

# Arachidonic acid metabolism in TNF, CD178 (CD95 ligand) and ceramide-mediated cytotoxicity

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## Abstract

**Introduction:** Arachidonic acid release has been reported to play a significant role in TNF-induced cytotoxicity. However, the role of this signalling cascade in CD178-(CD95 ligand)-mediated cytotoxicity remains unclear. The aim of the study was to investigate the role of arachidonic acid metabolism in TNF- and CD95-mediated cytotoxicity.

**Material and methods:** Naturally TNF-sensitive A9 and CD178-sensitive A9-CD95 (the cells stably transfected with human CD95) cell lines were subjected to inhibitors of phospholipase A2 (AACOCF3, PACOCF3), lipoxygenase (NDGA) and cyclooxygenase (indomethacin).

**Results:** We showed that the cytotoxic activity of TNF could be inhibited by AACOCF3 and PACOCF3, inhibitors of phospholipase A2 as well as by NDGA, displaying anti-lipoxygenase and anti-oxidant inhibitory activity. In contrast, none of the tested inhibitors of arachidonic acid metabolism affected CD178-mediated cytotoxicity in A9-CD95 cells stably expressing CD95. Cytotoxicity mediated by C6-ceramide, a possible mediator of TNF- and CD178-mediated cytotoxicity, was also unaffected by these inhibitors.

**Conclusions:** The above facts strongly suggest that CD95- as well as C6 ceramide-induced cell death does not depend on arachidonic acid metabolism.

**Key words:** arachidonic acid, TNF, CD178, CD95, ceramide.

## Introduction

TNF (tumour necrosis factor) is a potent mediator of a variety of immune reactions leading to elimination of damaged, infected and tumour cells. TNF-mediated cytotoxicity mostly depends on the induction of pro-apoptotic caspases [1]. However, activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) releasing arachidonic acid (AA) from membrane phospholipids has also been reported to play a role in TNF-induced cytotoxicity [2]. AA may subsequently be converted into active lipid mediators, such as leukotrienes and prostanoids by lipoxygenases or cyclooxygenases, respectively [3]. Interestingly, the pro-apoptotic activity of another AA-liberating enzyme, calcium-independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>), remains poorly understood [4, 5].

The question whether the mechanisms of TNF-mediated cytotoxicity are related to a direct effect of AA and/or its metabolites still awaits elucidation. Some reports have suggested a role of lipoxygenase (LOX) metabolites in TNF cytotoxicity [6, 7, 8], while others have demonstrated direct pro-apoptotic activity of AA [9, 10, 11].

Mechanisms leading to cPLA<sub>2</sub> activation in cells transducing cytotoxic signals also remain unclear. Activation of cPLA<sub>2</sub> in response to TNF has been proposed to be a consequence of CAPK (ceramide-activated protein kinase) and MAPK (mitogen-activated protein kinase) activity [12]. On the other hand, some recent reports suggest that TNF-induced cPLA<sub>2</sub> activation does not require ceramide generation. It seems to involve a TNF receptor which may activate the MAP kinase pathway directly through its adapter proteins [12].

CD178 (CD95 ligand, FAS ligand, APO-1 ligand) shares some functional similarities with TNF, such as an ability to induce signal transduction pathways leading to cell death [13]. However, the role of cPLA<sub>2</sub> and arachidonic acid in CD178-mediated cytotoxicity remains unclear. In CD95 expressing L929 cells, the CD95-mediated cytotoxicity was reported to be independent of cPLA<sub>2</sub> activity [14, 15]. The level of cPLA<sub>2</sub> in these cells did not correlate with sensitivity to anti-human CD95 antibodies [15]. Similarly, activation of cPLA<sub>2</sub> was insufficient for CD95-dependent apoptosis of HuT78 lymphoma cells [16]. However, other reports concluded that cPLA<sub>2</sub> plays a role in CD95-induced apoptosis of L929 cells [17] as well as in MCF7 human breast carcinoma cells [18], and mouse Sertoli cell line [19].

Considering the above, the aim of the present study was to investigate the role of arachidonic acid metabolism in CD95-mediated cytotoxicity as compared to TNF. Furthermore, a possible role of ceramide in the induction of arachidonic acid-dependent cytotoxicity was also examined.

## Material and methods

### Target cells lines

A9 cell line (ATCC CCL-1.4), a derivative of L929, stably transfected with pEF-BOS expression vector encoding human CD95 (A9-CD95) as well as the mock-transfected A9 cells were kindly provided by Dr S. Smola-Hess (Cologne, Germany). Both A9 cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) containing 10% FCS, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml). Prior to experiments A9 transfectants were expanded for at least two weeks in media containing 800 µg/ml hygromycin B (Calbiochem, San Diego, USA). At least two weeks prior to experiments the selecting antibiotic was withdrawn from the culture media.

### Reagents

Recombinant TNF produced in *Escherichia coli* was from BD Pharmingen (Belgium). Anti-CD95 antagonistic antibody (IgG clone ZB4) and anti-CD95 agonistic antibody (IgM clone CH-11) were obtained from Immnotech (France). C6-ceramide, nordihydroguaiaretic acid (NDGA), indomethacin (INDO) and

ActD (actinomycin D) were purchased from Sigma. AACOCF<sub>3</sub> (arachidonyl trifluoromethyl ketone) and PACOCF<sub>3</sub> (palmitoyl trifluoromethyl ketone) were from Calbiochem (San Diego, USA).

Serving as a source of soluble CD178 was cell-free supernatant collected from BHK-21 cells (ATCC CCL-10) stably expressing human CD178 (BHK/CD178 conditioned medium). Serving as controls were supernatants from BHK-21 mock transfectants. These cell lines were kindly provided by Dr S. Smola-Hess (Cologne, Germany).

Cytotoxicity assay and assessment of specificity of cytotoxicity mediators

For cytotoxicity assays, the cells were seeded in 96-well plates (10<sup>4</sup> cells/well) and allowed to attach overnight. Next, the cells were preincubated for 30 minutes at 37°C with AACOCF<sub>3</sub>, PACOCF<sub>3</sub> and indomethacin at concentrations ranging from 5 to 50 µM and with NDGA at concentrations ranging from 1 to 10 µM. These amounts were found to be the highest concentrations that were not cytotoxic for the cells. Then the cells were exposed either to TNF or CH-11 antibody or BHK/CD178 conditioned medium or C6-ceramide for 16 h in the absence or presence of 1 µg/ml ActD. Following incubation, viable cells were stained for 15 min at room temperature with 1% crystal violet solution in 30% methanol. Dye uptake was measured by the absorbance at 570 nm in a microtiterplate reader [20].

In experiments with inhibitors TNF was added to a final concentration of 1 ng/ml and CH-11 antibody was used at final concentration of 0.5 µg/ml. The above doses were chosen as they were found to induce 50% cytotoxicity of respective cells. BHK/CD178 conditioned medium was used at a concentration of 1 U/ml that corresponded to a dilution resulting in half-maximal killing of A9-CD95 cells. Exposure of A9 cells to increasing concentrations of C6-ceramide (10-40 µM) led to dose-dependent cell death (data not shown). For subsequent experiments the 40 µM C6-ceramide concentration was used as this concentration led to almost 80% cytotoxicity.

Addition of TNF to A9 or A9-CD95 cells resulted in a specific dose-dependent cytotoxicity (data not shown). Both cell lines required co-exposure of TNF and ActD, an inhibitor of RNA synthesis, for induction of cytotoxicity. Only A9-CD95 cells were killed in a dose-dependent manner either by CH-11 antibody or BHK/CD178 conditioned medium. This effect was completely blocked by ZB4 antibody, an anti-CD95 antagonistic antibody (data not shown). Supernatant collected from BHK-21 mock transfectants did not influence the viability of A9-CD95 cells (data not shown).

### Statistical analysis

Statistical differences between groups were evaluated by the nonparametric Wilcoxon's test. All results are expressed as mean ± standard deviation (SD).

## Results

### Effects of inhibitors of arachidonic acid cascade on CD178-mediated cytotoxicity

As shown in Figure 1, cytotoxic activity of TNF was blocked by addition of AACOCF<sub>3</sub> and PACOCF<sub>3</sub> or by NDGA at their maximal non-toxic concentrations. Effects of AACOCF<sub>3</sub> and NDGA were dose-dependent (data not shown). An inhibitory effect of PACOCF<sub>3</sub> was found at a concentration of 50 μM ( $p < 0.05$  by Wilcoxon's matched-pair test). Lower doses of PACOCF<sub>3</sub> did not affect TNF cytotoxicity. Maximal concentration of indomethacin, a cyclooxygenase inhibitor, did not influence the TNF-mediated cytotoxicity (Figure 1).

Contrary to TNF, the cytotoxic effect induced by either CH-11 antibody or BHK/CD178 conditioned medium on A9-CD95 cells was not influenced by any of the tested inhibitors of arachidonic acid metabolism (Figure 1), irrespectively of the concentration applied.

Lack of sensitivity of A9-CD95 cells to CD95-mediated cytotoxicity was not affected by addition of ActD.

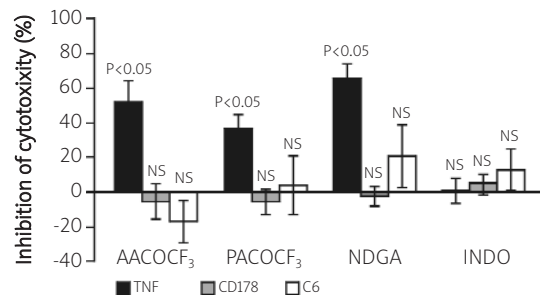
### Effects of inhibitors of arachidonic acid cascade on C6-ceramide mediated cytotoxicity

AACOCF<sub>3</sub>, PACOCF<sub>3</sub> and indomethacin could not prevent the C6-ceramide-mediated cell death of A9 cells, even when the maximal 50 μM concentration of inhibitors was used. However, pretreatment of the cells with 10 μM NDGA led to slight but reproducible inhibition of C6-ceramide induced cytotoxicity. As shown in Figure 1, NDGA could reduce the C6-ceramide mediated cytotoxicity up to 20%. This effect, however, was not statistically significant.

## Discussion

The results of our study support previous findings that AACOCF<sub>3</sub> inhibits TNF-mediated cytotoxicity [21]. Inhibition of TNF-mediated cytotoxicity was also observed at the highest non-toxic dose of PACOCF<sub>3</sub>. To our best knowledge the effect of PACOCF<sub>3</sub> on susceptibility to TNF cytotoxicity has not been studied so far. AACOCF<sub>3</sub> is a potent inhibitor of cPLA<sub>2</sub>, which at higher concentration was also shown to inhibit the calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>). In contrast, PACOCF<sub>3</sub> is considered to be predominantly an inhibitor of iPLA<sub>2</sub>. At higher concentration, however, it also may inhibit cPLA<sub>2</sub> [22]. PACOCF<sub>3</sub>-mediated inhibition of TNF was visible only at the highest non-toxic dose, having no effect at lower concentrations. However, discrimination between effects mediated by cPLA<sub>2</sub> and iPLA<sub>2</sub> would require further studies with highly specific inhibitors.

Contrary to TNF, CD178-mediated cytotoxicity was inhibited by neither AACOCF<sub>3</sub> nor PACOCF<sub>3</sub>, thus suggesting that CD178-mediated cell death is both cPLA<sub>2</sub>- and iPLA<sub>2</sub>-independent. This observation



**Figure 1.** Effect of the inhibitors of arachidonic acid cascade on TNF-, CD178- and C6-ceramide-mediated cytotoxicity. A9-CD95 cells were incubated for 16h with TNF (1ng/ml) (black bars) or BHK/CD178 conditioned medium (striped bars) or C6-ceramide (40 μM) (white bars) with AACOCF<sub>3</sub> (50 μM), PACOCF<sub>3</sub> (50 μM), NDGA (10 μM) or indomethacin (50 μM). All results show mean percentage of inhibition of the cytotoxic effect ± standard deviation (SD) calculated from six independent experiments. NS – not significant

supports the study of Enari et al. [15], which demonstrated that the cross-linking CD95 receptor induces cell death equally well in both cPLA<sub>2</sub>-deficient and -expressing L929 cells. On the other hand, our results are inconsistent with previous results showing that CD95-mediated cytotoxicity of U937 cells depends on iPLA<sub>2</sub> activation and AA release [23]. This difference might be explained by promonocytic origin of U937 cells and may reflect the ability of monocytes/macrophages to participate in induction of a proinflammatory response.

TNF-mediated cytotoxicity against A9 cells was also inhibited by NDGA, which is consistent with several previous observations [8, 24, 25]. NDGA was initially used as a nonselective LOX inhibitor with antioxidative properties. However, subsequent studies did not support a role for LOX or their products in TNF-induced cell death [24, 26]. This suggests that the effect of NDGA on TNF-induced cytotoxicity may be attributable instead to its antioxidant activity. In addition, NDGA effects may also be dependent on inhibition of cPLA<sub>2</sub> activation due to its ability to influence calcium signalling [27].

NDGA was also previously shown to inhibit CD95-mediated apoptosis in human glioma cells [28]. However, NDGA did not affect CD95-mediated cytotoxicity in L929 cells [14]. Our present study supports the latter observation, thus implying that neither AA nor its metabolites are involved in cytotoxicity mediated by CD178. Similarly, one could speculate that neither of the reactive oxygen species seems to be involved in CD95-induced cell death in A9 cells.

Both TNF and CD178 are known to activate sphingomyelinases leading to ceramide generation, which are involved in cell cytotoxicity. Indeed, C6-ceramide, a synthetic analogue of natural ceramides, exerted cytotoxic effects on A9 cells. This

effect, however, was not affected by any of the tested inhibitors, thus suggesting that signalling pathways activated by ceramides are either independent or are located downstream from PLA<sub>2</sub> or COX (cyclooxygenase). This is in agreement with the currently proposed model for c PLA<sub>2</sub> activation, which involves its phosphorylation by ERK1/2 activated directly by the TNF receptor regardless of ceramide generation [29].

### Conclusions

TNF-induced cytotoxicity is cPLA<sub>2</sub>-dependent. At the same time, CD95- as well as C6 ceramide-induced cell death does not depend on arachidonic acid metabolism.

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