dr. N de Tribolet (Lausanne, Switzerland). Glioma cells stably expressing $p53^{V135A}$ were transfected as described before[25] using the $p53^{V135A}$ hygro vector kindly provided by MF Clarke (Pittsburgh, PA, USA). MCF-7 human breast carcinoma cells were kindly provided by S Wesselborg (Tübingen, Germany). Crm-A-expressing cell lines were obtained by transfection using the Flag-crm-A-puro construct and were compared with puro control cells.[26] These cell lines were maintained in DMEM containing 10% fetal calf serum, 2 mM glutamine and penicillin (100 IU/ml)/streptomycine (100 μg/ml). The human colon carcinoma cell line HCT116 and its derivative, $80S14^{p21-/-}$, were a gift from B Vogelstein (Baltimore, MD, USA). These cells were cultured in McCoýs 5A supplemented with 10% fetal calf serum and antibiotics. SV40-FHAS human immortalized astrocytes were kindly provided by D Stanimirovic (Institute of Biological Sciences, National Research Council of Canada, Ottawa, Canada). Rat cerebellar granule neurons were prepared as described.[57]

Viability assay

Cell viability was measured by crystal violet staining. The cell culture medium was removed and surviving cells were stained with 0.5% crystal violet in 20% methanol for 10 min. The plates were washed extensively under running tap water, air-dried and optical density values were read in an ELISA reader at 550 nm wavelength.

Caspase-3-like enzymatic activity

The cells were seeded in 96-well plates (10 000 cells per well) and allowed to attach for 24 h. Then the cells were treated with CD95L or Apo2L/TRAIL as indicated. The cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 8.0), 60 mM NaCl, 2.5 mM EDTA and 0.25% NP40 for 10 min at 37°C. Then the substrate Ac-DEVD-amc (12.5 μM), diluted in phosphate-buffered saline (PBS), was added and incubated at 37°C for 15 min. Caspase-3-like activity was measured every 15 min for 1 h using a CytoFluor 2350 Millipore fluorimeter at 360 nm excitation and 480 nm emission wavelengths.

Flow cytometry

For CD95 staining, the cells were washed in PBS and then incubated in flow cytometry buffer (1% bovine serum albumin, 0.01% sodium azide in PBS) containing 10% sheep serum for 20 min at 4°C. After centrifugation, the cells were resuspended in flow cytometry buffer containing anti-CD95 antibody (1 μg/ml, mouse IgG_1, Immunotechnology, Hamburg, Germany) or mouse IgG_1 as a control. After 1 h incubation the cells were washed in flow cytometry buffer and then incubated in buffer containing sheep anti-mouse IgG, FITC-labeled (Sigma), diluted 1 : 256 for 20 min at 4°C. The cells were washed, fixed in 1% formaldehyde and analyzed by flow cytometry. The level of expression was calculated as the specific fluorescence index derived from the ratio of fluorescent signal obtained with the specific antibody and an isotype control antibody.

For Apo2L/TRAIL receptor measurement, the cells were detached with accutase, harvested with the supernatants in PBS, washed and resuspended in flow cytometry buffer containing 10% rabbit serum for 30 min at 4°C. After centrifugation, the cells were resuspended in flow cytometry buffer with Apo2L/TRAIL receptor 1 2, 3 or 4 antibodies (10 μg/ml) or mouse IgG_1 as a control. After 1 h incubation, the cells were washed in flow cytometry buffer and then incubated with biotinylated rabbit anti-mouse IgG (Sigma) diluted 1 : 200 in flow cytometry buffer for 20 min at 4°C. Cells were again washed and incubated with streptavidin–phycoerythrin (1 : 20, Sigma) in 100 μl flow cytometry buffer for 20 min at 4°C. The cells were washed, fixed in 1% formaldehyde and analyzed by flow cytometry.

TNFR-1 expression was measured accordingly. Blocking serum was sheep serum (10% in flow cytometry buffer), the cells were incubated with mouse TNF-R1 antibody or mouse IgG2a as an isotype control (10 μg/ml), labeled with FITC-conjugated anti-mouse IgG (1 : 256) and analyzed by flow cytometry.

Cotransfection assay and analysis of the sub-$G_{0/1}$ peak by flow cytometry

The cells were transfected with plasmids encoding IκBdn, or pcDNA3 as a control, and encoding membrane-bound GFP in a ratio of 2 : 1 using FuGene transfection reagent. After 24 h, the cells were treated with SS, CD95L or Apo2L/TRAIL for 16 h. Cells were detached with accutase, harvested with the supernatants in PBS, washed, fixed in 70% ethanol (20–30 min on ice), stained with propidium iodide (50 μg/ml) in the presence of RNase A (100 μg/ml) for 30 min on ice and subjected to cell cycle analysis using flow cytometry. The sub-$G_{0/1}$ peak was quantified and represented the nonviable cell population.

Immunoblot analysis

Subconfluent glioma cells were treated with SS, CD95L or Apo2L/TRAIL as indicated. Soluble protein lysates were obtained and SDS-PAGE with electroblotting performed as described.[27] Enhanced chemoluminescence (Amersham, Braunschweig, Germany) was used for detection.

Measurement of cytochrome *c* release

The cells were washed with PBS and lysed with MSH (mannitol, sucrose, HEPES) buffer plus digitonin (210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 200 μM EGTA, 5 mM succinate, 0.15% BSA, 40 μg/ml digitonin) at 4°C. After lysis, the supernatant was removed and centrifuged immediately for 10 min at 13000 $\times$ *g*. An equal volume of 10% trichloroacetic acid was added to the supernatant. Samples were kept at –20°C for at least 30 min. After another centrifugation (15 min at 13 000 $\times$ *g*), the pellets were dissolved in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and analyzed for cytochrome *c* content by SDS-PAGE and immunoblot.

Preparation of cytoplasmic and nuclear extracts

The cells were washed with ice-cold PBS, harvested by scraping into PBS and pelleted in a 1.5 ml microcentrifuge tube. The pellet was suspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After 20 (LN-18) or 30 (LN-229) min incubation on ice, 25 μl of 10% Nonidet P-40 was added, and the samples were vortexed for 10 s and then centrifuged briefly. The supernatant represented the cytoplasmic extracts. The nuclear pellet was resuspended in 30–50 μl of buffer C (20 mM HEPES, pH 7.9, 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerine, 1 mM DTT, 0.5 mM PMSF) and incubated at 4°C with shaking for 30 min. Nuclear debris was removed by centrifugation at 4°C. The protein concentration was determined with Bio-Rad Protein assay reagent.

Electrophoretic mobility shift assay

Nuclear proteins (15–20 μg) were incubated for 25 min at room temperature in a total volume of 20 μl with 1.4 μl of poly(dI-dC) (1 μg/μl) (Amersham Pharmacia Biotech), 2 μl BSA (10 μg/μl), 4 μl buffer F (20% Ficoll 400, 100 mM HEPES, pH 7.9, 300 mM KCl, 10 mM DTT, 1 mM PMSF), 2 μl buffer D + (20 mM HEPES, pH 7.9, 4% glycerine, 100 mM KCl, 0.5 mM EDTA, 0.625‰ NP-40, 2 mM DTT, 1 mM PMSF) and ^{32}P-labeled double-strand NF-κB binding oligonucleotide (10^5 cpm). The NF-κB oligonucleotide had the sequence: 5′-AGTTGAGGGGACTTTCC-CAGGC-3′. For supershift assays, 2–4 μg of polyclonal antibodies to different subunits of NF-κB were incubated with nuclear proteins for 30 min on ice prior to addition of ^{32}P-labeled NF-κB probe. The samples were resolved on 4% polyacrylamide gels that were dried and evaluated by autoradiography.

NF-κB luciferase assay

The cells were transfected in a 96-well plate with the PathDetect® NF-κB *cis*-reporter gene plasmid (#219077, Stratagene) using FuGene. The NF-κB-responsive enhancer was $(TGGGGACTTTCCGC)_5$. Cotransfection with a plasmid encoding MEKK, which activates the NF-κB pathway, was included as a positive control. At 24 h after transfection, the cells were treated with SS, CD95L or Apo2L/TRAIL as indicated, washed with PBS and lysed using 30 μl Cell Lysis Buffer (8 mM tricine, pH 7.8, 10 mM NaCl, 0.4 mM EDTA, 0.2 mM $MgSO_4$, 1 mM DTT, 0.2% Triton X-100). After one freeze–thaw cycle, the lysates were transferred to a LumiNuncTM plate (Nunc, Roskilde, Denmark), 60 μl luciferase assay reagent (40 mM tricine, 10 mM $MgSO_4$, 0.5 mM EDTA, 10 mM DTT, 0.5 mM ATP, 0.5 mM coenzyme A, 0.5 mM luciferin) was added automatically, and the luminescence was measured in a LumimatPlus (EG&G Berthold, Pforzheim, Germany). The background was subtracted from all values.

Statistical analysis

The data are usually representative of experiments performed at least three times with similar results. Viability assays were tested for significance by *t*-test ($^*P<0.05$, $^{**}P<0.01$).

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