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Research Article

Phenylarsine oxide interferes with the death inducing signaling complex and inhibits tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis

Xiao-Ming Sun[a,1], *Ana Canda-Sánchez*[a,1], *Ganesh R. Manjeri*[a], *Gerald M. Cohen*[a], *Michael J. Pinkoski*[a,b,*]

[a]*Medical Research Council Toxicology Unit, University of Leicester, Hodgkin Building, PO Box 138, Leicester LE1 9HN, UK*
[b]*Department of Infection, Immunity and Inflammation, University of Leicester, Hodgkin Building, PO Box 138, Leicester LE1 9HN, UK*

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ABSTRACT

The mechanism by which tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces death is the subject of intense scrutiny due to its preferential targeting of transformed cells for deletion. Based on recent findings that the TRAIL-dependent death inducing signaling complex (DISC) forms and signals at the plasma membrane without being internalized, we investigated the possibility that agents that prevent endocytosis may stabilize the surface bound DISC and thereby enhance TRAIL-dependent signaling. We utilized phenylarsine oxide (PAO), a trivalent arsenical that has been reported to inhibit endocytosis and to induce mitochondrial permeability transition. Therefore PAO could, by two separate and independent activities, enhance TRAIL-induced killing. Paradoxically, we found that rather than synergizing with TRAIL, PAO was an effective inhibitor of TRAIL-induced killing. Recruitment of FADD and caspase-8 to the TRAIL-dependent DISC was diminished in a concentration-dependent manner in cells exposed to PAO. The effects of PAO could not be reversed by washing cells under non-reducing conditions, suggesting covalent linkage of PAO with its cellular target(s); however, 2,3-dimercaptoethanol effectively overcame the inhibitory action of PAO and restored sensitivity to TRAIL-induced apoptosis. PAO inhibited formation of the TRAIL-dependent DISC and therefore prevented all subsequent apoptotic events.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of death ligands that trigger apoptosis in human target cells by ligation of its corresponding death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5). TRAIL has received considerable attention since the discovery that transformed cells are preferentially targeted for death by TRAIL (reviewed in [1,2]). As with the other death ligands, formation of the TRAIL-associated death inducing signaling

* *Corresponding author.* MRC Toxicology Unit, PO Box 138, Leicester LE1 9HN, UK. Fax: +44 116 252 5516.
E-mail address: mp191@le.ac.uk (M.J. Pinkoski).

Abbreviations: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DISC, death inducing signaling complex; FADD, Fas-associated death domain; PTPase, protein tyrosine phosphatase; CRD, cysteine rich domain; ANT, adenine nucleotide transporter; PS, phosphatidylserine; PAO, phenylarsine oxide; $\Delta\Psi m$, mitochondrial transmembrane potential; TMRE, tetramethylrhodamine ethyl ester; CHX, cycloheximide

[1] These authors contributed equally to this manuscript.

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complex (DISC) is the critical first step in the delivery of its apoptotic signal [3]. Following receptor engagement the adaptor molecule, FADD, interacts with the receptor via homotypic domains in each respective protein. Recruitment of FADD facilitates recruitment of procaspase-8, forming the DISC, and subsequent dimerization and activation of caspase-8 and engagement of the death pathway [3–5].

Death receptors of the TNF receptor (TNF-R) superfamily include CD95 (Fas, Apo-1), TRAIL-R1, TRAIL-R2 and TNF-R1 that share common signaling events such as recruitment of accessory molecules and activation of apical caspases. Different mechanisms have been reported to explain how different death receptors and their corresponding DISCs activate caspase-8. For example, CD95L and CD95 (Fas, Apo-1) have been reported to be internalized following ligand–receptor interaction prior to formation of a functional DISC through recruitment of DISC components, FADD and procaspase-8 and activation of caspase-8 [6,7]. However, CD95L-CD95 have also been demonstrated to induce apoptosis in the absence of internalization [8] and internalization may reflect an amplification step in some cells [7]. In the case of TNF-R1, after ligand binding, adaptor molecules TRADD, TRAF and RIP1 are recruited, followed by complex internalization. Dissociation of TRADD from TNF-R1 occurs prior to recruitment of FADD and activation of caspase-8 [9–11]. In contrast, we recently discovered that the TRAIL and TRAIL-R1/R2 DISC forms rapidly at the cell surface and needs not be internalized for activation of caspase-8 to occur and hence propagation of an apoptotic signal [12]. Internalization of TRAIL and TRAIL-R1/R2 has been reported [12,13] and although internalization is not necessary for apoptosis [12], it may reflect a down-regulation of surface receptors during low level ligand/receptor binding events that negatively regulate TRAIL-induced apoptosis [14]. Enforced internalization of TRAIL-R1 by the adenovirus RID proteins reduces susceptibility to TRAIL-induced apoptosis by lowering the availability of surface receptors [14]. We propose that internalization of TRAIL-R1, even when bound by ligand, can reduce the signaling capacity of the ligand–receptor complex. A corollary is that inhibition of receptor internalization may sustain signaling capacity of the DISC, whether by a simple stochastic mechanism or by a feedback amplification loop.

Phenylarsine oxide (PAO; phenoxyarsine) is a trivalent arsenical that reacts with free (reduced) vicinal protein thiols from spatially neighboring cysteine residues [15]. PAO is a small molecule that passes freely across membranes, giving it access to potential targets throughout the cell, including mitochondria where it has been shown to react with mitochondrial adenine nucleotide transporter (ANT), and proteins regulated by cellular redox state [16,17]. At sufficiently high concentrations, PAO, like other arsenicals, has been used as an uncoupler of oxidative phosphorylation and electron transport [16,18,19]. Its cellular targets also include protein tyrosine phosphatases [20–22]. PAO's biochemical properties have made it useful as an inhibitor of receptor-mediated endocytosis [23,24] and as a disruptor of mitochondrial function [16,18,19].

We investigated the possibility that PAO could potentiate TRAIL-induced apoptosis by two possible mechanisms: stabilization of the surface bound DISC by blocking internalization and sensitization of cells to mitochondrial disruption. Paradoxically, rather than synergizing with TRAIL, we found that PAO effectively inhibited TRAIL-induced apoptosis. The inability to form a stable precipitable DISC is likely to be the predominant mechanism of inhibition as it represents a block at the first stage of apoptosis initiation.

Materials and methods

Cell lines and reagents

BJAB (Burkitt Lymphoma) cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and Glutamax® at 37 °C and 5% CO_2. Anti-caspase-8 and -3 antisera were generated and utilized as previously described [25]. Anti-FADD (Clone 1) was obtained from BD Transduction Laboratories (Oxford, UK). Horseradish peroxidase-conjugated secondary antibodies (anti-mouse-HRP (#A8924) and anti-rabbit HRP (#A0545)), PAO, tetramethylrhodamine ethyl ester (TMRE) and propidium iodide (PI) were purchased from Sigma Aldrich (Gillingham, UK) and protease inhibitor cocktail from Roche Applied Science (Mannheim, Germany). Annexin V-FITC was purified as described earlier [26]. Recombinant dual His-6 and T7 tagged TRAIL was purified on nickel-nitroacetic acid beads as described previously [12,27] and strep-tagged TRAIL (strep-TRAIL) was purified on streptactin beads (Inoue, Harper and Cohen, submitted). Phycoerythrin-conjugated anti-DR4 (DJR-1), anti-DR5 (DJR2-4) and immunoglobulin isotype control (#12-4714) were purchased from eBioscience, Inc. (San Diego, CA). Agonistic anti-Fas (CH-11; Upstate Biotechnologies (Temecula, CA)) was used at 100 ng/ml for the times indicated. Specific killing, as determined by loss of $\Delta\Psi_m$, was calculated relative to cells treated with PAO alone. TNFα was purified as previously described [9] and used at a concentration of 200 ng/ml with CHX (5 μM).

Affinity precipitation and protein analysis

Following treatment, cells were washed in cold PBS and whole cell lysates were prepared in DISC precipitation buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100) containing protease inhibitors [4]. For DISC precipitation following treatment with strep-TRAIL, lysates were incubated overnight at 4 °C in the presence of streptavidin beads. The beads were washed, dissociated at 95 °C in SDS-loading buffer and analyzed by western blotting as described previously [12].

Assessment of apoptosis

Loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed using the potentiometric dye, TMRE. DNA fragmentation was measured using sub-G1 analysis of propidium iodide stained cells and PS externalization was assessed by annexin V-FITC labeling as described previously [12]. Briefly, $\Delta\Psi_m$ was measured following addition of TMRE to a final concentration of 50 nM in 200 μl cultures 20–30 min prior to the experimental endpoint and analyzed by flow cytometry. Loss of membrane asymmetry, as indicated by PS extrernalization, was assayed by labeling with FITC-conjugated annexin V at 4 °C for 30 min in annexin V-binding buffer [26]. Propidium iodide was added to a final concentration of 500 ng/ml immediately prior to data acquisition by flow cytometry in order to indicate necrotic cells. DNA fragmentation was assessed on ethanol fixed and permeabilized cells treated first with RNase A for 20 min at room temperature followed by