TNF–related apoptosis-inducing ligand (TRAIL) and erythropoiesis: a role for PKCε

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The regulation of the hematopoietic stem cell pool size and the processes of cell differentiation along the hematopoietic lineages involve apoptosis. Among the different factors with a recognized activity on blood progenitor cells, TRAIL - a member of the TNF family of cytokines - has an emerging role in the modulation of normal hematopoiesis.
PKCÂ levels are regulated by EPO in differentiating erythroid progenitors and control the protection against the apoptogenic effect of TRAIL. EPO-induced erythroid CD34 cells are insensitive to the apoptogenic effect of TRAIL between day 0 and day 3, due to the lack of specific surface receptors expression. Death receptors appear after day 3 of differentiation and consequently erythoid cells became sensitive to TRAIL up to day 9/10, when the EPO-driven up-regulation of PKCε intracellular levels inhibits the TRAIL-mediated apoptosis, *via* Bcl-2. In the time interval between day 3 and 9, therefore, the number of erythroid progenitors can be limited by the presence of soluble or membrane-bound TRAIL present in the bone marrow microenvironment.

Key words: PKCε; erythropoiesis; EPO; CD34; apoptosis; TRAIL.

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Apoptosis in the regulation of hematopoiesis

The regulation of the hematopoietic stem cell pool size (Domen, 2001) and the processes of cell differentiation along the hematopoietic lineages involve apoptosis. Fetal erythropoiesis can be negatively regulated by Fas ligand (Barcena *et al.*, 1999; Schneider *et al.*, 1999), although bone marrow hematopoiesis does not appear to be affected by Fas deficiency (Schneider *et al.,* 1999). Relatively little is known on the effects of other members of the TNF family on hematopoietic progenitors. Tumour necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines, which are structurally related proteins playing important roles in regulating cell death, immune response and inflammation (Wiley *et al.,* 1995; LeBlanc and Ashkenazi, 2003). TRAIL induces apoptosis in a variety of malignant cells both *in vitro* and *in vivo,* displaying minimal toxicity on normal cells and tissues (Smyth *et al.,* 2003; Takeda *et al.,* 2002). It interacts with 4 high affinity transmembrane receptors; TRAIL-R1 (DR4) and TRAIL-R2 (DR5) transduce apoptotic signals on binding of TRAIL, whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are homologues to DR4 and DR5 but they lack the intracellular death domain and apoptosis inducing capability, suggesting that TRAIL-R3 and TRAIL-R4 function as decoy receptors protecting normal cells from apoptosis (Sheridan *et al.,* 1997; Ashkenazi and Dixit, 1999). The earliest biochemical event following engagement of TRAIL death receptors by their ligand is the recruitment of proteins to the intracellular death domain of the receptor to form a structure known as the *death-inducing signaling complex* (DISC) (McFarlane, 2003).

The role of TRAIL in hematopoiesis

It has been previously demonstrated that TRAIL is involved in erythroid maturation. In fact, TRAIL acts as a negative regulator of adult erythropoiesis, selectively reducing the number of erythroblasts in liquid culture, as well as reducing the number and size of erythroid colonies in semisolid assays (Zamai *et al.,* 2000). Moreover, TRAIL inhibited the generation of mature erythroblasts in liquid culture through the activation of an ERK 1/2-mediated signaling pathway (Secchiero *et al.,* 2004).

Protein kinase Cε prevents apoptosis

It has been demonstrated that protein kinase Cε (PKCε) is selectively post-transcriptionally down-modulated in the EPO-dependent murine 32D-Epo.1 cells, while it is expressed in the parental cell line 32D as well as in the 32D-GM1 and 32D-G1 cells with granulomacrophagic and granulocytic phenotype, respectively. The pharmacological inhibition of PKCε increased the number of erythroid colonies *in vitro,* strongly suggesting a relevant role for this isoform of PKC in erythropoiesis (Bassini *et al.,* 1999). Previous observations (Baxter *et al.,* 1992) had already established a link between PKCε and apoptosis in different model systems. Gubina *et al.,* (1998) demonstrated that PKCε prevents apoptosis of the factor-dependent TF-1 cells cultured in the absence of cytokines *via* Bcl-2 up-regulation.

PKCe protects hematopoietic cells against TRAIL-mediated apoptosis

In differentiating erythroid progenitors, PKCε levels are regulated by EPO and control the protection against the apoptogenic effect of TRAIL. PKCε is important for cytokine and growth factor receptor-mediated signalling and is one of the PKC isoforms modulated during human and mouse erythroid cell differentiation (Bassini *et al.,* 1999). EPO promotes in human CD34 cells a series of events that modify the sensitivity of these cells to TRAIL. TRAIL induces apoptosis in HeL, K562 and TF-1 cell lines after 48 hours treatment with 50 ng/mL of TRAIL. The phenotypic analysis with anti TRAIL-Receptors (TRAIL-Rs) MoAbs reveal that the three cell lines have a very neat expression of death receptors R1 and R2, while decoy receptors R3 and R4 are expressed with a variable intensity (Figure 1A). The over-expression of PKCε in these cell lines, using as negative control a kinase inactive PKCε K522M mutated (PKCε$_m$) (Ivaska *et al.,* 2002) induces a significant reduction of TRAIL-mediated apoptosis in cell lines overexpressing PKCε and treated with 50 ng/mL TRAIL for 48 hrs (Figure 1B).

EPO-mediated up-regulation of PKCε is responsible for TRAIL resistance of human CD34-derived erythroblasts

Freshly purified CD34 cells - isolated from PBL of healthy donors by immunomagnetic positive selection using the CD34$^+$ cell isolation Kit (Milteny Biotech, Gladbach, Germany) - do not express the four TRAIL-Rs. However, when cultured in serum free medium in the presence of IL-3, SCF and EPO, starting from day 3 CD34 cells show a progressive increase of the surface expression of TRAIL-R1 and R2, similarly to what observed in hematopoietic cell lines. Accordingly, when TRAIL is added to EPO-cultured CD34 cells, they are resistant to apoptosis at day 0. From day 3 onward, differentiating erythroid progenitors express surface death receptors TRAIL-R1 and -R2 and become sensitive to TRAIL. At this stage PKCε levels are still undetectable and over-expression of PKCε abrogates TRAIL induced apoptosis. Surprisingly however, the sensitivity to TRAIL of EPO-differentiating CD34 cells decreased progressively from day 7 onward, and from day 10 erythroblasts became resistant to TRAIL, while control cultures of normal CD34 cells in the absence of EPO were always resistant to the apoptogenic effect of TRAIL (Figure 1C-D), thus independently confirming that the sensitivity to TRAIL of normal CD34 cells between day 3 and day 7 was EPO-dependent.

Around day 6/7, EPO-induced PKCε levels increase rapidly confering to erythroid cells resistance to TRAIL, notwithstanding the surface expression of death receptors. Figure 1E shows the kinetic of PKCε induction in primary CD34 cell cultures in the presence or absence of EPO. Primary day-10 CD34 cell cultures transfected with siRNAs targeting PKCε mRNA (specific targeting of PKCε mRNA was evaluated by western blot analysis of control PKCδ expression) became sensitive to TRAIL-induced apoptosis, as CD34 cells treated with PKCε inhibitor (Figure 1F). On the contrary, over-expression of PKCε in CD34 cells differentiated with EPO for 3 days induces resistance to the apoptogenic effect of TRAIL, while control PKCεm-transfected CD34 cells did not (Figure 1F). (Double-strand siRNAs (dsRNA), corresponding to nt 223-244, 429-450, 942-963 and 1158-1179 on

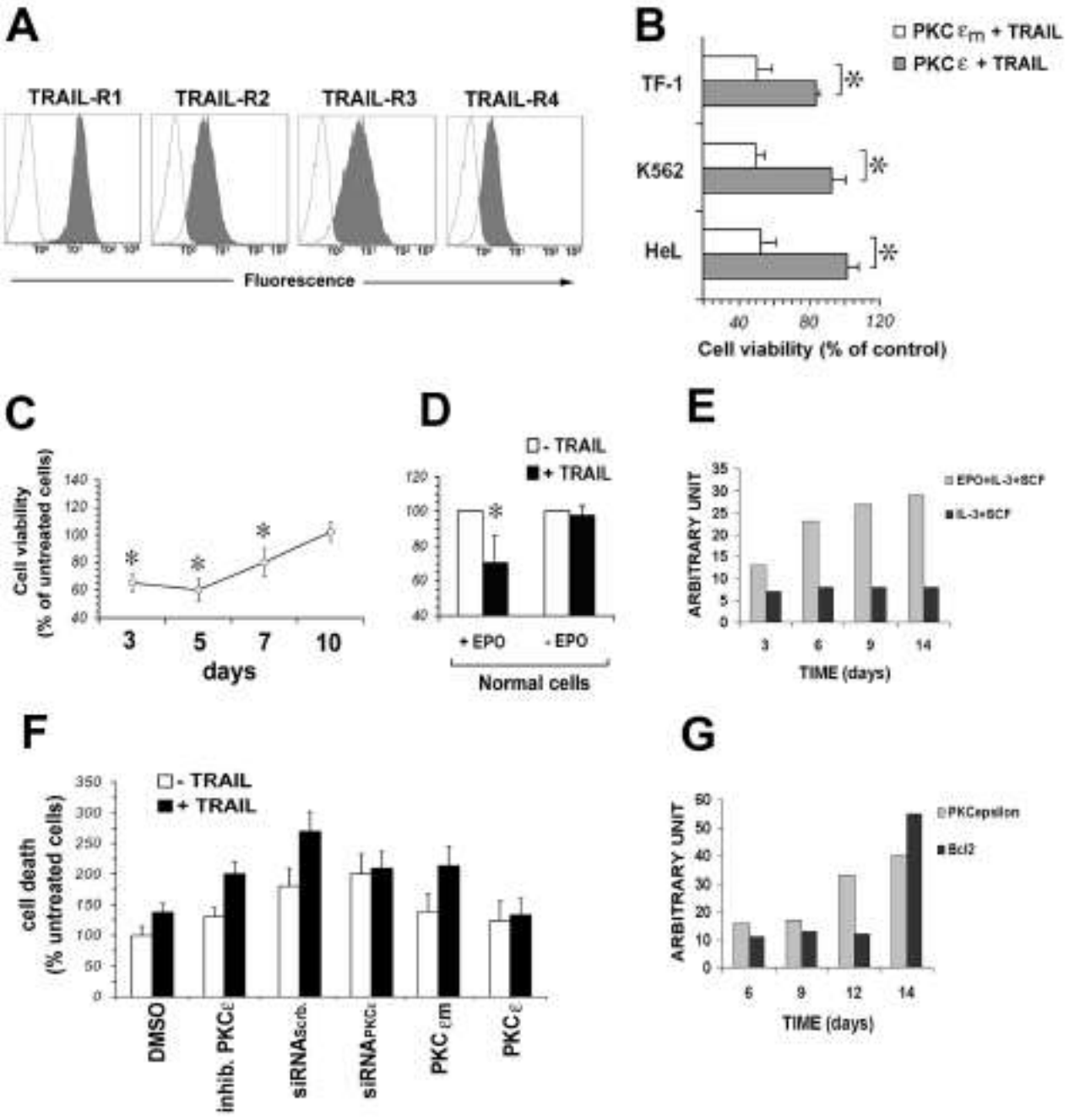

Figure 1. (A) Phenotypic profile of HeL cells. Similar profiles were obtained from K562 and TF-1 cells. The cells were stained with specific MoAbs to TRAIL-R1, -R2, -R3, -R4 (1 μg of anti-TRAIL-Rs mAbs – Alexis Biochemical, San Diego, CA, USA - followed by PE-labeled goat anti-mouse IgG – Immunotech, Beckman Coulter, Miami, FL, USA). Specific fluorescence histograms (grey) are superimposed to negative controls (empty histograms). (B) K562, HeL and TF-1 cell lines were transfected with PKCε (filled bars) or with PKCεm (empty bars) and 48 hour later were treated with 50 ng/mL of TRAIL. Residual cell viability was analyzed by flow cytometry, staining cells with Anexin V-FITC (ACTIPLATE, Valter Occhiena, Torino, Italy) and PI. The mean of three independent experiments is reported as percentage of control. *p<0.05. (C,D) Sensitivity to TRAIL-induced apoptosis of CD34-derived erythroblasts. At the indicated time intervals cells were treated for 48 hours with 50 ng/mL of TRAIL and the percentage of residual cell viability was monitored by staining apoptotic cells with annexin V-FITC and PI. CD34 cells obtained from donors were cultured 3 days with IL-3 and SCF in serum free medium with (+EPO) or without (-EPO) EPO in the presence or absence of TRAIL. Quantification of cell viability expressed as percentage of control. Each histogram is the mean of 3 independent experiments expressed as percentages of control. *p<0.05. Panel (E) Densitometric histogram of western blot detection of total PKCε protein expression in CD34 cells cultured in serum free medium with the indicated cytokines. (F) TRAIL-induced apoptosis in CD34 cells pretreated with specific PKCε inhibitor (250 μg/mL for 48 hr, Calbiochem) or transfected with GFP-PKCε, GFP-PKCεm or PKCε-specific siRNA. Forty-eight hours later cells were treated with 50 ng/mL of TRAIL. Apoptosis was monitored 48 hours after TRAIL treatment, staining cells with Annexin V-FITC and PI. Controls were represented by CD34 cells transfected as above but not treated with TRAIL. TRAIL-induced cell death is reported as percentage of controls. *p<0.05. (G) CD34-derived erythroblasts were cultured with IL-3, SCF and EPO up to 14 days and total Bcl-2 (1:50, Santa Cruz, Ca, USA) and PKCε protein expression levels were monitored at the indicated times by Western blot. Densitometric histogram is shown.

human PKCε mRNA (NM005400), were synthesized by Silencer siRNA Construction Kit (Ambion, Austin, Texas) (Mirandola *et al.,* 2004) and transfected (100nM each) using the Amaxa nucleofection technology™ (Amaxa, Koeln, Germany)).

EPO induces Bcl-2 up-regulation in human CD34-derived erythroblasts

From the data obtained by Gillespie and co-workers (Gillespie *et al.,* 2005) it appears that PKCε might have a general role in the fine tuning of the signalling emanating from death receptors triggering. Given that the general activation of PKC by PMA does not affect TRAIL-Rs aggregation at cell surface (Harper *et al.,* 2003), and that over-expression of PKCε does not modulate TRAIL-Rs surface density, it has been supposed the presence of down-stream anti-apoptotic mediators possibly modulated by PKCε.

A possible candidate involved in the protection from TRAIL-mediated apoptosis is Bcl-2, whose levels have been demonstrated to be sensitive to PKCε (Gubina *et al.,* 1998). Kinetic analysis of Bcl-2 expression during EPO-driven erythroid maturation showed an up-regulation of the protein expression at day 14 (Figure 1G). The data therefore hint at Bcl-2 as one possible candidate down-stream PKCε that might mediate erythroid cell resistance to TRAIL. In fact, Bcl-2 levels are up-regulated in erythroid progenitors with a kinetic that is compatible with that of EPO-driven PKCε induction, even it is not possible to exclude that other antiapoptotic intermediates could counteract the effects of TRAIL. These data parallel those from Gubina *et al.,* (1998) that demonstrated that the over-expression of PKCε in the TF-1 cell line was able to induce Bcl-2 expression.

In conclusion, PKCε is protective against TRAIL-induced apoptosis. Early erythroblasts are insensitive to TRAIL due to the lack of surface receptors. By day 3 the receptors are expressed and erythroblasts can be killed by TRAIL, up to day 7/8. in this time period, any soluble or membrane-bound TRAIL can limit the number of maturing erythroid cells. PKCε, that is up-regulated upon EPO stimulation in erythroblasts protects from apoptosis the surviving cells *via* Bcl-2 up-regulation. Hematological disorders characterized by alterations of the number of differentiating erythroid cells can recognize a new molecular pathogenetic role to an umbalanced induction of PKCε.

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