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## Letter to the Editor

# Induction of annexin-1 during TRAIL-induced apoptosis in thyroid carcinoma cells

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*Dear Editor*,

Annexin-1 (ANXA1, lipocortin-1) is the first characterized member of the annexin superfamily of proteins, so called since their main property is to bind (i.e. to *annex*) to cellular membranes in a $Ca^{2+}$-dependent manner. Originally described as a phospholipase A2-inhibitory protein, ANXA1 can affect many components of the inflammatory reaction besides the metabolism of arachidonic acid. ANXA1 has recently been shown to have a role in the apoptosis of inflammatory cells.[1] The overexpression of ANXA1 in U-937 cells[2] and in broncho-alveolar epithelial cells[3] promoted apoptosis associated with caspase-3 activation. Exogenous ANXA1 stimulated transient rise in intracellular calcium concentrations accompanied by dephosphorylation of the proapoptotic protein Bad and apoptosis of neutrophils.[4] ANXA1 may also represent an endogenous ligand mediating engulfment of apoptotic cells. The protein is recruited to the phosphatidylserine (PS)-rich domains of apoptotic cell surface. This recruitment requires caspase activity and the release of intracellular calcium. Importantly, silencing ANXA1 protein by small interference RNA (siRNA) resulted in defective tethering and engulfment of apoptotic cells.[5]

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family of proteins including FasL, and TNF-$\alpha$. TRAIL effectively kills most thyroid cancer cell lines tested, including those originating from anaplastic carcinomas.[6] Given the interest of TRAIL as a promising new agent against thyroid cancer and the importance to understand the mechanisms of TRAIL-induced death, we have investigated the role of ANXA1 in TRAIL-induced apoptosis in the follicular undifferentiated (FRO) thyroid carcinoma cell line.

First, we checked the ability of TRAIL to induce apoptosis of FRO cells. Time-course experiments showed that 5 ng/ml TRAIL induced a significant apoptosis at 8 h with a peak at 24 h (Figure 1a). Apoptosis was measured as percent hypodiploid nuclei by flow cytometry. To confirm the pro-apoptotic role of TRAIL, the activity of caspase-3 was measured by fluorimetric assay. Figure 1b shows that a robust stimulation of caspase-3 activity was caused by 5 ng/ml TRAIL as early as 3 h with a peak at 24 h incubation. Next, the effects of TRAIL on ANXA1 expression were investigated by Western blot analysis. TRAIL (5 ng/ml) stimulated the cytosolic expression of ANXA1 in a time-dependent fashion with a peak at 24 h (3.3-fold increase *versus* 0 time, $P<0.001$; Figure 1c). Interestingly, between 0 and 8 h after TRAIL, no ANXA1 signal could be detected on the membrane of FRO cells. Conversely, a remarkable ANXA1 expression on FRO cell membrane was observed at 24 h after TRAIL (Figure 1c). As ANXA1 may induce apoptosis through Bad dephosphorylation,[4] the levels of both Bad and phospho-Bad proteins were measured by Western blot after TRAIL stimulation. Figure 1c shows that phospho-Bad levels decreased with time and were barely detectable at 24 h after TRAIL. Bad levels were not modified by the TRAIL treatment. In order to further investigate the role of ANXA1 in TRAIL-induced apoptosis, the technique of siRNAs was utilized. FRO cells were transfected using oligofectamine with siRNAs designed against ANXA1 or with a scrambled siRNA and then challenged with 5 ng/ml TRAIL for 8 h (Figure 1d). In control cells, TRAIL upregulated ANXA1 expression and inhibited phospho-Bad expression (lane 2). siRNAs directed against ANXA1 strongly inhibited ANXA1 expression in both unstimulated and TRAIL-treated cells (lanes 5 and 6). In these cells, the downregulation of ANXA1 expression by siRNAs was accompanied by increased expression of phospho-Bad. In fact, phospho-Bad levels in cells transfected with siRNAs against ANXA1 (lane $5=76.7\pm1.2$; lane $6=81.6\pm0.7$), measured by densitometry of the ratio phospho-Bad/Bad, were significantly increased compared to TRAIL-stimulated control cells (lane $2=21.3\pm0.9$, $P<0.001$) and scramble siRNA-transfected cells (lane $4=21.3\pm1.5$, $P<0.001$). Scrambled siRNAs had no effect on ANXA1 and phospho-Bad expression. These results strongly suggest that Bad phosphorylation/dephosphorylation may be modulated by ANXA1 expression levels.

We then analyzed the effects of ANXA1 siRNAs on TRAIL-induced apoptosis, measured as appearance of hypodiploid nuclei. Cells were transfected with or without scrambled or ANXA1 siRNAs as described and then challenged with 5 ng/ml TRAIL for 24 h. Figure 1e shows that TRAIL-induced cell death was unaffected in cells transfected with scrambled siRNAs. Conversely, in FRO cells transfected with siRNAs against ANXA1, TRAIL-induced apoptosis was partially inhibited ($-26\%$, $P<0.05$). The effects of siRNAs were confirmed by assessment of caspase-3 activity measured 3, 6 and 24 h after TRAIL stimulation (Figure 1f). Again transfection with scrambled siRNAs had no effect on caspase-3 activity at all times. On the other hand, TRAIL-induced caspase-3 activity was partially reduced in FRO cells transfected with siRNAs against ANXA1 at all times considered: 3 h, $-22\%$, $P<0.01$; 6 h, $-24\%$, $P<0.001$; 24 h, 25%, $P<0.001$.

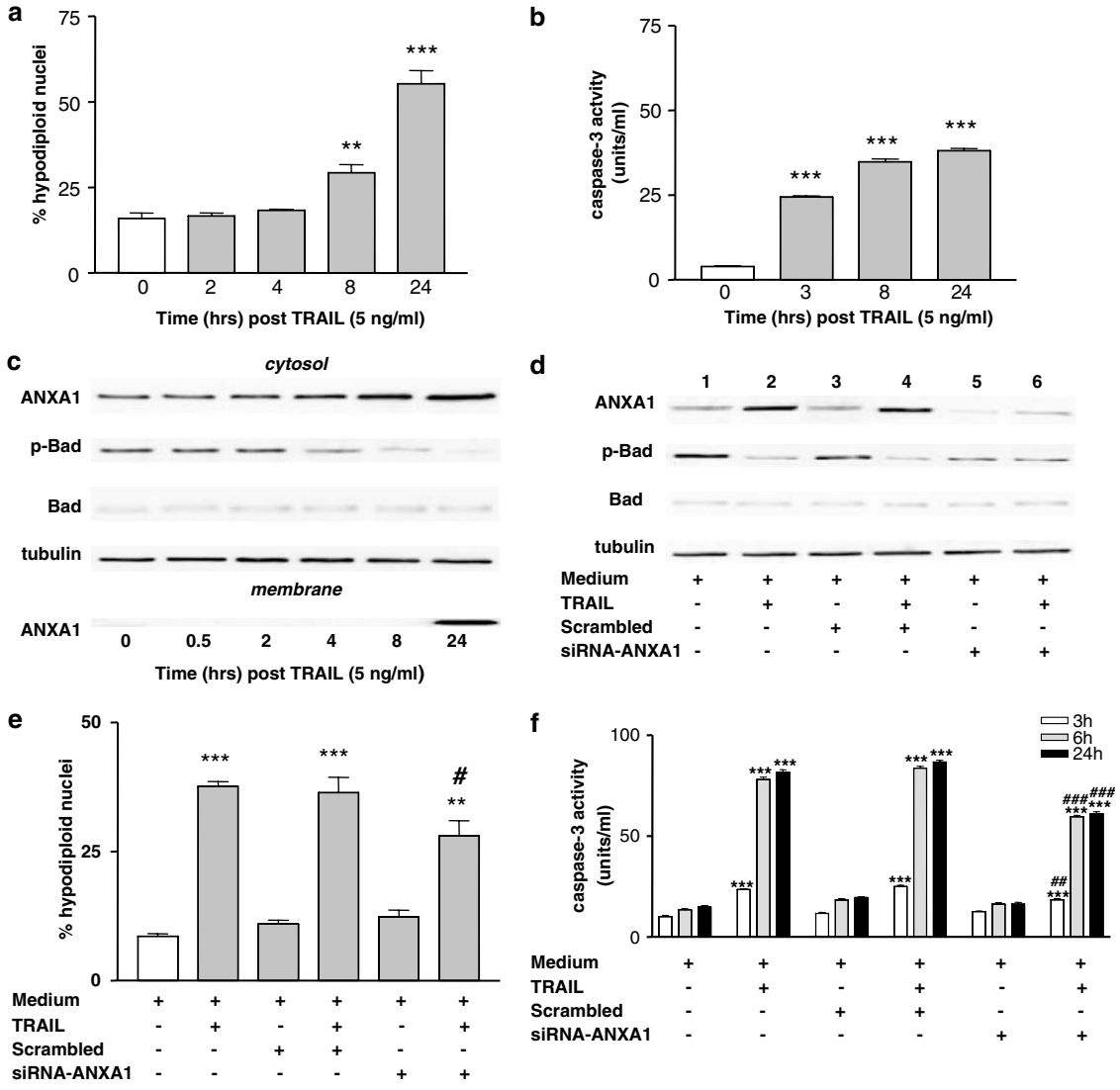


**Figure 1** (**a**) Time-dependent apoptosis induced by TRAIL (5 ng/ml) in FRO thyroid carcinoma cells. Apoptosis was measured as percentage of hypodiploid nuclei by propidium iodide staining. Cells ($2 \times 10^5$) were washed in phosphate-buffered saline (PBS) and resuspended in 500 $\mu$l of a solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 $\mu$g/ml propidium iodide. After incubation at 4°C for 30 min in the dark, cell nuclei were analyzed with Becton Dickinson FACScan flow cytometer. (**b**) Time-dependent activation of caspase-3 by TRAIL (5 ng/ml). Caspase-3 activity was measured by fluorimetric assay. Cells ($2 \times 10^6$) were lysed in a buffer containing 10 mmol/l Tris (pH 7.5), 130 mmol/l NaCl, 1% Triton X-100, 10 mmol/l NaPi and 10 mmol/l NaPPi; 20 $\mu$g protein was then incubated with 20 $\mu$mol/l Ac-DEVD-AMC (Becton Dickinson, Milan, Italy) in a buffer containing 20 mmol/l HEPES (pH 7.5), 10% glycerol and 2 mmol/l dithiothreitol (DTT) at 37°C for 2 h. The release of AMC was monitored in a spectrofluorimeter with an excitation wavelength of 380 nm and emission wavelength range of 430–460 nm. (**c**) Effect of TRAIL on the expression of ANXA1, Bad and phospho-Bad (p-Bad) in FRO cells. Cells were plated at a density $5 \times 10^6$/ml and incubated with or without TRAIL (5 ng/ml) at different times. A 30 $\mu$g portion of proteins from the cell extracts was analyzed by Western blot using polyclonal anti-ANXA1 antibodies or commercially available anti-p-Bad or anti-Bad polyclonal antibodies (Cell Signaling, CELBIO, Milan, Italy). Immunoreactive bands were quantified by densitometry. Membrane proteins were obtained in 5 mM EDTA-Tris-HCl, which acts as a $Ca^{2+}$ chelating agent and removes ANXA1 attached to cell surface. A 30 $\mu$g portion of proteins was then analyzed by Western blot as above. (**d**) Effect of siRNAs against ANXA1 on the expression levels of ANXA1, Bad and phospho-Bad (p-Bad) in unstimulated and TRAIL-stimulated FRO cells. siRNAs with two thymidine residues (dTdT) at 3′ end of the sequences were designed for two sequences for ANXA1 – Annexin 1(A) (sense 5′-CAGCGUCAACAGAUCAAAG-3′) and Annexin 1(B) (sense 5′-CCGAUCUGAGGACUUUGGU-3′) – gene along with their corresponding antisense RNA oligonucleotides as described (Dharmacon Research Inc., Lafayette, CO, USA). siRNA oligo scrambled KROAA-006461 (sense 5′-CAGUCGCGUUUGCGACUGG-3′) (Dharmacon Research Inc., Lafayette, CO, USA) was used as control of siRNA-ANXA1 at 100 nM final concentration. Approximately, $1.5 \times 10^5$ cells were plated in six-well plates at 30–50% confluency in media containing 10% FBS. Transfection of RNA oligonucleotides (siRNAs) was performed using oligofectamine (Invitrogen, Milan, Italy), following the protocols provided by the manufacturer, to result in a final RNA (**a**, **b**) mixture of concentration 100 nM. At 72 h after transfection, the cells were treated with TRAIL (5 ng/ml) for 8 h. Western blot analysis was performed on 30 $\mu$g protein extracts as described above. Immunoreactive bands were quantified by densitometry. (**e**) siRNAs against ANXA1 partially inhibited TRAIL-induced apoptosis in FRO cells. Transfection was performed as above. Apoptosis was analyzed by propidium iodide staining as described previously after 24 h incubation with or without TRAIL (5 ng/ml). (**f**) siRNAs against ANXA1 partially inhibited TRAIL-induced caspase-3 activity in FRO cells. Transfection was performed as above. Caspase-3 activity was measured by fluorimetric assay as described above at the indicated times with or without TRAIL (5 ng/ml). All data are means $\pm$ S.E.M. of three experiments performed in triplicate. Bonferroni test: $^{**}P<0.01$ *versus* 0 time (**a**) or *versus* medium (**e**); $^{***}P<0.001$ *versus* 0 time (**a**, **b**) or *versus* medium (**e**, **f**); $^{\#}P<0.05$ *versus* TRAIL and TRAIL + scrambled (**e**); $^{\#\#}P<0.01$ *versus* 3 h TRAIL and TRAIL + scrambled (**f**); $^{\#\#\#}P<0.001$ *versus* TRAIL and TRAIL + scrambled (**d**) or *versus* 6–24 h TRAIL and TRAIL + scrambled (**f**)

These results show for the first time that in FRO cells TRAIL is able to upregulate ANXA1 expression and induce the translocation of the protein to the membranes of apoptotic cells. The time course of the upregulation of ANXA1 expression by TRAIL correlates well with the stimulation of both caspase-3 activity and cell death caused by TRAIL. Furthermore, the inhibition of ANXA1 expression by siRNAs leads to a partial, but statistically significant reduction of TRAIL-induced apoptosis, suggesting that ANXA1 expression may contribute to the proapoptotic effects of TRAIL. The upregulation of ANXA1 expression by TRAIL may be due to transcriptional activation. ANXA1 promoter has an AP-1 site that can be stimulated by phorbol esters to induce gene expression of the protein.[7] It is also known that DR5 ligation by TRAIL mediates AP-1 activation to induce gene expression of proteins like IL-8 that possess AP-1 motifs in their promoters.[8] The mechanism(s) of ANXA1 gene transcriptional activation by TRAIL are currently under investigation in our laboratory.

It has been shown that exogenous ANXA1 is able to induce neutrophil death through intracellular calcium increase and BAD dephosphorylation. This allows BAD to associate with mitochondria, heterodimerize with Bcl-XL and promote apoptosis.[4] The present data support the hypothesis that in FRO cells ANXA1 may promote apoptosis by reducing the phosphorylation levels of Bad. No evidence is currently available as to which phosphatase, if any, might be activated by ANXA1 to dephosphorylate Bad.

Finally, the membrane expression of ANXA1 after 24 h TRAIL (Figure 1c) confirms previous results in Jurkat cells[5] and suggests that the exposure of ANXA1 on the surface of apoptotic cells may be a widespread mechanism for the recognition and removal of apoptotic cells not restricted to a single cell type. Further work is necessary to clarify the role of ANXA1 as 'eat me' signal in FRO cells.

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