Protein Kinase C Inhibits CD95 (Fas/APO-1)-Mediated Apoptosis by at Least Two Different Mechanisms in Jurkat T Cells

Carmen Ruiz-Ruiz, Gema Robledo, Jovita Font, Manuel Izquierdo and Abelardo López-Rivas

*J Immunol* 1999; 163:4737-4746; http://www.jimmunol.org/content/163/9/4737

**References**

This article cites 84 articles, 36 of which you can access for free at: http://www.jimmunol.org/content/163/9/4737.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
We have recently reported that activation of protein kinase C (PKC) plays a negative role in CD95-mediated apoptosis in human T cell lines. Here we present data indicating that although the PKC-induced mitogen-activated protein kinase pathway could be partially implicated in the abrogation of CD95-mediated apoptosis by phorbol esters in Jurkat T cells, the major inhibitory effect is exerted through a PKC-dependent, mitogen-activated protein kinase-independent signaling pathway. Furthermore, we demonstrate that activation of PKC diminishes CD95 receptor aggregation elicited by agonistic CD95 Abs. On the other hand, it has been reported that UV radiation-induced apoptosis is mediated at least in part by the induction of CD95 oligomerization at the cell surface. Here we show that activation of PKC also inhibits UVB light-induced CD95 aggregation and apoptosis in Jurkat T cells. These results reveal a novel mechanism by which T cells may restrain their sensitivity to CD95-induced cell death through PKC-mediated regulation of CD95 receptor oligomerization at the cell membrane. The Journal of Immunology, 1999, 163: 4737–4746.

A proapoptotic function of PKC in CD95-induced death in Jurkat cells was recently reported (14). We have previously shown that PKC plays a negative role in CD95-mediated apoptosis in Jurkat T cell lines. Here we present data indicating that although the PKC-induced mitogen-activated protein kinase pathway could be partially implicated in the abrogation of CD95-mediated apoptosis by phorbol esters in Jurkat T cells, the major inhibitory effect is exerted through a PKC-dependent, mitogen-activated protein kinase-independent signaling pathway. Furthermore, we demonstrate that activation of PKC diminishes CD95 receptor aggregation elicited by agonistic CD95 Abs. On the other hand, it has been reported that UV radiation-induced apoptosis is mediated at least in part by the induction of CD95 oligomerization at the cell surface. Here we show that activation of PKC also inhibits UVB light-induced CD95 aggregation and apoptosis in Jurkat T cells. These results reveal a novel mechanism by which T cells may restrain their sensitivity to CD95-induced cell death through PKC-mediated regulation of CD95 receptor oligomerization at the cell membrane. The Journal of Immunology, 1999, 163: 4737–4746.
mediated by CD95/CD95L interactions (43). Although ceramide-mediated CD95L up-regulation may be responsible for the apoptosis induced under certain stress conditions, there are recent published results that suggest the existence of CD95L-independent mechanisms of stress-induced apoptosis (44). Furthermore, it has been reported that UV light induces clustering of CD95 receptors in the cell membrane by an unknown mechanism in the absence of CD95L or anti-CD95 Abs (45, 46). This results in the recruitment of the death adapter molecule FADD/MORT1 and the induction of caspase 8-mediated apoptosis.

In this report we have tried to determine more precisely the mechanism by which activation of PKC inhibits apoptotic cell death induced upon CD95 ligation in the membrane of human Jurkat T cells. We show that the inhibition of PKC-mediated MAPK activation either by a specific inhibitor or by a dominant inhibitory mutant of MEK-1 only partially blocks PKC attenuation of CD95-mediated apoptosis. Furthermore, our results indicate for the first time that activation of PKC inhibits anti-CD95- and UV light-induced CD95 receptor aggregation through a MAPK-independent mechanism. We also present data indicating that activation of PKC reduces apoptosis triggered by UVB irradiation of Jurkat T cells.

Materials and Methods

Materials

RPMI 1640 medium and FCS were obtained from Life Technologies Europe (Paisley, U.K.). PDBu, carbobal, and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO). BIM, Ro-318220, and H-89 were obtained from Calbiochem (La Jolla, CA). CH-11 mAb (IgM) reaact- ing with CD95 was purchased from Medical & Biological Laboratories (Nagoya, Japan). Anti-Apo-1 (IgG3) was obtained from Kamiya Biomedical (Thousand Oaks, CA). Anti-CD95 rabbit polyclonal IgG Ab (C-20), anti-CD95L rabbit polyclonal IgG Ab (Q-20), and anti-Myc mAb (9E10; IgG1) were pur- chased from Santa Cruz Biotechnology (Santa Cruz, CA). MEK1 inhibitor, PD098059, was purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antisera against poly (ADP-ribose) polymerase (PARP), anti-FIL, was provided by Dr. G. de Murcia (Ecole Superieure de Biologie Medicale, Strasbourg, France). Wild-type MEK (pEXV3 MAPKKwt), domi- nant inhibitory mutant MEK (pEXV3 MAPKKK221A), and constitutively active MEK (pEXV3 MAPPK 217E2/221E) plasmids were gifts from Dr. C. J. Marshall (Institute of Cancer Research, London, U.K.). We are grateful to Dr. D. Cantrell (Imperial Cancer Research Fund, London, U.K.) for the pEF Bos ERK-2-Myc tag and CMV-raf CD2 plasmids and the anti- ratCD2 Ab (OX-34). We are also grateful to Dr. Michael Hahe (Univer- sity of Lausanne, Lausanne, Switzerland) for the gift of recombinant hu- man CD95L and CD95L cross-linker.

Cell culture

Cells of the human leukaemic T cell lines Jurkat and J-HMMI-2.2 Jurkat expressing the human masucarin acetylcholine type 1 (HMI) receptor and the human Raji B lymphoblastoid cell line were maintained in culture in RPMI 1640 medium containing 10% FCS and 1 mM L-glutamine at 37°C in a humidified 5% CO_2/95% air incubator.

UVB irradiation of Jurkat cells

Jurkat cells were irradiated at room temperature in complete growth me- dium with a UVB transilluminator source (310 nm, Fotodyne, New Berlin, WI). Briefly, Jurkat cells (10^7/ml) were seeded in plates and exposed from below to a UVB light source at a distance of 2.5 cm for different times. The energy applied in these experiments ranged from 2-10 J. After irradiation, the cells were incubated for the indicated time.

Determination of apoptotic cells

Hydoploid apoptotic cells were determined by cytofluorometric analysis of DNA content after extraction of the degraded DNA from apoptotic cells (47).

DNA transfections

Cells were transfected by electroporation (BTX Electroporation System, San Diego, CA). Cells (2 × 10^7) were resuspended in 0.65 ml of medium with the indicated concentration of plasmid DNA and then pulsed at 960 μF, 186 Oh, and 300 V. Transfected cells were diluted with culture me- dium to 10^7 cells/ml and cultured for 20 h. After incubation, cell suspensions were subjected to Ficoll gradient separation to remove dead cells.

Analysis of ERK kinase activity

Cells were transfected by electroporation with pEF-Bos ERK2-Myc tag and incubated with the appropriate stimuli. Immunoprecipitates of ERK2-Myc tag and in vitro kinase assays to analyze ERK2 activity were con- ducted as described previously (48). Briefly, cell lysates were precleared with insoluble protein A-Sepharose suspension and incubated for 1 h with 2 μg of 9E10 mAb and then for another hour with 20 μl of a 50% sus- pension of protein G-Sepharose beads (Sigma). After several washes, in vitro kinase assays were conducted for 30 min at room temperature in 20 μl of a kinase assay buffer supplemented with 10 μM ATP, 5 μM of [γ-32P]ATP, and 10 μg of MBP as a substrate. The reaction was stopped with 2× sample buffer, and samples were run in 15% SDS-PAGE minigels. Quantitation of 32P incorporated into the MBP protein band was performed by electronic autoradiography (InstantImager, Packard Instrument, Meriden, CT).

Cytofluorometric analysis of CD2 expression and apoptosis

Jurkat cells (2 × 10^7) transfected with the rat CD2 plasmid were washed and resuspended in 100 μl of PBS, and 6 μg/ml of anti-CD2 mAb was added. Conditioned medium from myeloma X63 was used as a negative control. After 30 min on ice, cells were washed once with cold PBS and incubated with FITC-conjugated rabbit anti-mouse Igs (Dako, Carpenteria, CA) for 30 min on ice. After this incubation, cells were treated as described above for the analysis of apoptotic cells. CD2-positive cells and hydop- loid apoptotic cells were analyzed in a FACScan cytoflurometer (Becton Dickinson, San Jose, CA).

Immunoblot detection of PARP

Cells (5 × 10^6) incubated under the indicated conditions, were pelleted, resuspended in 20 μl of sample buffer (50 mM Tris-HCl (pH 6.8), 6 M urea, 2% ME, 3% SDS, and 0.003% bromophenol blue) and sonicated. Proteins were resolved on SDS-10% polyacrylamide minigels and transferred onto Immobilon membranes (Millipore, Bedford, MA). The blot was blocked with 5% milk powder in PBS/0.1% Tween 20 (PBS/Tween) for 1 h, washed with PBS/Tween, and incubated with antiserum anti-F11 (1/ 2000) for 1 h. The blot was again washed with PBS/Tween and developed with HRP-coupled goat anti-rabbit (1/2000; Dako) followed by enhanced chemiluminescence (Amersham, Aylesbury, U.K.).

Immunoblot detection of CD95L

Acetone-precipitated proteins from cell lysates corresponding to 5 × 10^6 cells were resolved on 10% SDS-PAGE minigels. The blots were probed with rabbit polyclonal anti-CD95L Ab (Q-20; Santa Cruz Biotechnology). To detect immuno complexes, the blots were probed with HRP-coupled goat anti-rabbit IgG followed by enhanced chemiluminescence.

Analysis of monomeric and aggregated CD95 receptors

To detect CD95 aggregation upon anti-CD95 mAb stimulation, Jurkat cells (5 × 10^6) were incubated in complete growth medium at 37°C with 1 μg/ml CH11 IgM anti-CD95 for 10 min, washed with PBS buffer, and incubated with 1 μl of ice-cold lysozyme buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM NaF, 10 mM iodoacetamide, 200 μM NaVO_3, and 1% Nonidet P-40) for 10 min. Postnuclear supernatants were resolved on SDS- 7.5% PAGE minigels.

For the detection of CD95 oligomerization after UVB light exposure, cells (5 × 10^6) were irradiated for 5 min, incubated at 37°C for 5 min, washed with PBS, and treated as described previously (45). In brief, cells were resuspended in 500 μl of PBS and incubated with 2 mM cleavable cross-linker 3,3'-dithiobis-[sulfosuccinimidyl propionate] (Sigma) for 15 min on ice. The reaction was quenched with 10 mM ammonium acetate for 10 min, and cells were washed with PBS and lysed in 150 μl of ice-cold lysis buffer for 10 min. Postnuclear supernatants were incubated at 4°C for 1 h with either 0.1 μg/ml (Ab limiting) or 2 μg/ml (Ab excess) Apo-1 IgG3 anti-CD95 mAb, and immune complexes were precipitated using protein A-Sepharose (Sigma). After washing three times in lysis buffer, immuno- precipitates were resuspended in Laemmli buffer, boiled for 5 min, and resolved on SDS-10% polyacrylamide minigel.
Resolved samples were transferred onto Immobilon membranes, and the blots were treated as described previously (immunoblot detection of PARP) using a rabbit polyclonal anti-CD95 Ab (0.2 μg/ml) followed by HRP-conjugated goat anti-rabbit (1/2000; Dako) for the detection of CD95.

Results

Activation of MEK1 is partially involved in the prevention of CD95-induced apoptosis by a PKC activator in Jurkat T cells

Several reports have described that T cell activation can inhibit apoptosis induced by ligation of CD95 in the cell surface with CD95 Abs (36, 37). It has also been suggested that the mechanism underlying this inhibitory effect involves the activation of the MAPK pathway (36, 37). T cell activation induces the expression of FLIP (FLICE inhibitory protein), a potent inhibitor of CD95-induced apoptosis (37, 49). Induction of FLIP in Jurkat T cells was dependent on the stimulation of the MAPK pathway and could be observed after several hours of activation (37). Our previous results indicated that inhibition of CD95-induced signaling is observed immediately after addition of PKC activators (33), thus making unlikely the involvement of a newly synthesized inhibitor. In this report we have tried to determine the mechanism of PKC-mediated prevention of CD95-induced apoptosis and to define the step(s) in CD95 signaling at which PKC exerts its inhibitory role.

Inhibition of CD95-mediated apoptosis by PKC activation occurs in a dose-dependent manner (Fig. 1A). Thus, when Jurkat cells were incubated in the presence of an anti-CD95 Ab, apoptosis was clearly prevented at PDBu concentrations ranging from 2–20 ng/ml. Because this inhibitory action of PKC has been mainly studied in apoptosis induced by CD95 Abs (29–31, 33) we further investigated this effect, and here we show that PDBu also inhibited CD95L-induced apoptosis in Jurkat cells (Fig. 1B).

To analyze the role of the MAPK pathway in mediating the inhibition of CD95-induced apoptosis by PDBu, PD 098059, a rather specific inhibitor of the activation of MEK1/2 (50), was used. As shown in Fig. 2A, treatment of Jurkat cells with either 2 or 20 ng/ml PDBu prevented CD95 Ab-induced cell death. In these experiments, pretreatment of cells with PD 098059 significantly reduced the anti-apoptotic effect of 2 ng/ml PDBu (Fig. 2A). Interestingly, this inhibitor of MEK1/2 activation did not affect the prevention of CD95-induced apoptosis by 20 ng/ml PDBu (Fig. 2A). These results suggested that aside from MEK1/2 activation, other signals generated upon PKC activation might be involved in the inhibition by phorbol esters of Fas-mediated cell death. However, it could also be possible that PD 098059 was not able to inhibit MEK1/2-mediated MAPK activation at high PDBu concentrations, because it has been reported that the extent of inhibition by PD 098059 might depend on how potently MEK1/2 was activated by agonists (50). To determine whether this was the case, we analyzed the activation of the MAPK pathway in cells transiently transfected with ERK2 tag (48) and stimulated with two different concentrations of PDBu. As shown in Fig. 2B, ERK-2 was activated by the phorbol ester in a dose-dependent manner. Moreover, the presence of PD 098059 completely inhibited ERK2 activation induced by PDBu even when a high concentration of phorbol ester was used (Fig. 2B). These results indicated the existence in Jurkat cells of at least two different PKC-dependent mechanisms that inhibit Fas-mediated cell death: a MAPK-dependent mechanism, as we have shown in this study and has been recently proposed (36), that can be of significance under conditions of suboptimal stimulation of PKC by agonists, and a MAPK-independent pathway that is activated when PKC is maximally stimulated.

To confirm the importance of MEK1 activation in the apoptosis inhibitory signals induced at a submaximal dose of PDBu, we transiently cotransfected Jurkat cells with a plasmid encoding the rat CD2 receptor and a plasmid encoding either a dominant inhibitory mutant of MEK1 or the wild-type protein. After incubation with the appropriate stimuli we determined the percentage of apoptotic cells within the population of transfected cells (CD2+ cells). As shown in Fig. 2C, when cells transfected with wild-type MEK1 were treated with CD95 mAb, they underwent apoptosis (32% sub-G1 cells). In the presence of 2 ng/ml PDBu there was a marked
inhibition of the apoptotic program (12% sub-G₁ cells). However, cells transfected with a dominant inhibitory mutant MEK₁ were more susceptible to CD95-mediated apoptosis (45% sub-G₁ cells) than those transfected with wild-type MEK₁ and PDBu only partially protected these cells from CD95-mediated apoptosis (33% sub-G₁ cells). These results confirmed those obtained using the inhibitor of MEK₁ activation PD098059 and suggested that activation of MEK₁ could play a certain role in the abrogation of CD95-induced apoptosis by PDBu.

**FIGURE 2.** Inhibition of MEK₁ reduced the abrogation of CD95-induced apoptosis by PDBu. A, Jurkat cells were incubated for 2 h with or without 50 µM of the MEK₁ inhibitor PD 098059. Following this treatment, cells were incubated for 8 h either without or with 100 ng/ml CH11 in the absence or the presence of two different concentrations of PDBu (2 and 20 ng/ml). The percentage of apoptotic cells in the population was determined by flow cytometry. B, PD 098059 blocked the activation of ERK2 caused by PDBu. Jurkat cells transfected with 10 µg of pEFP-BOS ERK2 tag were either unstimulated or stimulated for 30 min with 2 or 20 ng/ml PDBu after 1 h of preincubation in the presence or the absence of 50 μM PD 098059. MBP kinase assays were performed on the 9E10 immunoprecipitates from cell lysates. ERK activation is expressed as fold induction of MBP phosphorylation as determined by electronic autoradiography. C, Jurkat cells were cotransfected with 10 µg of rat CD2 DNA and either 30 µg of dominant inhibitory mutant MEK (pEXV3 MAPKK 221A; DN) or 30 µg of wild-type MEK (pEXV3 MAPKKwt; WT) as described in Materials and Methods. Twenty hours after transfections cells were incubated with or without 100 ng/ml CH11 in the presence or the absence of 2 ng/ml PDBu for 8 h. Hypodiploid apoptotic cells in the CD2⁺ population were determined by flow cytometry. Results are presented as the percentage of apoptotic cells in the population after subtracting the values of apoptosis in untreated cultures. The levels of apoptosis in untreated WT and DN cells were 17 ± 2% and 14 ± 3%, respectively. D, Jurkat cells were cotransfected with 10 µg of rat CD2 DNA and either 30 µg of constitutively active MEK (pEXV3 MAPKK 217E/221E; CA) or 30 µg of wild-type MEK (pEXV3 MAPKKwt; WT) and incubated for 20 h. Apoptotic cells in the CD2⁺ population were determined by flow cytometry after stimulation without or with 100 ng/ml CH11 for 8 h. In all experiments error bars represent the SD from at least three independent experiments. E, Cell extracts from Jurkat cells treated as described in A were subjected to Western blotting, and the formation of the 29-kDa fragment of PARP was determined as described in Materials and Methods. Results are representative of at least two different experiments.
apoptosis by suboptimal doses of PKC activators. To determine whether the activation of MEK1 is sufficient to block CD95 mAb-induced apoptosis, we cotransfected Jurkat cells with the CD2 DNA and with DNA encoding either a constitutively activated MEK1 or the wild-type protein. After analysis of the CD2 population we observed that the percentage of apoptotic cells after stimulation with CD95 mAb was reduced in those expressing constitutively activated MEK1 (Fig. 2D). These data support the idea that the inhibitory effect of low doses of PDBu on CD95-induced cell death is mediated in part by activation of MEK1.

A key event in CD95-mediated apoptosis is the sequential activation of several members of the family of Cys proteases, caspases, which, in turn, induce the cleavage of different substrates (51, 52). One of these substrates is the 113- to 116-kDa nuclear enzyme PARP, which is proteolitically cleaved by caspases within the bipartite nuclear location signal to produce two fragments of ~85 and 29 kDa (53). In a previous study we have demonstrated that PKC activation inhibited caspase-3 activation and PARP cleavage induced by anti-CD95 mAb in Jurkat cells (33). In this report, we have analyzed the possible involvement of MEK1 activation in mediating the inhibitory action of PKC on CD95-induced activation of caspase-3-like proteases. To this end we have determined the generation of the 29-kDa fragment of PARP as previously described (33). The experiment shown in Fig. 2E indicates that the phorbol ester, PDBu, inhibited CD95-mediated cleavage of PARP in Jurkat cells in a dose-dependent manner. Similar to what was observed in cell death experiments (Fig. 2A), the inhibitor of MEK1 activation, PD 098059, was able to prevent the inhibitory effect on PARP cleavage of different concentrations of PDBu (Fig. 2E). Furthermore, in agreement with the experiments analyzing apoptosis (Fig. 2A), PD 098059 did not affect PDBu-induced halt of CD95-mediated PARP cleavage at PDBu 20 ng/ml (Fig. 2E).

**PKC activation inhibits the aggregation of CD95 receptors induced by CD95 mAbs or UVB radiation**

In a previous report we reported that PKC activation blocked CD95-elicited signals that occurred immediately after CD95 receptor ligation by specific mAb (33). It has been demonstrated that the first event upon CD95 stimulation is the trimerization of the intracellular death domain, which, in turn, recruits FADD and FLICE to form the DISC (18, 21, 22). On the other hand, it was reported that high molecular mass (>200 kDa) CD95 aggregates are formed immediately after receptor cross-linking with CD95 Abs in different human B and T cell lines (54). To further characterize the inhibition of CD95 signaling pathway by PKC activation we have analyzed CD95 aggregation in Jurkat cells. As shown in Fig. 3A, stimulation of Jurkat cells with an IgM CD95 mAb induced the formation of SDS-stable high m.w. aggregates of CD95 as previously reported (18, 54). These aggregates were not seen if the cells were incubated with the Ab at 4°C, which is below the transition temperature of the membrane (results not shown). Moreover, CD95 aggregation by Ab required an intact cell, as it was not observed when the Ab was added to lysed cells (not shown).

Interestingly, in the presence of PDBu, CD95 mAb-induced receptor aggregation was significantly reduced (Fig. 3A). We observed the same results when Jurkat cells were stimulated with IgG3 CD95 mAb (unpublished results). Importantly, this acute treatment with PDBu did not affect the expression of CD95 Ag in the membrane of Jurkat cells (33). To determine whether the effect of PDBu was due to PKC activation, we used the PKC inhibitor BIM. The presence of BIM during the incubation with phorbol ester abrogated the reduction in CD95 aggregates formation caused by the PKC activator (Fig. 3B). Similar results were obtained with the PKC inhibitor Ro-318220 (not shown). However, we did not observe a prevention of the suppressor effect of PDBu on CD95 aggregation when using the PKA inhibitor H-89 (results not shown). The role of the MAPK pathway in mediating PDBu-induced attenuation of CD95 aggregation was assessed with the inhibitor of MEK1 activation, PD 098059. The results obtained indicated that the PDBu effect was independent of MEK1 activation (Fig. 3B). All these experiments were performed at the concentration of PDBu (20 ng/ml) that caused maximal reduction in aggregate formation. At lower concentration of PDBu (2 ng/ml), which partially inhibits CD95-induced apoptosis in a MAPK-dependent way (Fig. 2A), PDBu was not able to inhibit CD95 aggregation induced by anti-CD95 mAb (not shown). These results suggested that PKC activation, following treatment with maximal concentrations of phorbol ester, may prevent CD95-induced apoptosis by inhibiting the first step in CD95 signaling pathway, that is, receptor multimerization.

It has been reported that UVB radiation induces the oligomerization of CD95 receptors, which triggers the downstream caspase
We investigated in two different cell lines, Jurkat and Raji, whether UVB light induced CD95 aggregation and if it could be inhibited by PKC activation. To this end, we followed an experimental design previously described (45). The basic principle is that at limiting concentrations (0.1 μg/ml) of IgG3 anti-APO-1 mAb more molecules of the CD95 receptor will be immunoprecipitated in the presence of a reversible cross-linker reagent if aggregation has taken place upon UVB irradiation of cells. The results presented in Fig. 4, A and B, indicate that in UVB-treated Jurkat and Raji cells there was a marked increase in the amount of CD95 protein immunoprecipitated by limiting Ab compared with that in untreated cells. These results indicated that UVB irradiation of Jurkat and Raji cells caused aggregation of CD95 receptors as previously demonstrated in other cell lines (45, 46). Interestingly, UVB radiation-induced CD95 receptor oligomerization was significantly inhibited in the presence of 20 ng/ml PDBu (Fig. 4, A and B).

**Activation of PKC reduces UV irradiation-induced apoptosis in Jurkat T cells**

Based on the above data, we tried to determine whether the activation of PKC by phorbol ester was also able to inhibit UVB light-induced apoptosis in Jurkat cells. When UVB irradiated for different times, Jurkat T cells underwent apoptotic cell death, as determined by the generation of hypodiploid cells. The percentage of apoptotic cells after radiation was a function of the irradiation time length (Fig. 5A). Similar to what was observed in Jurkat cells treated with CD95 mAb, we found that in the presence of PDBu there was a marked reduction in the percentage of apoptotic cells induced upon UVB irradiation (Fig. 5A). Moreover, the effect of PDBu on UVB light-induced apoptosis was due to the activation of PKC. As shown in Fig. 5B, the suppression of apoptosis caused by the phorbol ester was not observed in the presence of the PKC inhibitor BIM. The PKC inhibitor Ro-318220 produced similar results as BIM (not shown). In contrast, the PKA inhibitor H-89 did not abolish the PDBu effect on apoptosis (results not shown). We also examined the role of the MAPK pathway in the prevention of UVB light-induced apoptosis by PKC activation. As shown in Fig. 5B, PD 098059 did not abolish the PDBu effect on apoptosis (results not shown).
However, we cannot exclude the possibility that other CD95-independent mechanisms might also contribute to UV radiation-induced apoptosis (42).

**Induction of CD95L expression upon UVB irradiation of Jurkat T cells does not mediate UVB-induced apoptosis**

It has been recently demonstrated that certain stress treatments such as anticancer drugs and gamma irradiation up-regulate CD95L expression and induce apoptosis through a CD95-dependent pathway involving the formation of a CD95/CD95L complex (43). In addition, it was shown that ceramide, which accumulates in response to stress, mediates the up-regulation of CD95L expression and apoptosis observed following drug addition or gamma irradiation. However, more recent data have indicated that not all stress stimuli use the same cell death pathways (44). It was shown that UV, gamma irradiation, and anisomycin stimulated c-Jun N-terminal kinase activity and induced CD95L expression in Jurkat cells. Nevertheless, only anisomycin-induced apoptosis was dependent on CD95/CD95L interactions (44). To further investigate the role of CD95L in UVB-induced apoptosis we used J-HM1-2.2 Jurkat cells expressing the human muscarinic acetylcholine type 1 (HM1) receptor. We have recently shown that these cells undergo apoptosis upon activation of the muscarinic receptor with the agonist carbachol (55). This cell death process involved the up-regulation of CD95L (Fig. 6A) and required the formation of a CD95/CD95L complex, as it could be blocked by either an antagonistic CD95 Ab (DX2) or an anti-CD95L Ab (NOK-1; Fig. 6B). Therefore, these cells provide a positive control for the Abs used to prevent CD95/CD95L interactions. We observed that UVB irradiation induced the expression of CD95L in J-HM1-2.2 Jurkat cells (Fig. 6A) as previously reported in Jurkat cells (44). However, the cell death resulting from UVB irradiation was not prevented by blocking CD95/CD95L interactions with antagonistic CD95 Ab or CD95L Ab (Fig. 6B), which suggests that UV-induced CD95L is not involved in UV-mediated apoptosis of J-HM1-2.2 cells. Similar results were obtained in Jurkat cells (not shown).

**Discussion**

**Mechanisms of cellular resistance to CD95-mediated apoptosis in T cells: role of protein kinase C and receptor oligomerization**

Cellular expression of CD95 receptor and ligand is not always sufficient to elicit an apoptotic response. There are examples of CD95-resistant cells that express high levels of CD95 receptor in their plasma membrane and yet they are not killed by CD95L or CD95 mAbs (27, 28). This is the case of peripheral T lymphocytes, which in the initial phases of antigenic stimulation up-regulate the expression of CD95 and CD95L, but proliferate instead of dying. These cells undergo CD95-mediated apoptosis only after they are repetitively stimulated through the TCR (56). Mechanisms involving the up-regulation of FLIP (49) or Bcl-xL (57) during the early stage of T cell activation have been proposed to explain the resistance of mature T cells to CD95-mediated apoptosis. Lack of recruitment of FLICE to the CD95 death-inducing signaling complex has also been observed in these cells (57). Resistance can be overcome by treatment with inhibitors of macromolecular synthesis (58), which suggests the presence of short-lived inhibitory proteins. In this respect, it is interesting to mention the existence of proteins associated with the cytosolic part of CD95 Ag that may regulate the capacity of this receptor to transmit apoptotic signals (28).

The human T cell line Jurkat has been widely used as a model to study activation-induced cell death and Fas-mediated apoptosis. Jurkat T lymphocytes are normally sensitive to CD95-mediated apoptosis when CD95 receptor is cross-linked by anti-CD95 Abs (24, 33, 51, 52). We and others have recently shown that activation of PKC antagonizes CD95-mediated signaling and apoptosis in Jurkat cells and in other cell lines (29, 30, 33, 59). Furthermore, inhibitors of this kinase enhance the susceptibility of cells to anti-CD95 mAbs (59, 60). PKC activates the MAPK pathway by stimulating p21”^W and Raf kinase in different cell types (61, 62). It has been proposed that activation of the ERK pathway prevents apoptosis and promotes cellular survival (35). On the other hand, CD95 ligation can induce the activation of JNK/SAPK (63, 64), which may contribute to the induction of apoptosis. These observations suggest that different MAP kinase family members could contribute in different ways to the decision for cellular life or death. In this report we have demonstrated that under certain circumstances, the PKC-induced ERK pathway may be important in negatively controlling CD95-mediated cell death. Our results confirmed recently published data indicating that the MAPK kinase (MEK1) is a negative regulator of CD95-mediated apoptosis in T cells (37). This protective mechanism may have a role in the early
PKC ACTIVATION INHIBITS CD95 RECEPTOR AGGREGATION

4744

stages of T cell activation, preventing the onset of CD95-dependent, activation-induced cell death (36). How activation of the MAPK pathway leads to resistance to CD95-mediated apoptosis is not known, although members of this cascade can phosphorylate Bad and inhibit apoptosis (65). Moreover, as MAPK signals seem to inhibit the initial phases of CD95-mediated apoptosis (36), it may potentially interfere in the steps proximal to formation of the death complex (18, 21, 22). In this respect, induction of FLICE inhibitory protein by stimulation of the MAPK pathway was recently reported in T cells activated with Con A (37). However, our data demonstrated that the dependence on MEK1 activation for survival was only observed when submaximal concentrations of PKC activator were used. In this report we show that in the presence of maximal concentrations of phorbol ester, a PKC-regulated, MAPK-independent pathway is activated that inhibits CD95 aggregation in the membrane and CD95-mediated apoptosis. It is interesting that apoptosis induced by a human CD40/CD95 chimeric receptor is not inhibited by PKC activation (66). This receptor contains the CD95 transmembrane/intracellular domain fused to the CD40 extracellular domain. As the extracellular domain could play an important role in regulating multimerization of receptors at the cell membrane (67), its replacement by a heterologous receptor domain might suppress specific constraints derived from intracellular signaling, such as PKC activation. On the other hand, it remains to be demonstrated whether PKC directly modulates CD95 receptor oligomerization or requires additional effector activities. A recent report (68) has indicated that phosphatidylinositol 3'-kinase can have a suppressor effect on CD95-mediated apoptosis in T cells. Moreover, it is known that phosphatidylinositol 3'-kinase is a downstream effector of p21ras (69), which, in turn, can be activated by PKC (61). Further investigation is needed to demonstrate the role of phosphatidylinositol 3'-kinase in PKC-dependent, MAPK-independent inhibition of CD95-induced apoptosis.

Regulation of UV light-induced CD95 aggregation and apoptosis by PKC

The apoptotic response of cells to UV radiation may include the accumulation of the tumor suppressor protein p53 and the transcriptional activation of p53-regulated genes (70). However, other mechanisms are also involved (39). Among these p53-independent mechanisms, the activation of the sphingomyelin pathway by UV light has been reported (42). This pathway, initiated by hydrolysis of sphingomyelin in cell membranes, generates the second messenger ceramide, which, in turn, stimulates a stress-activated protein kinase (SAPK/JNK) pathway. It has been shown that SAPK/JNK activation by various stress treatments is implicated in stress-induced apoptosis (42). Our unpublished observations indicate that the rapid activation by UVB light of the SAPK/JNK pathway, which is normally a result of ceramide generation, is not inhibited by phorbol ester pretreatment of Jurkat cells, suggesting that the attenuation of UVB-induced apoptosis by PKC should occur at a different point. More recent evidence has suggested a role of the CD95/CD95L system in stress-activated apoptotic cell death (43). However, there are contradictory findings about the role of CD95L in UV radiation-induced apoptosis (44, 71). Our data demonstrated that blocking CD95/CD95L interactions in Jurkat cells did not diminish UVB-induced apoptosis, although CD95L was induced after UVB irradiation. These results also implied that PKC activation must be interfering at a different step in the pathway(s), leading to apoptosis upon UVB irradiation of Jurkat T cells.

The activation of growth factor and cytokine receptors by UV light has been recently demonstrated (72, 73). These findings have prompted the hypothesis that the physical stress elicited at the plasma membrane level upon irradiation of cells may lead to the aggregation of receptors required for complete activation in the absence of ligands (73). More recently, it was reported that the apoptotic response of different cell types to UV irradiation is mediated at least in part by activation of CD95/Fas/APO-1 (45, 46). This activation occurs via CD95 receptor oligomerization, subsequent recruitment of the death adapter molecule FADD/MORT1, and induction of caspase-8 activity. In this report we have demonstrated that upon irradiation of Jurkat cells with UVB light there is aggregation of CD95 receptors. Furthermore, our results have shown for the first time that activation of PKC prevents UV light-induced aggregation of CD95 receptors and apoptosis. Similar results were obtained in the human lymphoma B cell line Raji. Consistent with these results was the finding that CD95 Ab-induced aggregation of CD95 receptors was also reduced in Jurkat cells incubated in the presence of a PKC activator. To confirm the early abrogation by PKC of CD95 signaling we have tried to determine the association of the adapter molecule FADD/MORT1 with CD95. However, we have failed to detect the recruitment of this adapter by CD95 aggregates. This is not unexpected in view of recent data (74) indicating the existence of two different CD95/Fas/APO-1 signaling pathways (type I and II cells). According to these authors Jurkat cells are type II cells in which DISC formation is strongly reduced, although they express high levels of FADD and caspase-8. However, although lower amounts of FADD and caspase-8 are recruited to the aggregated receptor in Jurkat cells compared with type I cells, it appears that this is sufficient to mediate apoptosis upon CD95 ligation (74).

Our results indicate that a likely target for PKC-mediated inhibition of CD95 mAb and UVB radiation-induced apoptosis is the mechanism of CD95 Ag oligomerization in the membrane (18). However, at present we should not exclude the possibility that other targets downstream of CD95 oligomerization might also cooperate in the regulation by PKC of CD95 signaling. In this respect, it has been proposed that expression of c-Myc sensitizes fibroblasts to CD95-induced apoptosis (75), and PKC-β could abrogate Myc-induced apoptosis in some cell types (76), although this blocking action seemed to be related to changes in the cell cycle. On the other hand, the inhibitory protein of CD95 signaling FLIP is induced upon T cell activation (37, 49). The role of FLIP in PKC-induced inhibition of CD95-mediated apoptosis remains to be elucidated.

Protein phosphorylation and the control of CD95 receptor clustering in the plasma membrane

Although CD95/APO-1 phosphorylation has not been observed upon activation of this receptor (18), phosphorylation sites have been found in the membrane-proximal cytoplasmic domain of CD95/APO-1 (77). Therefore we speculate that phosphorylation of these sites by either PKC or associated kinases (77) may generate negatively charged residues that could function to impede oligomerization of receptors and consequently prevent CD95-mediated signaling and apoptosis. Regulation of CD95 function and sensitivity by negatively charged molecules has been proposed in the case of the surface sialylation of CD95 (18).

On the other hand, agents that inhibit actin polymerization may have profound effects on receptors patching and capping (78). In this respect, it has been reported that nitric oxide, which may decrease filamentous actin formation (78), is an inhibitor of CD95-mediated apoptosis (79). PKC may induce drastic alterations in cell morphology and membrane dynamics, which correlates with the reorganization of submembranous actin (80). This action is mediated by phosphorylation and inhibition of the filamentous actin cross-linking protein MARCKS (81), a widely expressed PKC...
substrate (82). Therefore, an explanation for the observed effect of PKC activation on CD95-induced apoptosis would be that phosphorylation of cellular substrates involved in regulating actin cytoskeleton may change CD95 receptor mobility and clustering, as reported for other membrane receptors (83, 84). Reduced capping of death receptors may also be a mechanism used to escape from CD95-mediated apoptosis during tumor development (32). Whether PKC plays a role in inhibiting capping of CD95 receptors and mediating the resistance of tumor cells to CD95-induced apoptosis is unknown. Experiments are currently being conducted to define the role of cytoskeleton in the regulation by PKC of CD95/ APO-1 clustering and the induction of apoptosis.

Acknowledgments

We thank Dr. A. Weiss and Genentech, Inc. (South San Francisco, CA) for providing the J-HM1-2.2 cell line (Material Transfer Agreement 6009-121033).

References


