Lipoxygenase inhibitors block CD95 ligand-mediated apoptosis of human malignant glioma cells

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Abstract CD95 ligand is a cytotoxic cytokine that induces apoptosis. Here we report that CD95-mediated apoptosis of human malignant glioma cells is associated with arachidonic acid (AA) release. Inhibitors of phospholipase A_2, phospholipase C or diacylglycerol lipase have minor effects on AA release and fail to modulate apoptosis. Formation of two AA metabolites generated during CD95-dependent apoptosis is attenuated by the lipoxygenase inhibitor, nordihydroguaretic acid (NDGA). NDGA also blocks CD95 ligand-induced apoptosis. This effect is independent of antioxidant properties of NDGA. Lipoxygenase may thus play a critical role in CD95 ligand-induced apoptosis of human malignant glioma cells.

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Key words: Glioma; CD95; Nordihydroguaretic acid; Lipoxygenase; Apoptosis; Arachidonic acid

1. Introduction

CD95 (Fas/APO-1) and tumor necrosis factor receptor p55 (TNF-R1) belong to the nerve growth factor (NGF) receptor superfamily. Activation of both receptors by their respective ligands, CD95 ligand and TNF, triggers apoptosis in susceptible target cells [1,2]. CD95-dependent signaling involves FADD/MORT1 (Fas-associating protein with death domain) [3] which binds to an interleukin 1-converting enzyme (ICE)-like protease, FLICE (FADD-like ICE), an upstream component of a proteolytic cascade consisting of other proteases including CPP32 [4]. CD95 signaling also involves activation of acidic and neutral sphingomyelinase [5,6], resulting in generation of ceramide. Activation of neutral sphingomyelinase may be linked to cytoplasmic phospholipase A_2 ($cPLA_2$) [6,7]. Targets of ceramide include stress-activated protein kinase JNK [8], Ras [9] and ceramide-activated protein kinase [10].

The signaling pathways of the TNF receptor type I are similar and include interactions of TRADD (TNFR1-associated death domain) with FADD and TRAF2 (TNFR-associated factor 2) [11], ceramide generation through activation of sphingomyelinases [2], activation of ceramide-activated protein kinase [12], ICE-like proteases [13], NFκB [2], Ras [14], Raf-1 [15] and $cPLA_2$ [16]. Activation of $cPLA_2$ results in the release of arachidonic acid (AA) which is metabolized by lipoxygenases and cycloxygenases. The AA metabolites of 5-, 12-, and 15-lipoxygenase, produced by oxidation of AA, are hydroperoxy-, and hydroxy-eicosatetraenoic acids, dihydroxy-, epoxy-eicosatrienoic acids (HPETES, HETES, DHET, EET), and leukotrienes. TNF toxicity of L929 fibrosarcoma cells involves $cPLA_2$ [16]. The levels of $cPLA_2$ in L929 sublines correlate with sensitivity to TNF but not with sensitivity to anti-human CD95 antibodies after transfection of CD95 [17]. Further, inhibitors of AA metabolism prevent TNF toxicity but not that of CD95 antibodies [17]. Similarly, activation of $cPLA_2$ is insufficient for CD95-dependent apoptosis of HuT78 lymphoma cells [6]. Yet, other authors concluded that $cPLA_2$ did play a role in CD95 antibody-induced apoptosis of L929 cells expressing human CD95 [10]. The specific role of AA metabolites in the induction of apoptosis is unclear. Several observations suggest a central role of lipoxygenase metabolites in TNF toxicity of L929 and TA1 adipogenic cells [18,19]. 15-HPETE induces apoptosis in HIV-infected T cells [20].

We are interested in CD95 targeting as a novel approach of immunotherapy for human malignant glioma [21–23]. While expression of CD95 is a positive predictor of sensitivity to CD95-mediated apoptosis, variations in CD95 expression do not account for all heterogeneity of sensitivity to CD95-mediated apoptosis [23]. Thus, some glioma cell lines require the co-exposure to CD95 ligand and inhibitors of RNA and protein synthesis for sensitization to apoptosis, indicating the constitutive or induced expression of labile cytoprotective proteins which inhibit apoptosis. The signal transduction events during CD95-mediated apoptosis of human glioma cells have not been studied in detail. Here we examine the role of AA metabolism in CD95 ligand-induced apoptosis of these cells. AA release might be of particular interest since dexamethasone, an inhibitor of PLA_2, attenuates CD95-mediated glioma cell apoptosis [21].

2. Materials and methods

2.1. Chemicals

[^{3}H]Thymidine (185 Gbq/mmol), [5,6,8,9,11,12,14,15-^{3}H]AA (230 Ci/mmol) and 1-stearoyl-2-[1-^{14}C]arachidonyl-sn-glycero-3-phosphocholine (54 mCi/mmol) were obtained from Amersham (Braunschweig, Germany). Quinacrine, dexamethasone, aristolochic acid, nordihydroguaretic acid (NDGA), indomethacin, esculetin, *N*-tert-butyl-α-phenylnitrone (PBN), superoxide dismutase (SOD), *N*-acetyl-L-cysteine and butylated hydroxytoluene were purchased from Sigma (St. Louis, MO). Arachidonyl trifluoromethylketone ($AACOF_3$), D609 and RHC 80267 were from Biomol (Hamburg, Germany), 2′,7′-dihydrofluorescein diacetate ($DCF-H_2$) was obtained from Molecular Probes (Groningen, The Netherlands).

2.2. Cell lines and cell culture

Human malignant glioma cell lines LN-18, LN-229 and LN-308 were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) and maintained as described [21–23]. L929 mouse fibrosarcoma cells, kindly provided by Prof. P.H. Krammer (Heidelberg, Germany), were cultured in RPMI-1640 containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The murine neuroblastoma cell line Neuro-2A was maintained in MEM supplemented with 10% FCS, 2 mM glutamine, 1% non-essential amino acids and penicillin/streptomycin. Neuro-2A cells engineered to produce soluble murine CD95 ligand

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have been described [24]. One unit of cytotoxic activity of CD95 ligand in Neuro-2A supernatants was defined as the activity required for half-maximal killing of the CD95 antibody-sensitive glioma cell line, LN-18 [21]. The experiments using CD95 ligand-containing supernatants were performed using as control the supernatant from pooled neo vector control cells. LN-308 cells forced to express human CD95 driven by the CMV promoter of the BCMGS vector have been described [23].

2.3. Flow cytometry

CD95 expression at the cell surface was measured by flow cytometry [21,23]. The expression level was calculated as the specific fluorescence index (SFI) derived from the ratio of fluorescent signal obtained with the specific CD95 antibody and an isotype control antibody.

2.4. Assessment of viability and apoptosis

Membrane integrity was assessed by trypan blue exclusion or LDH release, using a commercial LDH assay kit (Boehringer, Mannheim, Germany). For most cytotoxicity assays, the cells were seeded in 96-well plates (10^4 cells/well) and allowed to attach for 24 h. In some experiments, the cells were pre-incubated with enzyme inhibitors for 2 h and then exposed to CD95 ligand for 16 h in absence or presence of cycloheximide (CHX). Growth and viability were assessed by crystal violet staining in most assays. Proliferation was also measured by [^{3}H]thymidine incorporation. DNA fragmentation was measured by quantitative DNA fluorometry [25]. Formation of reactive oxygen species was measured in the Cytofluor 2350 plate reader (Millipore, Bedford, MA) at 485 nm excitation and 530 nm emission after incubation of cells for 30 min with DCF-H_2 (1 μg/ml) at different time points after exposure to CD95 ligand [26].

2.5. Determination of AA release

Glioma cells seeded in 6-well plates were incubated for 24 h with [^{3}H]AA, washed, and exposed to CD95 ligand. Medium samples were collected at specified time points, centrifuged, and radioactivity measured in a liquid scintillation counter. The cells were lysed and organelles separated with differential centrifugation.

2.6. cPLA$_2$ assay

The cells were treated as indicated and the cPLA$_2$ assay performed as described [6].

2.7. Extraction and thin layer chromatographic (TLC) analysis of AA metabolites

The glioma cells were labeled with [^{3}H]AA as described above and stimulated with CD95 ligand (80 U/ml) in the absence or presence of CHX (10 μg/ml) for 8 h. The supernatants were centrifuged for 10 min at 4000 rpm and lipids extracted as described [27]. Separation of lipids was performed using a solvent system consisting of chloroform/methanol/glacial acetic acid/water (100:60:16:8, v/v). Iodine-stained bands comigrating with the respective standards were isolated and measured in a liquid scintillation counter.

3. Results

3.1. CD95-mediated apoptosis of human malignant glioma cells is associated with the release of AA

The role of AA metabolism in CD95-mediated apoptosis of human malignant glioma cells was examined in three glioma cell lines with different patterns of sensitivity to CD95 ligand (Table 1). LN-18 expresses moderate levels of CD95 and is highly sensitive to CD95 ligand. LN-229 exhibits high expression of CD95 but is rather resistant to CD95 ligand unless co-exposed to inhibitors of RNA and protein synthesis. LN-308 is resistant to CD95 ligand because of little CD95 expression at the cell surface. LN-308 cells engineered to express high levels of CD95 acquire sensitivity to CD95-mediated apoptosis [23]. Fig. 1 illustrates that CD95 ligand-induced apoptosis evolves more rapidly in LN-229 than in LN-18 cells but that co-exposure to CHX is required for apoptosis in LN-229 cells.

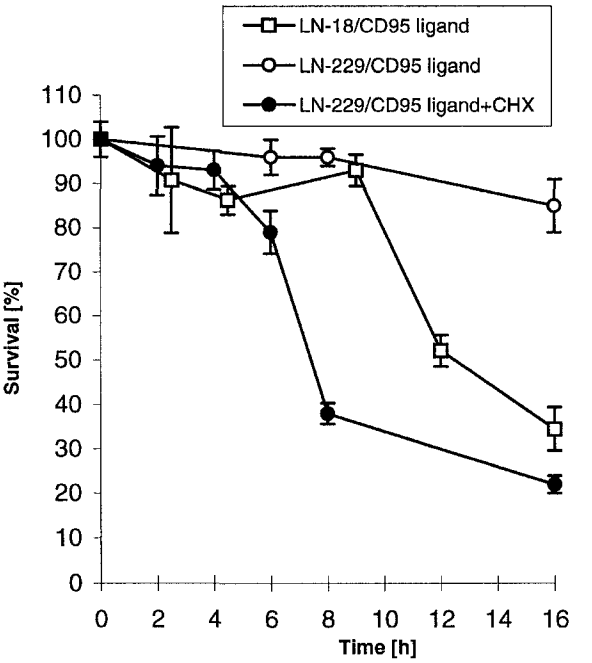

Fig. 1. Time-dependent induction of apoptosis in human glioma cells by CD95 ligand. LN-18 (□) or LN-229 (○) were exposed to CD95 ligand (80 U/ml) in the absence (LN-18, LN-229) or presence (LN-229, ●) of CHX (10 μg/ml) for various length of time. Survival was assessed by crystal violet staining. Data are expressed as mean ± SD ($n = 3$) percentage of survival compared with controls exposed to neo control supernatant without or with CHX.

To investigate a CD95 ligand-mediated AA release, glioma cells labeled with [^{3}H]AA were exposed to CD95 ligand in the absence or presence of CHX. Time-dependent changes in the levels of ^{3}H-labeled compounds were monitored in the cell culture medium (supernatant) as well as in cytosolic, nuclear and particulate cell fractions. There was an increase in AA in the cell culture medium peaking at 4–8 h after exposure to CD95 ligand correlating with the induction of cytotoxicity: (i) CD95 ligand induced AA release in LN-18 cells (Fig. 2A); (ii) CHX cotreatment increased AA release by CD95 ligand-treated LN-229 cells (Fig. 2D); while no AA was released from CD95 ligand-treated LN-308 neo cells, CD95-transfected LN-308 cells, which are sensitized to CD95-mediated apoptosis (Table 1), were induced to release AA by CD95 ligand (Fig. 2C). The differential quantification of radioactivity in supernatant, nucleus, cytoplasm and particulate fractions revealed that radioactive AA was released from the particulate fraction (Fig. 2A). To confirm that AA release was not an unspecific consequence of cell death, we performed parallel experiments to follow the time courses for AA release, DNA fragmentation and trypan blue dye exclusion. We observed AA release in LN-18 cells approximately 4 h before CD95 ligand-mediated apoptosis was detected by crystal violet staining (Fig. 1) and trypan blue uptake (Fig. 2A,B). In LN-229 cells, AA release precedes both DNA fragmentation and trypan blue uptake (Fig. 2D–F). Thus, enhanced AA release, induction of DNA fragmentation and loss of membrane integrity appear to be sequential steps during CD95-mediated apoptosis of LN-18 and LN-229 malignant glioma cells, confirming that AA release does not result from non-specific membrane damage.

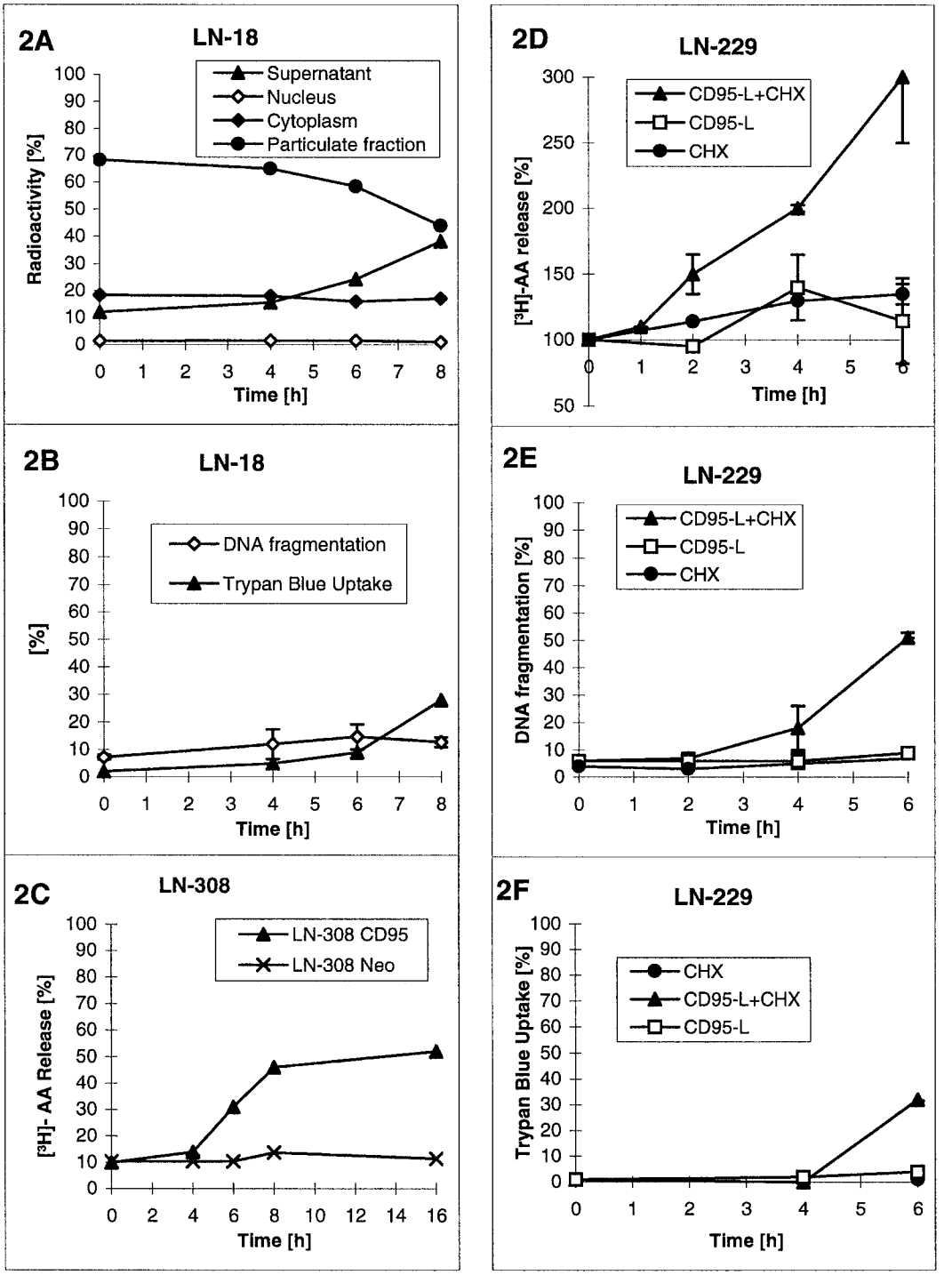

Fig. 2. AA release during CD95-mediated apoptosis of human malignant glioma cells. A: LN-18 cells prelabeled with [^{3}H]AA were exposed to CD95 ligand (80 U/ml). At the indicated times, supernatants were collected and cells lysed as described in Section 2. The radioactivity in the supernatant and in each cell compartment were measured in a liquid scintillation counter. B: LN-18 cells were exposed to CD95 ligand (80 U/ml). DNA fragmentation and trypan blue uptake were measured as described in Section 2. Note that glioma cells, notably LN-18 cells, fragment very little DNA when undergoing apoptosis, compared, e.g. with T cells [21,25]. C: LN-308 neo control cells or LN-308-CD95 clone C2 cells were prelabeled with [^{3}H]AA and exposed to CD95 ligand (80 U/ml). [^{3}H]AA release was measured as in (A). D-F: LN-229 cells were exposed to 80 U/ml CD95 ligand in the absence or presence of CHX (10 μg/ml). AA release (D), DNA fragmentation (E) or trypan blue uptake (F) were determined as described in Section 2. Data are representative and are expressed as mean ± SD percentages ($n = 3$).

3.2. *CD95-mediated apoptosis of human malignant glioma cells is unaffected by phospholipase inhibitors*

The generation of AA and AA metabolites during CD95 ligand-induced apoptosis suggested the involvement of phospholipases in the death pathway. Therefore we tested whether inhibitors of PLA_2, phospholipase C (PLC) or diacylglycerol lipase inhibited CD95 ligand-mediated cytotoxicity. We had previously noted a cytoprotective effect of the synthetic ste-

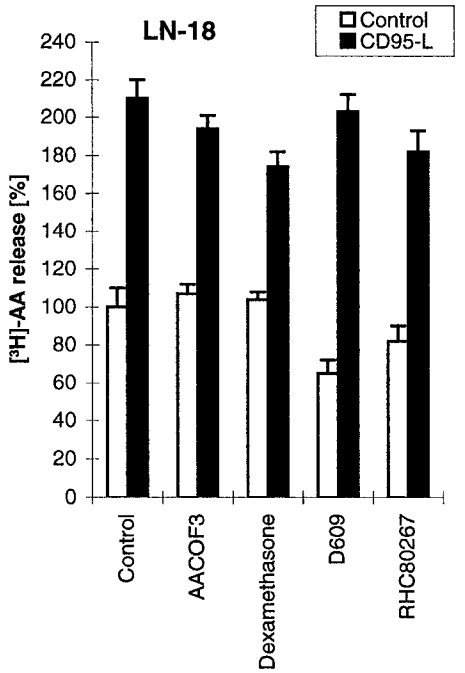

Fig. 3. Phospholipase inhibitors fail to prevent AA release from CD95 ligand-treated human malignant gliomas cells. LN-18 glioma cells were loaded with [^{3}H]AA, left untreated or pretreated for 2 h with $AACOF_3$ (100 μM), dexamethasone (10 μM), D609 (4 μg/ml) or RHC 80267(125 μM), and subsequently exposed to CD95 ligand (80 U/ml) for 8 h in the presence of the inhibitors. AA release was measured as described in Section 2. Data are expressed as mean ± SD percentages ($n = 3$).

roid, dexamethasone, a nonselective inhibitor of PLA_2, on CD95 antibody-induced apoptosis of human glioma cells [21]. Quinacrine, $AACOF_3$, dexamethasone and aristolochic acid were evaluated for the inhibition of PLA_2. D609 and RHC80267 were used to inhibit PLC and diacylglycerol lipase (Table 2). To ensure the efficacy of the inhibitors, we performed all studies in parallel with L929 cells, a model for the protective effect of phospholipase inhibitors from TNF-mediated apoptosis [16,17]. Quinacrine was cytotoxic to the glioma cells at concentrations previously reported to block PLA_2 activity in L929 cells [17]. None of the phospholipase inhibitors enhanced glioma cell survival after exposure to CD95 ligand. In contrast, most inhibitors attenuated TNF-α toxicity of L929 cells. Next we measured whether AA release during CD95 ligand-induced apoptosis (Fig. 2) resulted from PLA_2 activation. Basal AA release was unaffected by AACOF3 and dexamethasone but decreased significantly by D609 and RHC80267 ($P < 0.05$, t-test), suggesting a role for PLC and diacylglycerol lipase in basal AA generation. CD95 ligand-evoked AA release was attenuated significantly by dexamethasone and RHC80267 when considering drug effects on CD95-mediated AA release alone. However, in light of the decrease of basal AA release induced by RHC80267 in untreated cells, only dexamethasone had a significant specific effect on CD95-mediated AA release: absolute CD95-evoked increases in AA release were 110% in untreated cells, 87% with AACOF3, 70% with dexamethasone, 138% with D609, and 100% with RHC80267. Direct measurement of enzyme activity using ^{14}C-labeled phosphatidylcholine revealed a moderate induction of PLA_2 activity in L929 cells exposed to TNF-α but no consistent increase in glioma cells during CD95-mediated apoptosis (data not shown). Thus, the enzymatic source of AA generation in human glioma cells stimulated with CD95 ligand remains obscure.

3.3. *NDGA, a lipoxygenase inhibitor, blocks CD95 ligand-induced apoptosis of human malignant glioma cells*

To identify AA metabolites that might be involved in CD95-mediated apoptosis, lipids were extracted from LN-18 and LN-229 cells exposed to CD95 ligand or CD95 ligand plus CHX for 8 h and separated by TLC. Two AA-related compounds with *Rf* values of 0.7 and 0.2 were specifically released after CD95 ligand exposure and not detected in supernatant obtained from control cells. Using leukotriene C4 as a reference chemical, one of the compounds (*Rf* 0.7) was tentatively identified as an eicosanoide. Since leukotrienes are derived from AA by lipoxygenases, we assessed whether inhibition of such enzymes would interfere with the formation of the two AA metabolites. Preincubation of the cells with the lipoxygenase inhibitor, NDGA, for 2 h prior to CD95 ligation resulted in an attenuated signal for both compounds, notably for the *Rf* 0.7 derivative. A representative experiment is shown in Fig. 4A,B. Two metabolites migrating at *Rf* of 0.7 and 0.2 were also detected in L929 cells exposed to TNF plus CHX. Further, formation of these compounds was inhibited by NDGA (data not shown), suggesting a common pathway of CD95 and TNF receptor signaling.

To examine the biological role of AA metabolites in CD95-mediated apoptosis, we determined whether inhibitors of lipoxygenases or cycloxygenases prevented the cytotoxic effects of CD95 ligand. Both NDGA and esculetin provided protection from CD95-mediated apoptosis. In contrast, the cycloxygenase inhibitor, indomethacin, had no such effect. NDGA and esculetin inhibit the proliferation of glioma cells [28,29]. Here, complete growth arrest was not essential for the protective effect of NDGA since NDGA concentrations sufficient for rescue from CD95 ligand-induced cytotoxicity did

Table 1
Differential sensitivity of LN-18, LN-229, LN-308 and CD95-transfected LN-308 human malignant glioma cells to CD95 ligand-induced apoptosis

	CD95 expression (specific fluorescence index)	Cytotoxicity of CD95 ligand alone (% survival)	Cytotoxicity of CD95 ligand plus CHX (% survival)
LN-18	1.5	6 ± 1	4 ± 1
LN-229	2.5	91 ± 3	26 ± 2
LN-308	1.1	93 ± 5	91 ± 4
LN-308-CD95	6.4	39 ± 3	22 ± 3

CD95 expression was assessed by flow cytometry and expressed as SFI value (see Section 2).
For cytotoxicity assays, the glioma cells were exposed to CD95 ligand (80 U/ml) in the absence or presence of CHX (10 μg/ml) for 16 h. Survival was assessed by crystal violet staining. Data are expressed as mean ± SEM of percentage of survival compared with controls exposed to control supernatant, or control supernatant and CHX. These data confirm and extend previously published results [21–23].

not reduce proliferation in LN-229 cells as assessed by [^{3}H]thymidine incorporation (Fig. 4D). Moreover, these concentrations of NDGA were not cytotoxic as determined by LDH release (Fig. 4C,D).

NDGA is also an antioxidant [19]. However, antioxidant properties of NDGA were not involved in the protection of glioma cells from CD95-mediated apoptosis since (i) there was no formation of reactive oxygen species as assessed by DCF-H_2 fluorescence (data not shown) and since several antioxidants, including PBN (1 mM), superoxide dismutase (50 U/ml) and *N*-acetyl-L-cysteine (1 mM) failed to abrogate apoptosis (Table 2). In these experiments, the glioma cells were pretreated with the agents for 2 h and then co-incubated with the agents and CD95 ligand in the absence (LN-18) or presence (LN-229) of CHX (10 μg/ml), using concentrations of the antioxidants that have previously been shown to block potassium deprivation-induced apoptosis of cerebellar granule neurons in our laboratory [26].

4. Discussion

Human malignant gliomas are highly aggressive neoplasms which result in the death of affected patients within months. Cultured glioma cells are rather resistant to multiple proapoptotic stimuli including cancer chemotherapy drugs, gamma-irradiation, and TNF. In contrast, glioma cells are not resistant to CD95 ligand-induced apoptosis [21], suggesting that CD95 targeting may be a useful strategy to treat these tumors. Therefore, deciphering the signaling pathway activated during CD95-dependent apoptosis of glioma cells is not only of interest for basic research but may have clinical implications.

Here we report that CD95 ligand-induced apoptosis of glioma cells is associated with the release of AA. The enzyme responsible for this AA release could not be identified. CD95-evoked AA release has previously been reported in CD95-transfected MCF-7 mammary carcinoma cells [30]. These authors concluded that $cPLA_2$ was involved in the killing pathway since quinacrine and dexamethasone attenuated the cytotoxicity of TNF and CD95 antibodies. Similar conclusions were reached in a study on L929 cells expressing human CD95 [10]. CD95 ligation was associated with $cPLA_2$ induction in HuT78 lymphoma cells but that was not sufficient to cause cell death [6]. We failed to obtain direct evidence for $cPLA_2$ activation after CD95 ligation in glioma cells. Specific inhibitors of PLA_2 did not block CD95-dependent AA release or apoptosis (Fig. 3). These observations suggest cell type-specific cascades of CD95-mediated apoptosis. Whether the decrease in AA release (Fig. 3) is essential for the anti-apoptotic effect of dexamethasone [21], is unknown. Interestingly, dexamethasone also attenuates glioma cell cytotoxicity induced by several cancer chemotherapy drugs which have not been shown to kill via induction of AA generation [31].

Here we provide evidence for a critical role of a NDGA-sensitive step during CD95 ligand-induced apoptosis of human glioma cells (Fig. 4). The link between leukotrienes and glioma cell toxicity is not without precedent. PLA_2-mediated leukotriene synthesis has been reported to induce regression of experimental gliomas in rats [32]. On the other hand, lipoxygenase inhibitors interfere with the proliferation of glioma cells [28,29]. The protection from CD95-mediated apoptosis of glioma cells by NDGA reported here did not require a NDGA-induced cell cycle arrest (Fig. 4). Further, although NDGA and esculetin are antioxidants [19], such properties of both chemicals were not involved here since there was no formation of reactive oxygen species during CD95-mediated

Table 2
Effects of phospholipase, lipoxygenase, cycloxygenase inhibitors and radical scavengers on CD95 ligand-induced apoptosis of human malignant glioma cells

Compound	Concentration	Survival LN-18 (%)	LN-229 (%)	L929 (%)
None	–	23 ± 3	22 ± 2	18 ± 2
Inhibitors of $cPLA_2$				
Quinacrine	5 μM (10 μM)	8 ± 2 (toxic)	22 ± 2 (toxic)	25 ± 1 (35 ± 1)*
Aristolochic acid	1 mM	19 ± 3	22 ± 3	32 ± 1*
$AACOF_3$	100 μM	21 ± 4	18 ± 2	50 ± 3*
Dexamethasone	10 μM	35 ± 3*	24 ± 3	22 ± 2
Inhibitor of PLC				
D609	4 μg/ml	25 ± 2	28 ± 3	25 ± 1*
Inhibitor of diacylglycerol lipase				
RHC80267	125 μM	18 ± 1	18 ± 1	38 ± 3*
Lipoxygenase inhibitors				
NDGA	30 μM	70 ± 2*	102 ± 1*	100 ± 9*
Esculetin	1 mM	45 ± 4*	46 ± 4*	72 ± 2*
Cycloxygenase inhibitor				
Indomethacin	40 μM	23 ± 6	21 ± 2	21 ± 2
Radical scavengers				
PBN	1 mM	27 ± 1	21 ± 2	n.d.
SOD	50 U/ml	23 ± 2	23 ± 3	n.d.
N-acetyl-cysteine	1 mM	22 ± 1	18 ± 2	n.d.
Butylated hydroxytoluene	100 μM	21 ± 3	22 ± 4	n.d.

The glioma cells were pre-exposed to the compounds for 2 h.
LN-18 cells were then exposed to CD95 ligand at 80 U/ml alone, LN-229 to CD95 ligand (80 U/ml) plus CHX (10 μg/ml).
L929 cells served as a positive control for a protective effect of the inhibitors and were exposed to TNF-α (10 ng/ml) plus CHX (10 μg/ml).
Viability was measured by crystal violet assay at 16 h. Data are expressed as mean ± SD of percentage of survival compared with controls exposed to control supernatant or medium, in the absence or presence of the inhibitors or CHX ($n = 3$, $^*P < 0.05$, *t*-test, protective effect of enzyme inhibitor).

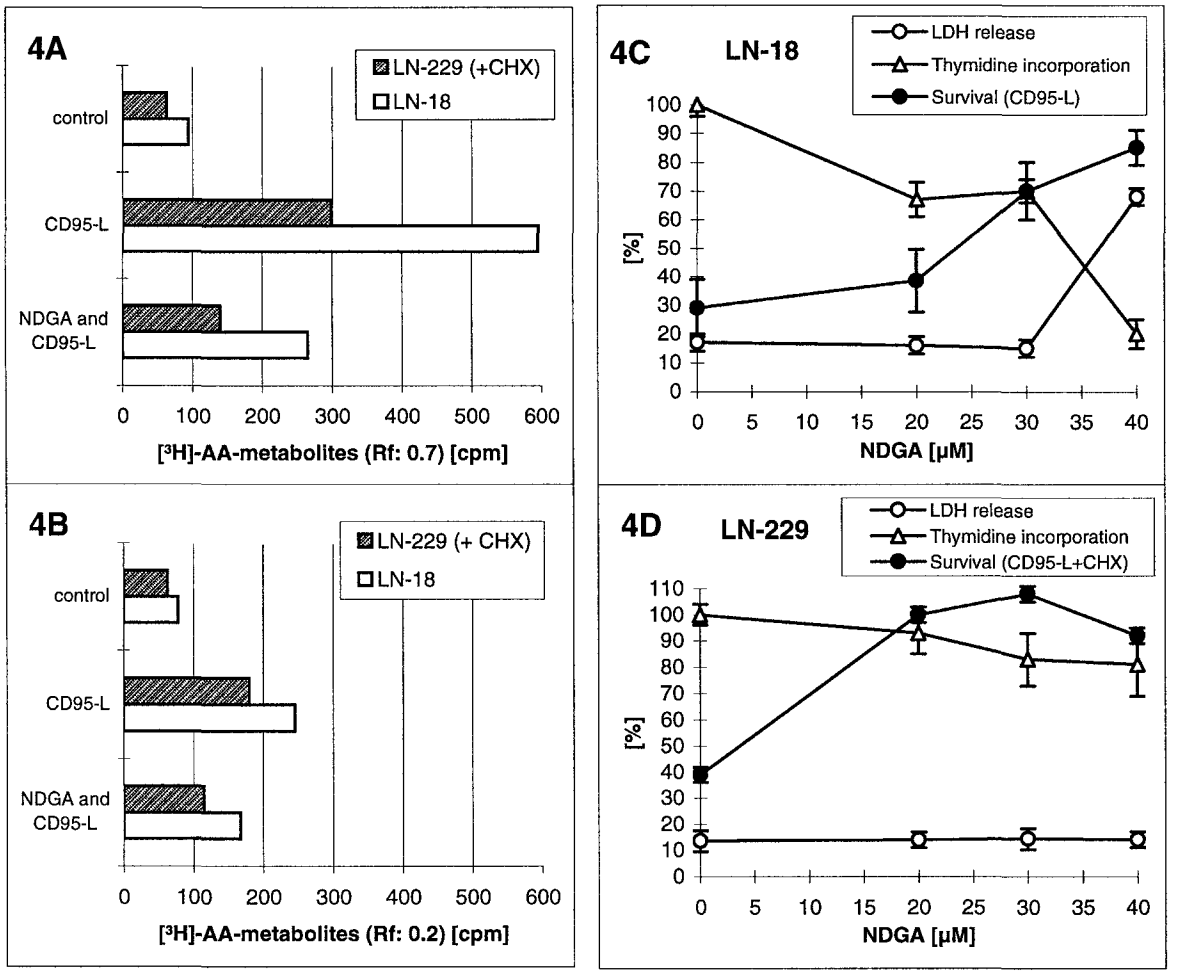

Fig. 4. NDGA inhibits CD95 ligand-induced AA metabolism and apoptosis of human malignant glioma cells. A,B: The glioma cells were labeled with [^{3}H]AA. LN-18 cells were exposed to CD95 ligand (80 U/ml), LN-229 cells to CD95 ligand (80 U/ml) plus CHX (10 μg/ml), in the absence or presence of NDGA (30 μM) for 8 h. ^{3}H-containing lipids were extracted, separated by TLC, and radioactivity measured in a liquid scintillation counter. A,B: AA metabolites with *Rf* values of 0.7 (A) and 0.2 (B). C,D: LN-18 or LN-229 cells were treated as in Fig. 1 with CD95 ligand alone (LN-18, (C)) or CD95 ligand plus CHX (LN-229, (D)) in the absence or presence of increasing concentrations of NDGA. Cytotoxicity induced by CD95 ligand, as well as [^{3}H]thymidine incorporation and LDH release induced by NDGA alone, were measured in parallel as described in Section 2, at 16 h in LN-18 and at 8 h in LN-229. [^{3}H]Thymidine labeling lasted 8 h. Data are expressed as mean ± SD of percentage of survival, [^{3}H]thymidine incorporation or LDH release compared with untreated controls.

apoptosis of glioma cells and since several antioxidants failed to block CD95-mediated apoptosis, as previously reported for non-glial cells [33,34]. Further studies are required to elucidate and dissect the subcellular biological effects of NDGA-like compounds which include at the same time prominent protection from CD95-mediated apoptosis and inhibition of proliferation.

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References

[1] N. Itoh, S. Yonehara, A. Ishii, M. Yonehara, S.I. Mizushima, M. Sameshima, A. Hase, Y. Seto, S. Nagata, Cell 66 (1991) 233–243.
[2] R.A. Heller, M. Krönke, J. Cell Biol. 126 (1994) 5–9.
[3] A.M. Chinnaiyan, K. O'Rourke, M. Tewari, V.M. Dixit, Cell 81 (1995) 505–512.
[4] M. Muzio, A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, M. Mann, P.H. Krammer, M.E. Peter, V.M. Dixit, Cell 85 (1996) 817–827.
[5] R.N. Kolesnick, A. Haimovitz-Friedman, Z. Fuks, Biochem. Cell Biol. 72 (1994) 471–474.
[6] M.G. Cifone, P. Roncaioli, R. De Maria, G. Camarda, A. Santoni, G. Ruberti, R. Testi, EMBO J. 14 (1995) 5859–5868.
[7] F. Ratter, M. Germer, T. Fischbach, K. Schulze-Osthoff, M. Peter, W. Dröge, P.H. Krammer, V. Lehmann, Int. Immunol. 8 (1996) 1139–1147.
[8] M. Verheij, R. Bose, X.H. Lin, B. Yao, W.D. Jarvis, S. Grant, M.J. Birrer, E. Szabo, L.I. Zon, J.M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks, R.N. Kolesnick, Nature 380 (1996) 75–79.
[9] E. Gulbins, R. Bissonnette, A. Mahboubi, S. Martin, W. Nishioka, T. Brunner, G. Baier, G. Baier-Bitterlich, C. Byrd, F. Lang, R. Kolesnick, A. Altman, D. Green, Immunity 2 (1995) 341–351.
[10] L.R. Ballou, S.J. Laulederkind, E.F. Rosloniec, R. Raghow, Biochim. Biophys. Acta 1301 (1996) 273–287.
[11] H. Hsu, H.B. Shu, M.P. Pan, D.V. Goeddel, Cell 84 (1996) 299–308.
[12] S. Mathias, K.A. Dressler, R.N. Kolesnick, Proc. Natl. Acad. Sci. USA 88 (1991) 10009–10013.
[13] M. Miura, R.M. Friedländer, J. Yuan, Proc. Natl. Acad. Sci. USA 92 (1995) 8318–8322.

[14] J.C. Trent, D.J. McConkey, S.M. Loughlin, M.T. Harbison, A. Fernandez, H.N. Ananthaswamy, EMBO J. 15 (1996) 4497–4505.
[15] C. Belka, K. Wiegmann, D. Adam, R. Holland, M. Neuloh, F. Herrmann, M. Krönke, M.A. Brach, EMBO J. 14 (1995) 1156–1165.
[16] M. Hayakawa, N. Ishida, K. Takeuchi, S. Shibamoto, T. Hori, N. Oku, F. Ito, M. Tsujimoto, J. Biol. Chem. 268 (1993) 11290–11295.
[17] M. Enari, H. Hug, M. Hayakawa, F. Ito, Y. Nishimura, S. Nagata, Eur. J. Biochem. 236 (1996) 533–538.
[18] E.M. Haliday, C.S. Ramesha, G. Ringold, EMBO J. 10 (1991) 109–115.
[19] V.B. O'Donnel, S. Spycher, A. Azzi, Biochem. J. 310 (1995) 133–141.
[20] P.A. Sandstrom, P.W. Tebbey, S. Van Cleave, T.M. Buttke, J. Biol. Chem. 269 (1994) 798–801.
[21] M. Weller, K. Frei, P. Groscurth, P.H. Krammer, Y. Yonekawa, A. Fontana, J. Clin. Invest. 94 (1994) 954–964.
[22] M. Weller, U. Malipiero, A. Aguzzi, J.C. Reed, A. Fontana, J. Clin. Invest. 95 (1995) 2633–2643.
[23] M. Weller, U. Malipiero, A. Rensing-Ehl, P.J. Barr, A. Fontana, Cancer Res. 55 (1995) 2936–2944.
[24] A. Rensing-Ehl, K. Frei, R. Flury, B. Matiba, S.M. Mariani, M. Weller, P. Aebischer, P.H. Krammer, A. Fontana, Eur. J. Immunol. 25 (1995) 2253–2258.
[25] M. Weller, D.B. Constam, U. Malipiero, A. Fontana, Eur. J. Immunol. 24 (1994) 1293–1300.
[26] J.B. Schulz, M. Weller, T. Klockgether, J. Neurosci. 16 (1996) 4696–4706.
[27] E.G. Bligh, W.J. Dyer, Can. Biochem. J. Physiol. 37 (1959) 911–917.
[28] H. Blomgren, G. Kling-Andersson, Anticancer Res. 12 (1992) 981–986.
[29] D.E. Wilson, A. DiGianfilippo, F.G. Ondrey, K.M. Anderson, J.E. Harris, J. Neurosurg. 71 (1989) 551–557.
[30] M. Jäättelä, M. Benedict, M. Tewari, J.A. Shayman, V.M. Dixit, Oncogene 10 (1995) 2297–2305.
[31] Weller, M., Schmidt, C., Roth, W. and Dichgans, J. (1997) Neurology, in press.
[32] D.H. Goddard, J.S. Bomalaski, S. Lipper, R.G. Shorr, M.A. Clark, Cancer Lett. 102 (1996) 1–6.
[33] K. Schulze-Osthoff, P.H. Krammer, W. Dröge, EMBO J. 13 (1994) 4587–4596.
[34] H. Hug, M. Enari, S. Nagata, FEBS Lett. 351 (1994) 311–313.