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Erk-dependent cytosolic phospholipase A_2 activity is induced by CD95 ligand cross-linking in the mouse derived Sertoli cell line TM4 and is required to trigger apoptosis in CD95 bearing cells

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Abstract

In the present study we demonstrated that CD95L cross-linking generated reverse signalling in the mouse derived Sertoli cell line TM4. Treatment of TM4 cells with mAb anti-CD95L induced activation of the cytosolic phospholipase A_2 ($cPLA_2$). Cytosolic PLA_2 activation was controlled by the MAPK pathway as indicated by the ability of the specific MEK inhibitor, PD098059, to abolish $cPLA_2$ activation. In addition, Western blot experiments showed a rapid increase in phosphorylated Erk1/2 following CD95L cross-linking, while no effect on the phosphorylation of other MAPK, p38 or JNK, was observed. CD95L cross-linking by mAb increased the levels of soluble CD95L and apoptotic activity of TM4 cell supernatants, which was blocked by co-incubation with the PLA_2 inhibitor, AACOCF3 or PD098059. Finally, pre-treatment of TM4 cells with $AACOCF_3$ or PD098059 completely abolished TM4-induced apoptosis of Jurkat T cells, thus indicating that the Erk/$cPLA_2$ pathway is required for CD95L-induced apoptosis. *Cell Death and Differentiation* (2000) **7,** 916–924.

Keywords: CD95L; reverse signalling; MAPK; PLA_2; apoptosis

Abbreviations: CD95L, CD95 ligand; PLA2, phospholipase A_2; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; DAG, diacylglycerol; TNF, tumour necrosis factor

Introduction

In the last few years, the importance of the CD95 (Fas, APO-1)/CD95L (FasL) system during development and tissue homeostasis has become increasingly clear.[1–3] In the adult the CD95 system has been demonstrated to play a major role in the modulation of the immune system such as the maintenance of the peripheral T and B cell tolerance and cell mediated cytotoxicity.[4,5] In addition, constitutive expression of CD95L in testicular Sertoli cells and in different ocular cell types has been proved to be a major determinant in maintaining the immune privilege of these tissues.[6–9] This also applies to several tumours, which have been found to express functional CD95L as a protective mechanism against the CD95 positive cells of the immune system.[10,11] The physiological relevance of the CD95 system is underscored in *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mice carrying mutations in the CD95 and CD95L genes, respectively. These animals exhibit dramatic lymphoproliferation and systemic autoimmunity as a consequence of altered lymphocyte apoptosis.[12,13] Within the testis, the CD95 system, beyond its role in immune privilege, has been identified as a paracrine signalling system by which Sertoli cells, expressing CD95L, may induce CD95-expressing germ cell apoptosis.[14,15] Indeed, in the mammalian testis physiological apoptosis occurs continuously to coordinate the number of differentiating germ cells to that of the supporting Sertoli cells and to ensure a quality control of the produced gametes.[16–20] In this context, J Lee and colleagues have recently demonstrated a direct involvement of the CD95 system in germ cell apoptosis under normal conditions or following testicular injury such as radiation exposure.[14,15,21]

CD95L is a type II membrane bound protein which belongs to the tumour necrosis factor (TNF) family, and which induces apoptosis in CD95 bearing cells.[22,23] CD95 is a transmembrane receptor protein, which shares a high degree of homology with the tumour necrosis factor/nerve growth factor receptor family. All are characterized by an intracellular domain, called the 'death domain', responsible for the activation of the multiple intracellular signalling pathways following CD95 interaction with CD95L or CD95 receptor cross-linking.[24–28] Interestingly, it has been recently proposed that CD95L may also transduce internal co-stimulatory or reverse signals upon interaction with CD95 in cytotoxic T lymphocytes.[29] This is further supported by the ability of other TNF family members to transduce internal signals, as well as by the cross-species conservation of the cytoplasmic domain of these ligands.[30–32] In this context, of particular interest is the recently reported identification of a casein kinase I motif within the cytoplasmic domain of six members of the TNF family, including CD95L.[33] In particular, Watt and colleagues demonstrated that the cytoplasmic domain of membrane bound TNF is phosphorylated under basal conditions and that binding to the TNF receptor induced rapid dephosphorylation.[33] In the present study, we first

show that the mouse-derived Sertoli cell line TM4 expresses functional CD95L at a higher level compared to that present in primary cultures of rat prepubertal Sertoli cells, thus providing a useful cell system to investigate CD95L-mediated reverse signalling as well as apoptosis. CD95L activation on TM4 cells, through cross-linking by monoclonal anti-CD95L antibody, results in a rapid and specific induction of Erk1 and Erk2 phosphorylation and in $cPLA_2$ activity. Importantly, inhibition of this signalling pathway completely abolished TM4 cell induced- and CD95L-mediated apoptosis.

Results

CD95 ligand expression in prepubertal rat Sertoli cells in culture and in the TM4 cell line

The aim of the present study was to investigate the ability of Sertoli cell-expressed CD95L to induce reverse signalling following its activation by specific mAb cross-linking. To this end, in the initial experiments we monitored the possibility of using the mouse derived Sertoli cell line, TM4. In Figure 1 we show the expression, at both protein and mRNA level, of CD95L in TM4 cells compared to CD95L expression in primary cultures of rat prepubertal Sertoli cells. Both Western blot (Figure 1A), obtained using the monoclonal Ab 33, and semi-quantitative RT–PCR (Figure 1B), showed higher expression of CD95L in TM4 cells with respect to that in prepubertal Sertoli cells after 24–36 h of culture. Immunofluorescence followed by flow cytometry analysis of CD95L expression in TM4 cells, using clone H11 anti-CD95L monoclonal antibody, showed that the majority of CD95L immunopositivity was intracellular (Figure 2). Similar results were obtained using mAb 33 (data not shown) while no signals were observed with the mouse anti-rat CD161 antibody used as negative control. In addition, measurement of soluble CD95L (sCD95L) by Elisa in the supernatants of TM4 cells also showed the presence of sCD95L (about 0.1 ng/10^6 cells). To assess the functionality of CD95L expressed by TM4 cells, JAM test experiments were performed using, as target, CD95 positive Jurkat cells. As reported in Figure 3, TM4 cells were able to induce DNA fragmentation in Jurkat cells at effector/target cell ratios as low as 3 to 1 (Figure 3). The specificity of this interaction was confirmed by the preincubation of Jurkat cells with a specific blocking monoclonal anti-CD95 antibody, which completely abolished TM4-induced apoptosis of Jurkat cells (Figure 3). In view of the presence of sCD95L in the surnatants of TM4 cells we also tested the presence of apoptotic activity in medium conditioned by 2×10^6 TM4 cells for 4 h incubated with 10^5 labelled Jurkat cells. The results showed the presence of only very weak apoptotic activity (1–2% of hypodiploid nuclei). To determine whether TM4 cells were also able to induce specific lysis of target cells, the ^{51}Cr-release assay was performed. The results showed that effector/target ratios as high as 100 : 1 were devoid of cytotoxic effects on Jurkat cells (insert of Figure 3), thus indicating that TM4-induced death of Jurkat

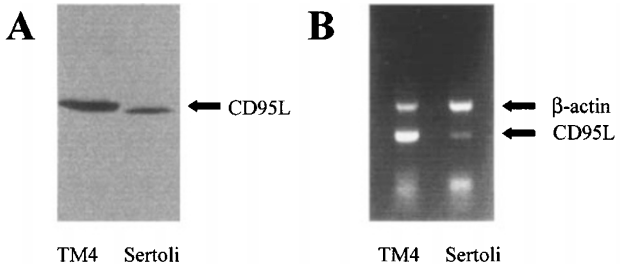

Figure 1 CD95L expression in the mouse derived Sertoli cell line, TM4, and primary culture of prepubertal rat Sertoli cells. (**A**) Expression of CD95L protein in TM4 cell line and in prepubertal rat Sertoli cells. Cells were plated on Petri dishes in 1% FCS DMEM (rat Sertoli cells) or on flasks in DMEM supplemented with 5% HS and 2.5% FCS (TM4 cell line) for 2 days; then, after a confluence of 90% was reached, cells were scraped in PBS and homogenised in lysis buffer. Protein extracts were processed for Western blotting, as described in the Materials and Methods section, using the mouse monoclonal anti-CD95L antibody Clone 33 as primary antibody. (**B**) CD95L mRNA expression in TM4 cell line and prepubertal rat Sertoli cells. Total RNA from TM4 and Sertoli cells culture was extracted as described in Materials and Methods, then 2 μg of each sample were reverse transcribed; cDNA obtained was amplified using CD95L primers and β-actin primers as internal control. Products of PCR were run on 2% agarose gel giving two specific bands: CD95L (239 bp) and β-actin (389 bp). Data presented are representative of three independent experiments

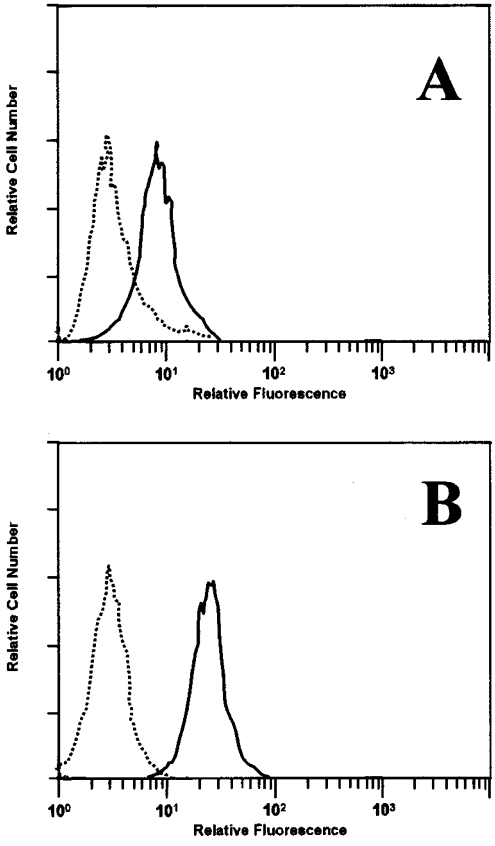

Figure 2 Immunofluorescence and flow cytometry analysis of CD95L expression in TM4 cells. About 1×10^6 cells, permeabilized (**B**) or not (**A**) by incubation for 10 min in PBS containing Triton X-100 (0.1%), were incubated for 1 h at 4°C with anti-CD95L antibody (Clone H11, solid lines), washed twice in PBS and incubated for 1 h with FITC-Gam prior FACS analysis as described in the Materials and Methods section. Data presented are representative of three independent experiments. Dotted lines represent cells incubated with FITC-Gam alone

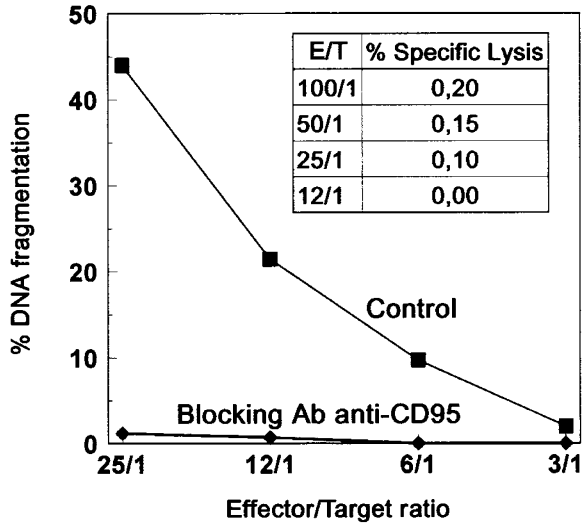

Figure 3 TM4 cells induce apoptosis of Jurkat T cells in a CD95-dependent manner. ^{3}H-TdR-labelled Jurkat cells were cultured with TM4 effector cells at the indicated effector/target (E/T) cell ratios. Jurkat cells were preincubated in the absence (control) or in the presence of mAb anti-CD95 ($5\,\mu g \times 10^{-6}$ cells) for 30 min at 4°C. Target cell death was determined at 4 h by measuring DNA fragmentation of ^{3}H-labelled target cell DNA (JAM assay). TM4 cells were also tested for lytic activity against ^{51}Cr-labelled target cells at the indicated E/T ratios (insert). Data presented are representative of three independent experiments

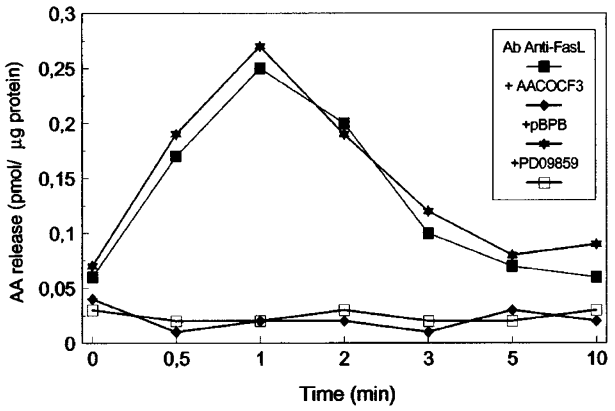

Figure 4 Time-dependent phospholipase A_2 (PLA_2) stimulation by CD95L cross linking in TM4 cells by specific mAb. TM4 cells were treated in the absence (control) or in the presence of mAb anti-CD95L (Clone H11, 1 μg/ml) for different period of time. At the end of the incubation time cell lysates were prepared and tested for PLA_2 activity against radiolabelled arachidonyl-PC vesicles as described in the Materials and Methods section. In parallel culture TM4 cells were pre-treated with $AACOCF_3$ (10 μM) or pBPB (20 mM) or PD09859 (50 μM). Data shown represent the mean of three independent experiments with S.E.M. lower than 10%

cells was totally due to triggering of the CD95-mediated apoptotic death pathway. In this regard it has to be noted that previous studies on T-cell mediated cytotoxicity have shown the ability of CD95L to induce ^{51}Cr-release from target cells.[34] The reason(s) for these discordant observations may lie in the different effector cells or in the different sensitivity of the target cells used and/or in the different experimental conditions. For example, one possibility is that a longer coincubation time of effector/target cells may lead to an increased release of ^{51}Cr from Jurkat cells.

These observations prompted us to adopt the TM4 cell line as a convenient and versatile cell system for all the following experiments.

Effects of CD95L engagement by monoclonal antibody on phospholipase A_2 (PLA_2) and phosphatidylinositol-specific phospholipase C (PI-PLC)

To assess the possible involvement of PLA_2 following CD95 cross-linking, cell extracts from control and mAb anti-CD95L (Clone H11, 1 μg/ml) treated TM4 cells, were tested for enzyme activity by analyzing arachidonic acid (AA) release from radiolabelled phospholipid 1-stearoyl-2-[1-^{14}C] arachidonyl L-3-phosphatidylcholine vesicles. As demonstrated in Figure 4, treatment of TM4 cells with anti-CD95L antibody induced a drastic and transient increase of PLA_2 activity. This stimulation was maximal after 1–2 min of incubation with the anti-CD95L antibody ($P<0.01$) and could be completely abolished by pre-treatment of TM4 cells with the $cPLA_2$ inhibitor $AACOCF_3$ (10 μM) but not by the $sPLA_2$ inhibitor pBPB (20 μM) (Figure 4). In addition, experiments showing that the reducing agent DTT, which can denature secretory but not cytosolic PLA_2, did not influence PLA_2 activity (data not shown), further confirming the involvement of $cPLA_2$ but not of $sPLA_2$ in the CD95L-induced AA release. Thus, together these findings demonstrate activation of $cPLA_2$ following CD95L cross-linking in TM4 cells. In contrast, we never observed any modification in PI-PLC activity in cell extracts tested against radiolabelled PI vesicles following CD95L cross-linking (data not shown). A similar outcome to the above experiments was obtained using the mouse monoclonal anti-CD95L antibody from Transduction (Clone 33). In the latter, an isotype-matched mouse monoclonal anti-rat CD161 antibody was used as control and did not show any effect on PLA_2 activity (data not shown). In the same Figure 4 we also show the effects of the MEK inhibitor PD098059 on PLA_2 stimulation following CD95L cross-linking by mAb (Clone H11). Pre-treatment of TM4 cells with PD098059 (50 μM) completely abolished $cPLA_2$ activation by CD95L cross-linking (Figure 4). The possible involvement of MAPK in the intracellular signalling activated by CD95L cross-linking in TM4 cells was further investigated by Western blot analysis of phosphorylated MAPK, Erk1, Erk2, p38 and JNK. Results reported in Figure 5 show a very rapid increase of the phosphorylated Erk1 and Erk2, temporally consistent with the activation of $cPLA_2$. On the other hand, in similar experiments, we did not observe any modulation of phosphorylated MAPK p38 or JNK (data not shown).

CD95L-induced reverse signalling is required for TM4 cell-induced apoptosis

We finally decided to analyze the possible involvement of Erk and $cPLA_2$ activation on TM4-induced and CD95L-mediated apoptosis of CD95 positive cells. To this end, TM4 cells were pre-treated or not for 4 h with the $cPLA_2$ inhibitor $AACOCF_3$

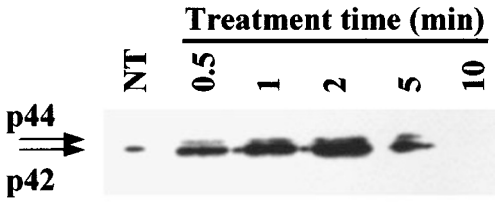

Figure 5 Erk1 and Erk2 (p44 and p42 MAPK) activation in TM4 cells following CD95L cross-linking. TM4 cells were treated in the absence (control) or in the presence of mAb anti-CD95L (Clone H11, 1 μg/ml) for different periods of time (0.5–10 min). At the end of the incubation time, cell proteins were prepared and analyzed for phospho-MAP kinase expression by Western blot as described in the Materials and Methods section. Data presented are representative of three different experiments

(10 μM) or the MEK inhibitor PD098059 (50 μM) and washed three times with PBS before running the JAM test. The results of these experiments, reported in Figure 6, demonstrated that inhibition of $cPLA_2$ activity, by either $AACOCF_3$ or PD098059, completely abolished the ability of TM4 cells to induce apoptosis of Jurkat cells.

The possible involvement of $cPLA_2$ on the expression of CD95L mRNA in TM4 cells was investigated by treatment of TM4 cells with exogenous arachidonic acid (10^{-6} M for 16 h). The results of the semiquantitative RT–PCR, where CD95L was coamplified with βactin as control, did not show any modulation of CD95L mRNA following arachidonic acid treatment (data not shown).

CD95L cross-linking increases the levels of soluble CD95L and the apoptotic activity of TM4 cell supernatants

In view of the latter results, we investigated the effects of AACOCF3 and PD098059 on the expression of membrane bound CD95L in TM4 cells as well as on release of soluble CD95L. To this end TM4 cells were treated with AACOCF3 (10 μM) or PD098059 (50 μM) for 2 h. At the end of the incubation time, levels of membrane bound CD95L on non-permeabilized cells were analyzed by immunofluorescence and flow cytometry. The results obtained showed the ability of PD098059 (see Figure 7) and AACOCF3 (data not shown) to increase the level of membrane bound CD95L in TM4 cells. In parallel experiments, the levels of soluble CD95L present in the supernatants of TM4 cells following CD95L cross-linking in the absence or in the presence of AACOCF3 or PD098059 were analyzed. In these experiments TM4 cells were pre-treated with anti-CD95L antibody (Clone H11, 1 μg/ml) for 30 min, in the absence or in the presence of AACOCF3 (10 μM) or PD098059 (50 μM), washed twice and incubated with fresh medium for further 2 h. At the end, levels of sCD95L in the supernatants were measured by Elisa. The results of this experiment demonstrated the ability of CD95L cross-linking to increase the level of sCD95L while both inhibitors, PD098059 and AACOCF3, drastically reduced this stimulation (Figure 8). We next decided to verify whether the increased levels of sCD95L observed following CD95L cross-linking in the supernatants of TM4 cells would correspond with increased apoptotic activity. With this aim, 2 h basal TM4 cell conditioned media or media from cells pre-

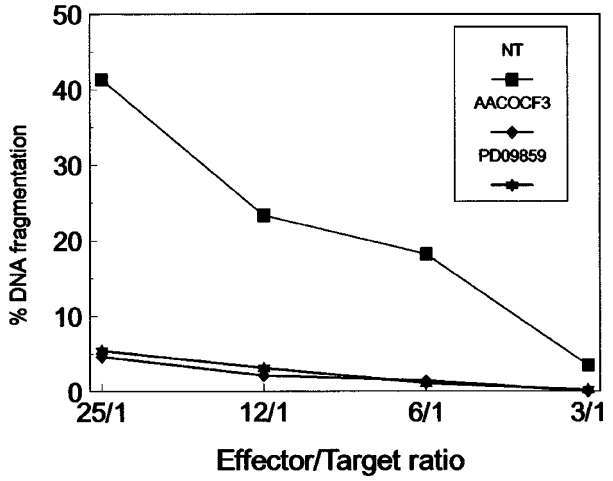

Figure 6 Effects of the PLA_2 inhibitor ($AACOCF_3$) and MEK inhibitor (PD098059) on TM4 cells induced apoptosis of Jurkat T cells. TM4 cells were pre-treated for 4 h with $AACOCF_3$ (10 μM) or PD098059 (50 μM) before running the JAM test as described in the Materials and Methods section at the indicated effector-target cell ratios. Data shown represent the mean of three independent experiments with S.E.M. lower than 10%

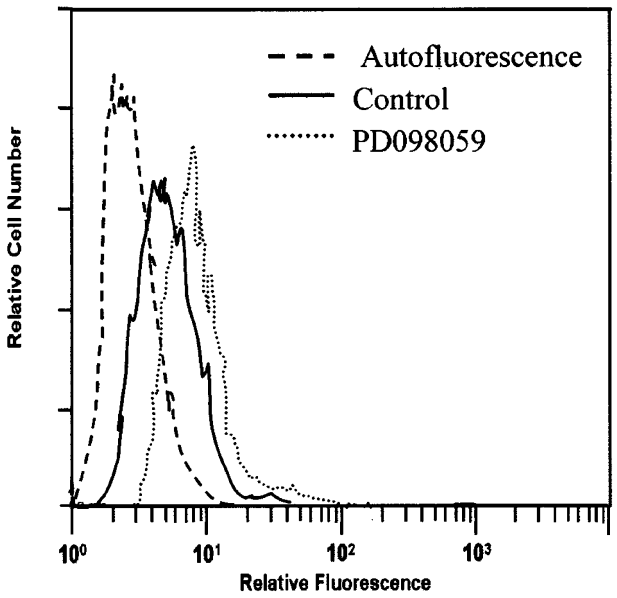

Figure 7 Effects of the MEK inhibitor (PD098059) on the level of membrane bound CD95L in TM4 cells. TM4 cells were treated with PD098059 (50 μM) or with $AACOCF_3$ (10 μM, data not shown) for 4 h. At the end of the incubation time non permeabilized cells were incubated for 1 h at 4°C with anti-CD95L antibody (Clone H11) washed twice in PBS and incubated for 1 h with FITC-Gam prior to FACS analysis as described in the Materials and Methods section. Data presented are representative of three independent experiments

treated with anti-CD95L antibody for 30 min, in the absence or presence of PD098059, were incubated overnight with labelled Jurkat cells. The results of these experiments (see Figure 9) showed an increase apoptotic activity of TM4 cell supernatants following CD95L cross-linking which was blocked by co-incubation with PD098059.

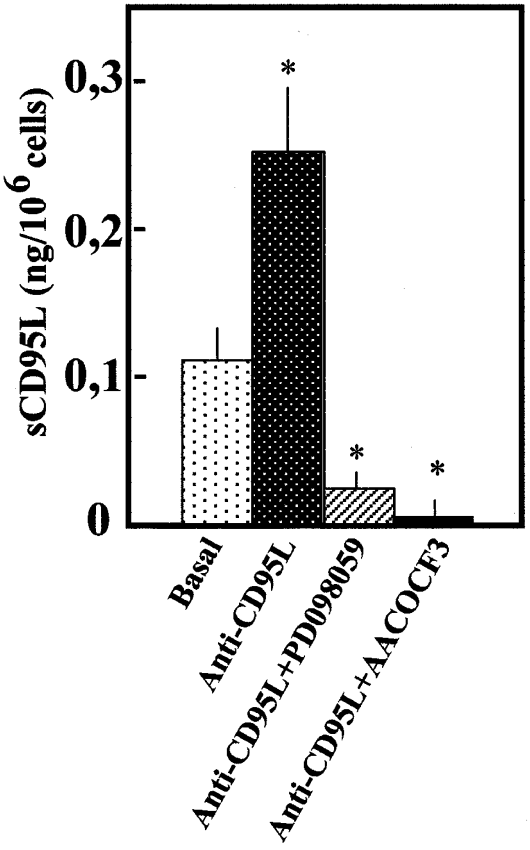

Figure 8 Effects of the MEK inhibitor (PD098059), the $cPLA_2$ inhibitor (AACOCF3) and CD95L cross-linking on the release of soluble CD95L in TM4 cell supernatants. To assess the effects of CD95L cross-linking on the levels of soluble CD95L TM4 cells have been pre-treated for 30 min with the anti-CD95L monoclonal antibody (Clone H11, 1 μg/ml) in the absence or in the presence of the MEK inhibitor PD098059 (50 μM) or the $cPLA_2$ inhibitor AACOCF3 (10 μM), washed and incubated for further 2 h. At the end, levels of soluble CD95L in the suranatants were quantitated by Elisa accordingly to the kit instructions. Data represent the mean $\pm$ S.E.M of three independent experiments. *$P<0.01$, Anti-CD95L vs Basal and Anti-CD95L+PD098059 or Anti-CD95L+AACOCF3 vs Anti-CD95L

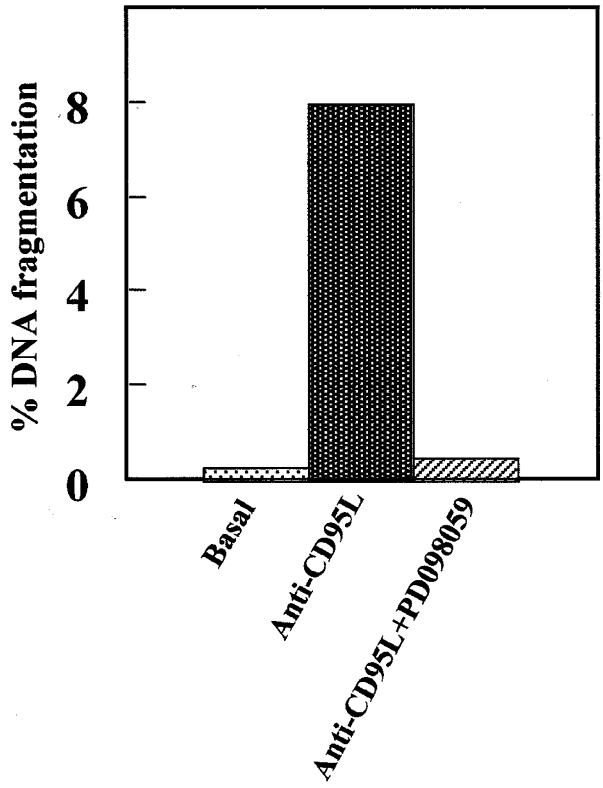

Figure 9 Increased apoptotic activity in TM4 cells supernatants following CD95L cross-linking. The apoptotic activity of TM4 cells conditioned media for 2 h in basal conditions or following pre-treatment with anti-CD95L antibody (Clone H11, 1 μg/ml) for 30 min, in the absence or in the presence of PD098059 (50 μM), were tested by JAM test as described in the Materials and Methods section. Data are representative of three independent experiments

Discussion

In the present study we provide evidence suggesting that reverse signalling through CD95L in the mouse derived Sertoli cell line, TM4, involves sequential activation of the MEK, MAPK Erk1/2 and the $cPLA_2$ pathways, which are required for CD95L induces apoptosis of CD95 bearing cells.

A growing number of reports have recently underscored the importance of reverse signalling in the physiology of diverse TNF family members such as CD30L, CD40L, OX40L as well as CD95L.[29,31,32,35] In particular, CD30L activation by mAb cross-linking has been shown to induce a rapid oxidative burst and increased IL-8 production in freshly isolated neutrophils,[32] while reverse signalling through CD95L has been demonstrated to be necessary to achieve maximal proliferation of cytotoxic T lymphocytes in response to alloantigens.[29] The existence of biological function(s) associated with the cytoplasmic tail of the TNF family members is also suggested by the high level of conservation of this domain in individual members across species, although the cytoplasmic domains are very different between family members.[30–32] However, the mechanisms or biochemical pathways involved in reverse signalling elicited by different TNF family members are yet to be unravelled. Of particular relevance are the recent findings of Watt and colleagues[33] showing the presence of a putative casein kinase I (CKI) consensus sequence in the cytoplasmic domain of six members of the TNF family, including CD95L. In particular, Watt and colleagues demonstrated that the cytoplasmic domain of TNF is phosphorylated in basal conditions, and that binding to the TNF receptor induced a rapid dephosphorylation. This, as predicted by the same Authors, could represent the initial event of a signalling pathway activated by the binding of this family of cytokine with their cognate receptor.

The aim of the present study was to investigate CD95L-induced reverse signalling in Sertoli cells well known to constitutively express CD95L. To this end we used the mouse derived Sertoli cell line TM4 as a convenient and versatile experimental cell system. Indeed, this cell line expresses functional CD95L molecules, as judged by JAM test experiments (see Figure 3), at a level about threefold

higher than those present in primary culture of prepubertal Sertoli cells as evidenced by Western blot (Figure 1A) and semi-quantitative RT–PCR (Figure 1B) experiments. In addition, as reported for other cell types,[36,37] in TM4 cells we also observed a major intracellular localization of CD95L molecules (Figure 2). Even though the physiological meaning for this cellular distribution is still unclear, it is worth noting that Bossi and Griffiths[37] have recently demonstrated that the cytoplasmic tail of CD95L contains a putative signal sequence that sorts the newly synthesized protein to secretory lysosomes of $CD4^+$, $CD8^+$ T cells and natural killer cells.

Within the seminiferous epithelium, Sertoli cells perform several physiological functions essential to normal spermatogenesis. Among these, the Sertoli cell has a scavenger role or a macrophage like-activity necessary to remove, from the tubular lumen, all residual bodies left by the differentiating spermatids as well as to remove apoptotic germ cells.[16–19] In normal macrophages, PLA_2 and PI-PLC activities have been shown to play a major role in modulating cellular functions following activation.[38] We thus surmised that these two enzymes could be involved in reverse signalling following CD95L cross-linking in Sertoli cells. Indeed, CD95L cross-linking by specific monoclonal antibody in TM4 cells induced a rapid and specific increase of the cytosolic, but not of the secretory, PLA_2 activity (Figure 4). In contrast, no effects were observed on the activity of the phosphatidylinositol-specific PLC. Phosphorylation represents a key step in the activation of the $cPLA_2$ in many types of cells.[39,40] Kinases involved in the phosphorylation of the $cPLA_2$ include Erk 1 and 2, which can phosphorylate $cPLA_2$ *in vitro* on serine 505, and the p38 MAPK.[39,41–43] Our findings showing the ability of the specific MEK inhibitor, PD098059, to abolish $cPLA_2$ activation (Figure 4) and the rapid phosphorylation of the MAPK Erk1/2 (Figure 5) following CD95L cross linking strongly suggesting the involvement of the MAPK pathway in CD95L induced reverse signalling in TM4 cells. Thus, even though different reports suggest that kinases other than Erk 1/2 may be involved in phosphorylation of $cPLA_2$,[44,45] in TM4 cells the sequential activation of MEK and the MAPK Erk 1/2 appears to play a major role in the phosphorylation and consequent activation of $cPLA_2$ following CD95L cross linking. Further studies will, however, be required to unravel the steps leading to MEK activation following CD95L cross-linking or binding to CD95. Of particular interest is the putative SH3 (Src homology region[46]) binding site identified in the cytoplasmic tail of human, mouse and rat CD95L by Takahashi and colleagues.[47] Indeed, this region has been demonstrated to bind strongly to the SH3 domain of the Fyn tyrosine kinase, while weak binding was observed with the N- and C-terminal SH3 domains of Grb2.[48–50]

The observation that pharmacological inhibition of either MEK or $cPLA_2$ in TM4 cells completely abolished the ability of TM4 cells to induce apoptosis of Fas positive target cells (Figure 6) could have physiological significance. This appears to corroborate the findings of Suzuki and Fink[29] mentioned above, thus suggesting that reverse signalling through CD95L plays an essential role in the physiology of this cytokine. The results of the experiments reported here (see Figures 7–9) suggest that the sequential activation of the MEK, MAPK and $cPLA_2$ pathways following CD95L cross-linking may lead to increased levels of soluble CD95L in TM4 cell supernatants which in turn may contribute to TM4 cell-induced apoptosis of CD95 bearing cells. However, even if our observations suggest a role for sCD95L in TM4 cell induced apoptosis, in agreement with previous reports showing the ability of sCD95L to induce apoptosis in Jurkat and other cell types,[51,52] the real contribution of sCD95L in the apoptotis of CD95 positive cells under physiological conditions remains to be established. Indeed, evidence from several sources would suggest that sCD95L has *per se* a very weak apoptotic activity and is able to antagonize the apoptosis of membrane bound CD95L (mCD95L) which has been indicated as the functional form of CD95L.[23,53–55] To this end of particular interest are the observations of Martinez-Lorenzo and colleagues showing the ability of activated human T cells to release bioactive CD95L and APO2 ligand in microvesicles as intact nonproteolyzed molecules.[56]

All together these evidences suggest that bi-directional signalling for TNF family members and their cognate receptor could be of physiological relevance requiring highly coordinated cellular functions in ligand and receptor expressing cells. Indeed, the data here presented indicate that the initial CD95/CD95L interaction, in our cellular system, is required but not sufficient to trigger the apoptotic events in the target cells.

Materials and Methods

Materials

DMEM, trypsin (type I), collagenase (type II), EDTA, antimouse IgG biotin-conjugated, extravidin peroxidase-conjugated, 3′-3′ diaminobenzidine and arachidonic acid were purchased from Sigma Chemical Co (St. Louis, MO, USA). RPMI 1640 medium was purchased from GIBCO-BRL (Life Technologies Inc., USA). The Bradford protein assay kit, electrophoresis and Western blotting reagents, and molecular weight markers were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Foetal calf serum (FCS) and horse serum (HS) were purchased from Mascia Brunelli (Milan, Italy). Blocking monoclonal anti-CD95 antibody (clone ZB4) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Two different anti-CD95 ligand (CD95L) antibodies were purchased from Transduction Laboratories (monoclonal Ab 33, Lexington, KY, USA) and from Alexis (monoclonal Ab H11, San Diego, CA, USA). The mouse anti-rat CD161 (Clone MCA 1427) antibody was purchased from Serotek (Oxford, UK). Phospho-MAPK Antibody Sampler containing the primary antibody for Phospho-Erk 1/2 MAPK, Phospho-p38 MAPK and Phospho-SAPK/JNK and the anti-rabbit IgG secondary antibody (HRP conjugated) was purchased from New England Biolabs, Inc. (Beverly, MA, USA). First-strand cDNA synthesis kit was purchased from Amersham Biotech (Milan, Italy). Rat CD95L and β-actin primers were obtained from Labtek (Milan, Italy). Taq DNA polymerase was purchased from Perkin Elmer (Rome, Italy). [methyl-^{3}H]Thymidine 1 mCi/ml (specific activity 80 Ci/mmol) and sodium chromate in aqueous solution ($Na_2{}^{51}CrO_4$) 5 mCi/ml (specific activity 200–500 mCi/mg chromium) were purchased from Amersham (Little Chalfont, UK). DL-dithiothreitol (DDT) and p-bromophenacyl bromide (pBPB) were purchased from Sigma Chemical Co (St. Louis, MO,

USA). Trifluoromethyl ketone analogue of arachidonic acid ($AACOCF_3$) and MEK inhibitor (PD09859) were purchased from Calbiochem (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (FITC-Gam) was purchased from Monosan (Bio Division Milan, Italy) and the FITC-conjugated rabbit anti-rat IgG was purchased from Vector Laboratories (Burlingame, CA, USA). Elisa kit for the measurement of soluble CD95L was purchased from Medical & Biological Laboratories Co. (Naka-ku Nagoya, Japan).

Cell cultures

Wistar rats were reared in our Institute facilities and all the experimental protocols were approved by the local ethical committee. Purified Sertoli cells were prepared from 5–10-day-old Wistar rats, as previously described,[57] and plated in 1% FCS-DMEM. The mouse Sertoli cell derived cell line TM4,[58] was cultured in DMEM supplemented with 5% HS and 2.5% FCS at 37°C in 5% CO_2, as previously described.[58] Jurkat cell line (acute T cell leukaemia, human) was cultured in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin.

Immunofluorescence and flow cytometry analysis of CD95L expression

Expression of CD95L on TM4 was assayed by indirect immunofluorescence and flow cytometry. 1×10^6 cells, permeabilized or not by incubation with Triton X-100 (0.1% in PBS for 10 min), were incubated with anti-CD94L (Clone H11 or the mAb 33 or the mouse anti-rat CD161) antibody for 1 h at 4°C, washed twice in phosphate buffered saline and stained with FITC anti-rat IgG or anti-mouse IgG for 1 h. A FACScan 440 (Becton Dickinson, Mountain View, CA, USA) flow cytometer was used for analysis.

Western blot analysis

To study the expression of CD95L at the protein level, both Sertoli cells and TM4 cells were scraped in PBS, centrifuged at 1200 r.p.m. for 10 min, resuspended in lysis buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 2 μg/ml aprotinin) and subjected to three rounds of freeze–thaw lysis. Samples were then centrifuged at 14 000 r.p.m. at 4°C for 20 min and surnatants were collected and frozen to −80°C. About 50 μg of extracted cell proteins, were separated on 12% SDS–PAGE, under reducing conditions and proteins transferred electrophoretically to nitrocellulose membrane. Non-specific binding sites were blocked with 10% non-fat dry milk in Tris-buffered saline-Tween 20 (0.3%) (20 mM Tris, 137 mM NaCl, pH 7.6) for 2 h at room temperature and blots incubated overnight with a mouse monoclonal anti-CD95L antibody (Clone 33, 1 : 1000) diluted in blocking solution containing 5% non-fat dry milk. After washing in Tris-buffered saline-Tween 20, nitrocellulose membranes were incubated for 1 h with goat antimouse horseradish peroxidase-conjugated IgG (Bio-Rad, Hercules, CA, USA), diluted 1 : 3000 in blocking solution, and immunoreactivity assessed by chemiluminescence reaction using the ECL Western blocking detection system (Amersham, Buckinghamshire, UK). Immunopositive bands were quantified by scanning densitometry, using Molecular Analyst PC software for the Bio-Rad model 670 scanning densitometer. To assess the effects of CD95L cross-linking on phospho-MAP kinases, cells were treated for different period of time with the mAb antiCD95L. At the end of the treatment cell extracts were prepared and analyzed by Western blot, as above described, with the antibody anti-phospho-MAPK (Erk 1/2) or anti phospho p38 MAPK or anti phospho-SAPK/JNK, all diluted 1 : 1000.

RNA isolation and analysis

Total cellular RNA was extracted from cultured Sertoli and TM4 cells as previously described.[59] Levels of CD95L mRNA were determined by the semi-quantitative reverse transcriptase-PCR (RT–PCR) method. Two μg of total RNA were reverse transcribed and the obtained cDNAs were used as a template for the subsequent PCR co-amplification of CD95L (238 bp) and β-actin (389 bp) as internal control. The following primers have been used: rat CD95L, upstream 5′-GGAATGGGAAGACACATATGGAACTGC-3′ and downstream 5′-CATATCTGGCCAGTAGTGCAGTAATTC-3′; β-actin, upstream 5′-AGGCATCCTGACCCTGAAGTAC-3′ and downstream 5′-TCTTCATGAGGTAGTCTGTCAG-3′. Conditions for co-amplification for rat CD95L and β-actin were 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for 35 cycles for rat CD95L and 25 cycles for β-actin. cDNAs were amplified using a DNA thermal cycler (Perkin Elmer-PCR system 9700) and the Taq DNA polymerase (2 units per tube) with 15 pmoles of both upstream and downstream primers, 2.2 mM magnesium chloride in a final volume of 50 μl. For each sample 18 μl of the PCR amplification product were analyzed on 2% agarose gel, and stained with ethidium bromide. To monitor the specificity of the RT–PCR products the amplified DNAs were recovered from the agarose gel, purified and subject to sequencing reaction in presence of fluorescent labelled nucleotides and analyzed by ABI Prism™ DNA sequencer (Perkin Elmer, CA, USA). All the obtained sequences corresponded to the expected one (data not shown).

Cytotoxicity assay

Cytotoxicity assay was performed as previously described[60] by incubating serial dilutions of effector cells with 5×10^3 ^{51}Cr-labelled target cells (Jurkat cell line) in triplicate wells of round-bottomed microtiter plates (Sterilin Teddington, Middlesex, UK) in final volume of 0.2 ml. After 4 h of incubation at 37°C, the plates were centrifuged and 0.1 ml supernatant was removed and counted. The percentage of specific ^{51}Cr-release was calculated as follows: 100 × (experimental release-spontaneous release)/(maximum release-spontaneous release).

JAM test

Target Jurkat cell death, resulting from co-culture with effector TM4 cells or following incubation in TM4 cells conditioned media, was quantitated by measurement of target cell DNA fragmentation using the JAM test.[61] DNA fragmentation was assessed by labelling the target cells with 0.01 μCi/μl ([^{3}H]ThymidineTdR) for 18–24 h at 37°C. Effector cells (TM4) were co-cultured with ^{3}H-TdR-labelled target cells for 18 h at 37°C at E/T ratios of 25 : 1; 12 : 1; 6 : 1 and 3 : 1 in 96-wells microplates. At the end of the incubation period, the cells were harvested onto glass-fibre filters using a semiautomatic cell harvester (Skatron). The filters were dried, added to liquid scintillation fluid, and radioactivity of unfragmented DNA retained on the glass fibre filter was counted in a β-counter. The main percentage of DNA fragmentation was calculated from triplicate co-culture using the following formula: % DNA fragmentation = 100 × (S-E)/S, where S is retained DNA in the absence of effector cells (spontaneous) and E is experimentally retained DNA in the presence of effector cells. Where indicated the effector cells were pre-treated for 4 h with the MEK inhibitor PD098059 (50 μM) or with the $cPLA_2$ specific inhibitor $AACOCF_3$ (10 μM) or for 30 min with the monoclonal anti CD95L antibody (Clone H11, 1 μg/ml).

After pretreatment, effector cells were washed three times with PBS for 3 min each time before starting the coculture with the target cells.

Phospholipase A_2 (PLA_2) and phosphatidylinositol-specific phospholipase C (PI-PLC) activity assays

TM4 cells (5×10^6) were incubated with monoclonal anti-CD95L antibody (Clone H11, 1 μg/ml or the mAb 33, 1 μg/ml or the mouse anti-rat CD161, 1 μg/ml) at 37°C for different time periods (0.5–10 min). Where indicated, the cells were pre-treated with $AACOCF_3$ (10 μM) or pBPB (20 μM) for 4 h. Incubation was stopped by centrifugation at $500 \times g$ for 1 min at 4°C. Cell pellets were resuspended in 300 μl of 250 mM Tris-HCl buffer pH 8.5 or 7.4 for PLA_2 or PI-PLC activity assay, respectively, containing 10 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 100 μM bacitracin and 1 mM benzamidine. Cells were lysed by sonication and protein concentrations were determined using a Bio-Rad protein assay. For PLA_2 assay, radiolabelled PC vesicles were prepared by sonication of the radiolabelled phospholipid L-3-phosphatidylchline, 1-stearoyl-2-[1-^{14}C] arachidonyl (Amersham, Little Chalfont, UK) for the detection of released arachidonic acid (AA), in 50 mM Tris-HCl buffer pH 8.5, containing fatty acid-free BSA (0.01%) in ice bath (5 min, 5 W and 80% output). PI-PLC activity in cell extracts was determined *in vitro* by their ability to hydrolyse ^{14}C-labelled PI vesicles and generate diacylglycerol (DAG) and/or monoacyl glycerol (MAG). Vesicles were obtained by sonicating 26.7 mCi/mmole phosphatidylinositol L-α-1-stearoyl-2-[1-^{14}C] arachidonyl (NEN Life Science Product, Inc. Boston, MA, USA) in 20 mM Tris-HCl, pH 7.4, containing 5 mM CaCl2, 5 mM $MgCl_2$ and fatty acid-free 0.01% BSA (final concentration 1 μM). Whole cell lysate (60–100 μg) was added to 250 μl of reaction buffer containing the vesicles and incubated for 1 h at 37°C, and the reaction was stopped by the addition of 250 μl of chloroform/methanol/acetic acid (4 : 2 : 1). Phospholipids were extracted,[62] dried under nitrogen, resuspended in 200 μl of chloroform, and applied in duplicate to a silica gel TLC plate. Samples treated with PC vesicles were chromatographed in chloroform : methanol : acetic acid : water (100 : 60 : 16 : 8) to separate the labelled product of PLA_2 activity, i.e., AA. Samples treated with PI-vesicles were chromatographed in petroleum ether/diethlether/acetic acid (70 : 30 : 1) to separate the parent phospholipid PI from DAG and MAG. The radioactive spots were visualized by autoradiography, scraped from the plate, and counted by liquid scintillation. PLA_2 activity was quantitated and expressed as AA release (pmol/μg protein). In some experiments, cell lysate were pre-treated with the reducing agent DTT for 15 min at 4°C before performing the assay. PI-PLC activity was quantitated by the release of DAG generation from PI and expressed as picomoles of DAG produced/μg protein.

Statistical analysis

Data points represent the mean of three independent experiments unless otherwise specified. Data were analyzed by Student's *t*-test using the STATPAC computer program and differences between the means were calculated as P values with $P < 0.05$ considered statistically significant.

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