Actin dependent CD95 internalization is specific for Type I cells

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Abstract CD95 Type I and Type II cells differ in the requirement for mitochondrial contribution to the execution of apoptosis. Mitochondria are required to amplify the apoptosis signal only in Type II cells. Here we show that CD95 internalizes in an actin dependent manner only in Type I cells and that the two CD95 apoptosis cell types differ in their threshold of active caspase-8 required to execute apoptosis. The data suggest that CD95 is linked to the cytoskeleton in different ways in the two cell types and that they have adapted to different levels of active caspase-8 generated at the activated receptor.

Key words: Actin; Apoptosis; Caspase-8; Endocytosis; Fas

1. Introduction

CD95 (APO-1/Fas) is the best studied member of the death receptor family [1]. Activated CD95 recruits the adaptor molecule FADD and the initiator caspase-8 to form the death-inducing signaling complex (DISC) [2]. By characterizing tumor cell lines we previously described two different CD95 apoptosis pathways [3]. In Type I cells, caspase-8 is recruited to the DISC, resulting in the release of active caspase-8 in quantities sufficient to directly activate caspase-3. However, in Type II cells, despite similar expression of CD95 signaling molecules, formation of the DISC is inefficient and only very small quantities of caspase-8 are generated at the cell surface. These amounts of caspase-8 are insufficient to process caspase-3 which may be protected from activation by expression of IAP proteins [4], but are sufficient to cleave the BH3-only protein Bid [5]. The cleavage product of Bid, tBid, then triggers the apoptogenic activity of mitochondria, a step that involves oligomerization of Bax and/or Bak on the outer mitochondrial membrane [5]. It has become clear that death receptors generally activate the mitochondrial pathway, suggesting a cross talk between the extrinsic and the intrinsic apoptosis pathways [3]. However, in Type I cells the mitochondrial branch is not essential for the execution of apoptosis, since inhibition of the mitochondrial route by overexpression of Bcl-2 or Bcl-x_L does not affect the apoptosis sensitivity of these cells [3]. We have recently shown that upon stimulation by either agonistic anti-CD95 antibody or CD95 ligand CD95 is internalized in a caspase-8 and actin dependent fashion [6]. We now demonstrate that Type I and Type II tumor cell lines differ in the way the death signal is initiated at the cell surface. CD95 is linked to F-actin and triggering CD95 induces its internalization only in Type I cells. Inhibition of receptor internalization in Type I cells by treating cells with the inhibitor of actin polymerization latrunculin A (LtnA) blocks CD95 internalization in Type I cells and reduces the amount of DISC-generated active caspase-8 to the very low level of active caspase-8 generated in Type II cells and significantly inhibits apoptosis. In contrast, treatment of Type II cells, which do not internalize CD95, with LtnA has no effect on apoptosis sensitivity, suggesting that CD95 signaling in Type II cells is different and is independent of actin.

2. Materials and methods

2.1. Cell lines and reagents

The B lymphoblastoid cell line SKW6.4, the T cell lines H9, Jurkat (clone E6-1), and CEM cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained in 5% CO_2 at 37°C. The C15 monoclonal antibody recognizes the p18 subunit of caspase-8 [7]. Anti-APO-1 (anti-CD95) is an agonistic monoclonal antibody (IgG3, κ) recognizing an epitope on the extracellular portion of CD95 [8]. The anti-actin antibody (clone AC-40) was obtained from Sigma and the anti-hematopoietic lineage cell-specific protein 1 (HS1) monoclonal antibody from Transduction Laboratories. All other chemicals used were of analytical grade and purchased from Sigma, Molecular Probes or Calbiochem. The plasmid to produce the leucine zipper-tagged CD95L (LzCD95L) was described elsewhere [9].

2.2. CD95 internalization and liquid-phase endocytosis

Internalization of CD95 was determined as previously described [6]. To determine the endocytic activity of cells, 5×10^5 cells were resuspended in 1 mg/ml FITC-dextran. Cells were incubated at 37°C for the times indicated and then washed three times with cold phosphate-buffered saline–fetal bovine serum and co-stained with propidium iodide (PI; 125 μg/ml) to exclude dead cells. Endocytic uptake of FITC-dextran by living cells was determined using flow cytometry. To determine baseline fluorescence not attributed to endocytosis, cells were incubated in 1 mg/ml FITC-dextran on ice for 2 h in parallel and then analyzed as above.

2.3. Induction of apoptosis and cytotoxicity assay

10^5 cells in 200 μl of medium were incubated in 96 well plates with different concentrations of anti-CD95 with or without Protein A (PA) (1 ng PA per 1 μg of anti-CD95) or with the various CD95L at 37°C overnight. In some experiments, increasing concentrations of LtnA were added to cells for 1 h prior to anti-CD95 stimulation. Quantification of DNA fragmentation, as a measure of apoptosis, was carried out by nuclear staining with PI as previously described [3]. For Western blot analysis some cells after stimulation were lysed as above and 60 μg of protein per lane was separated by SDS–PAGE and immunoblotted with the indicated antibodies.

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2.4. Determination of filamentous actin (F-actin) in Type I and Type II cells

The amount of F-actin was determined using the G-actin/F-actin assay kit following the manufacturer's instructions (Cytoskeleton Inc., CO, USA). Briefly, unstimulated or 5 μM LtnA-treated cells were resuspended in lysis buffer (50 mM PIPES pH 6.9, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% (v/v) glycerol, 0.1% NP-40, 0.1% Triton X-100, 0.1% Tween 20, 100 mM ATP, and protease inhibitor cocktail) and gently homogenized to lyse the cells. Lysates were centrifuged at 100 000×*g* to separate F-actin from soluble G-actin. After centrifugation 10 μg of protein form the supernatant (G-actin) and the pellet (F-actin) were subjected to 12% SDS–PAGE and immunoblotted with anti-actin antibodies (clone AC-40).

2.5. Caspase activity assay

Caspase-3 and caspase-8 activity was determined from cell lysates as follows: cells were stimulated for the indicated times with 1 μg/ml of anti-CD95 in the presence or absence of PA or 1 μg/ml of LzCD95L. Lysates from 500 000 cells were incubated in cleavage buffer containing 40 μM of amino trifluoromethyl coumarin (AFC)-labeled caspase-3 specific peptide DEVD or caspase-8 specific peptide IETD for 1 h at 37°C. Caspase activity was determined fluorometrically using a fluorescence plate reader with a 400 nm excitation filter and 508 nm emission filter. Values of unstimulated cells were taken as background and subtracted from those obtained with stimulated cells.

3. Results and discussion

The anti-CD95 monoclonal antibody anti-APO-1 is an IgG3 isotype that tends to aggregate through Fc–Fc interactions [10]. The activity of this antibody varies depending on its aggregation state (unpublished observation). We previously reported that the four prototype Type I/Type II cell lines are equally sensitive to anti-APO-1 [3]. This was in contrast to a recent report that showed that anti-APO-1 had low cytotoxic activity on the Type II cells Jurkat and CEM but was fully active on Type I SKW6 cells [11]. Addition of PA to anti-APO-1-treated Type II cells rendered them as sensitive as the SKW6 cells, whose cell death could not be further increased by addition of PA [11]. We retested a number of anti-APO-1 preparations and also identified preparations with very low cytotoxic activity on Type II cells but high activity on the two Type I cells (Fig. 1A). Addition of very small amounts of PA to anti-APO-1 (1 ng/ml PA added to 1 μg/ml anti-APO-1) resulted in a similar sensitivity of all four cell lines and therefore in a dramatic increase of apoptosis only in the Type II cells (Fig. 1A). These data reinforce the hypothesis that there are differences in the way CD95 is activated in Type I and Type II cells and suggest that anti-APO-1 can be used to distinguish Type I from Type II cells. However, the differences between Type I and Type II cells are not only observed with the anti-APO-1 antibody. A severely reduced amount of effector caspase activity is observed in Type II cells compared to Type I cells when treated with a leucine zipper-tagged form of the CD95 ligand (LzCD95L) (Fig. 1B).

We recently showed that triggering of CD95 causes a caspase-8 dependent clustering followed by internalization of CD95 into an endosomal compartment [6]. We now tested whether both Type I and Type II cells internalize CD95 after stimulation (Fig. 2A). No significant internalization was detectable in Type II cells (2 h after addition of anti-CD95) whereas CD95 was efficiently internalized in Type I cells. Type II cells were not generally defective in endocytosis since they were able to uptake FITC-labeled dextran at the same rate as Type I cells (Fig. 2B). These data suggest a difference in the way CD95 signals in Type I and Type II cells.

We found that CD95 clusters in both Type I and Type II cells although this activity could be inhibited by addition of caspase inhibitors only in Type I cells ([6,12] and data not shown). Therefore only CD95 internalization but not the preceding clustering can be used to differentiate Type I from Type II cells. A difference in the internalization of CD95 between Type I and Type II cells has been suggested before [13]. However, assuming that sCD95L is internalized and mCD95L internalization is retarded, these authors predicted that mCD95L is more active because the DISC remains at the cell surface longer, generating more caspase activity. This led to the hypothesis that only Type II cells would internalize CD95. In contrast, we found that CD95 internalization occurs in Type I cells and it is significantly retarded in Type II cells.

We recently reported that treating Type I cells with LtnA

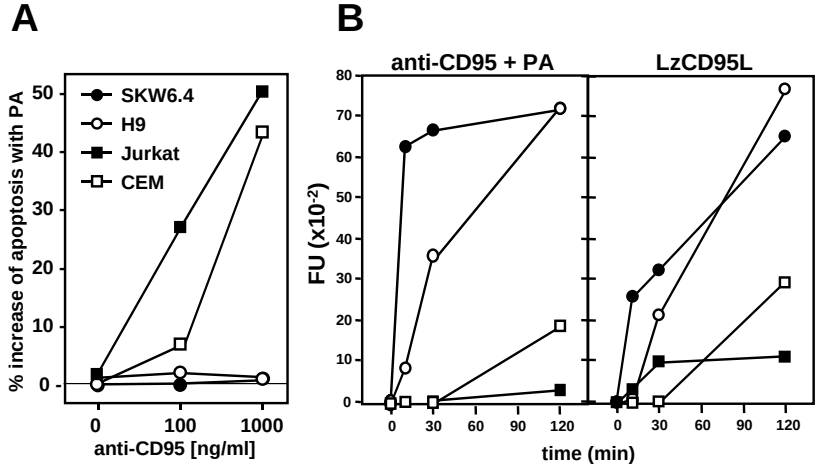

Fig. 1. Type II cells require a stronger activation stimulus for induction of apoptosis. A: Cells were incubated for 16 h with the indicated concentration of anti-CD95 in the presence or absence of PA. After incubation cells were harvested and analyzed by flow cytometry for DNA fragmentation using nuclear staining with PI. The data represent the percentage of increase of apoptosis in the presence of PA. The percentages of apoptosis without PA at 10, 100 and 1000 ng/ml respectively were: 19%, 50%, 67% SKW6.4; 11.8%, 43.97%, 74% H9; 9.97%, 14.73%, 21% Jurkat; and 15.4%, 22.1%, 27.6% CEM. B: Cells were treated with 1 μg/ml of anti-CD95 plus PA or with 1 μg/ml of LzCD95L for different times and caspase-3 activity was analyzed by the cleavage of the fluorogenic substrate DEVD-AFC.

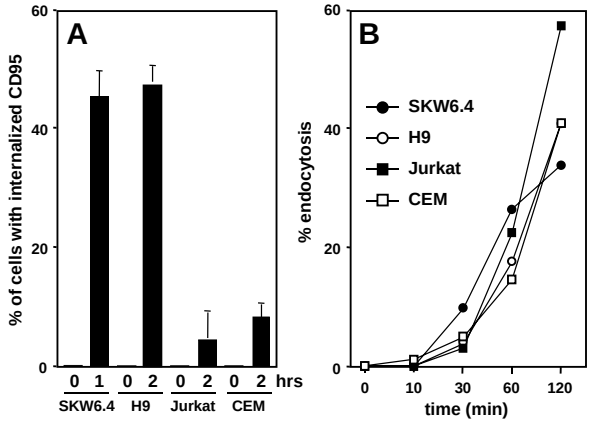

Fig. 2. CD95 internalization occurs in Type I but not Type II cells. A: Quantification of CD95 internalization at time point 0 and after 1 h of stimulation of SKW6.4 cells and 2 h stimulation of H9, Jurkat and CEM cells. The number of cells with 50% or more of CD95 internalized was determined as described in Section 2. The experiment was done in triplicate and the mean values with standard deviation are shown. B: Fluid-phase endocytosis of Type I and Type II cells was analyzed using FITC-dextran (molecular weight 40 000) as described in Section 2.

resulted in severely reduced recruitment of FADD and caspase-8 to the DISC, leading to reduced generation of caspase-8 [6] consistent with an earlier report that demonstrated that treatment with LtnA inhibits CD95 signaling [14]. We now show that this effect can be seen in Type I cells as exemplified by the analysis of the two prototype Type I cell lines SKW6.4 and H9 (Fig. 3B) but not in the two prototype Type II cell lines Jurkat and CEM (Fig. 3B). Type II cells form a very poor DISC and treatment with LtnA did not further decrease formation of the DISC in these cells (data not shown) nor did it cause a reduction in generation of caspase-8 (Fig. 3A). In fact, the amount of caspase-8 generated in LtnA-treated Type I cells was similar to the amount of caspase-8 produced in untreated Type II cells. This was also observed in the processing of procaspase-8 in Western blotting as shown for SKW6.4 and Jurkat cells (Fig. 3B). These data suggested that one of the reasons for the different responses of Type I and Type II cells to anti-CD95 triggering lies in the ability of these cells to form a DISC which in turn depends on F-actin. We therefore determined the ratio of globular (G) to filamentous (F) actin (Fig. 3C). Interestingly, SKW6.4 cells contained much more F-actin when compared to Jurkat cells and this amount was reduced when cells were treated with LtnA (Fig. 3C). Taken together the data suggest that Type II cells have a reduced ability to mobilize F-actin to form a DISC and to internalize CD95.

A number of recent data support a crucial role of the actin cytoskeleton in the CD95 apoptotic process. Actin filaments are involved in various steps of the CD95 apoptotic process such as receptor clustering, the formation of the DISC, and the CD95 internalization [15,6]. The actin binding protein ezrin has been demonstrated to bind to CD95 [15] and down-regulation of ezrin by antisense oligonucleotides renders cells resistant to CD95-mediated apoptosis [15]. Moreover, dramatic effects on ezrin and actin cytoskeleton occur following CD95 triggering [16]. We did not find a difference in the ex-

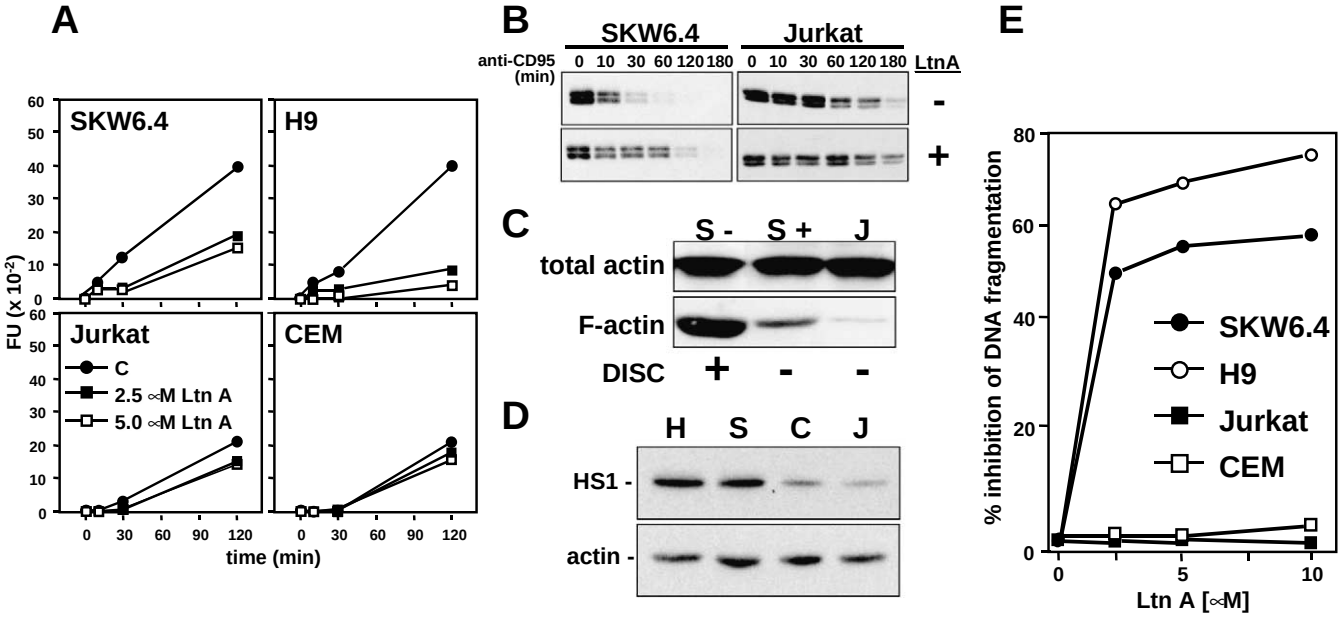

Fig. 3. Inhibition of actin polymerization prevents apoptosis only in Type I cells. A: Cells were treated with 1 μg/ml of anti-CD95 (+PA) for different periods of time in the absence or presence of LtnA. Caspase-8 activity was analyzed by cleavage of the fluorogenic substrate IETD-AFC. B: C15 immunoblot of cells treated with 1 μg/ml of anti-CD95 (+PA) for different periods of time in the absence (upper panel) or presence of LtnA (lower panel). C: Untreated SKW6.4 (S−), LtnA-treated SKW6.4 (S+) and Jurkat (J) cells were lysed and the F-actin and total actin contents were determined by Western blotting as described in Section 2. The ability of the cells to form (+) or not form (−) a DISC under the indicated conditions is indicated below the figure. D: Western blot analysis of total extracts of SKW6.4 (S), H9 (H), Jurkat (J) and CEM (C) cells for expression of HS1. E: Cells were incubated for 16 h with 1 μg/ml anti-CD95 (+PA) and different concentrations of LtnA. After incubation cells were harvested and analyzed by flow cytometry for DNA fragmentation using nuclear staining with PI. The data represent the percentage of decrease of apoptosis in the presence of the inhibitors. The percentage of specific apoptosis in the absence of inhibitors was 42% SKW6.4, 51% Jurkat, 58% H9, and 24% CEM. The data are representative of three independent experiments. In all experiment involving LtnA cells were preincubated with LtnA for 1 h.

pression level of ezrin in the tested Type I and Type II cells (data not shown). However, in a recent analysis of genes selectively expressed in Type I or Type II cells we noticed a number of actin binding and regulating proteins specific for Type I cells (unpublished data). One candidate protein, HS1, that has been shown to regulate F-actin polymerization [17] was found to be expressed at higher levels in the two Type I cell lines (Fig. 3D).

The activity of caspase-8 generated in LtnA-treated Type I and untreated Type II cells is comparable (Fig. 3A). We therefore tested whether Type I cells would respond differently to this level of active caspase-8. We found that apoptosis as measured by DNA fragmentation could be inhibited by LtnA only in Type I cells (Fig. 3E) whereas LtnA treatment had no effect on the apoptosis sensitivity of Type II cells. The latter data were confirmed using another actin binding compound swinholide A (data not shown). This suggests that Type I cells require higher concentrations of active caspase-8 to execute apoptosis compared to Type II cells.

Our data suggest that the actin dependent internalization of CD95 is specific for Type I cells. Consistent with our finding on the selective importance of actin for Type I cells we recently found by testing the 60 tumor cell lines of the anticancer drug screening panel of the NCI and searching the NCI data base on the activity of $>42\,000$ compounds to induce growth inhibition of these cells that Type I cells have a higher sensitivity to actin disrupting compounds whereas Type II cells are more sensitive to microtubule disrupting compounds [18]. This selective specificity was only found in CD95 apoptosis sensitive cell lines. Consistent with these findings it was recently shown that induction of apoptosis by actin disrupting reagents depends on functional CD95 expression [19].

Our data suggest that internalization is required for CD95 induction of apoptosis in Type I cells. Although specific inhibition of CD95 internalization has not been achieved it has been possible to block receptor internalization with LtnA in Type I cells. Consistent with our hypothesis, this treatment only affected apoptosis signaling in Type I cells and not in Type II cells which do not internalize CD95.

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References

[1] Peter, M.E., Scaffidi, C., Medema, J.P., Kischkel, F. and Krammer, P.H. (1999) Results Probl. Cell Differ. 23, 25–63.
[2] Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. and Peter, M.E. (1995) EMBO J. 14, 5579–5588.
[3] Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. and Peter, M.E. (1998) EMBO J. 17, 1675–1687.
[4] Salvesen, G.S. and Duckett, C.S. (2002) Nat. Rev. Mol. Cell Biol. 3, 401–410.
[5] Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J. and Schlesinger, P.H. (2000) Cell Death Differ. 7, 1166–1173.
[6] Algeciras-Schimnich, A., Shen, L., Barnhart, B.C., Murmann, A.E., Burkhardt, J. and Peter, M.E. (2002) Mol. Cell. Biol. 22, 207–220.
[7] Scaffidi, C., Medema, J.P., Krammer, P.H. and Peter, M.E. (1997) J. Biol. Chem. 272, 26953–26958.
[8] Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Möller, P., Falk, W., Debatin, K.M. and Krammer, P.H. (1989) Science 245, 301–305.
[9] Walczak, H., Degli-Esposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Timour, M.S., Gerhart, M.J., Schooley, K.A., Smith, C.A., Goodwin, R.G. and Rauch, C.T. (1997) EMBO J. 16, 5386–5397.
[10] Dhein, J., Daniel, P.T., Trauth, B.C., Oehm, A., Moller, P. and Krammer, P.H. (1992) J. Immunol. 149, 3166–3173.
[11] Huang, D.C., Hahne, M., Schroeter, M., Frei, K., Fontana, A., Villunger, A., Newton, K., Tschopp, J. and Strasser, A. (1999) Proc. Natl. Acad. Sci. USA 96, 14871–14876.
[12] Stegh, A.H., Barnhart, B.C., Volkland, J., Algeciras-Schimnich, A., Ke, N., Reed, J.C. and Peter, M.E. (2002) J. Biol. Chem. 277, 4351–4360.
[13] Tanaka, M., Itai, T., Adachi, M. and Nagata, S. (1998) Nat. Med. 4, 31–36.
[14] Subauste, M.C., Von Herrath, M., Benard, V., Chamberlain, C.E., Chuang, T.H., Chu, K., Bokoch, G.M. and Hahn, K.M. (2000) J. Biol. Chem. 275, 9725–9733.
[15] Parlato, S., Giammarioli, A.M., Logozzi, M., Lozupone, F., Matarrese, P., Luciani, F., Falchi, M., Malorni, W. and Fais, S. (2000) EMBO J. 19, 5123–5134.
[16] Kondo, T., Takeuchi, K., Doi, Y., Yonemura, S., Nagata, S. and Tsukita, S. (1997) J. Cell. Biol. 139, 749–758.
[17] Uruno, T., Zhang, P., Liu, J., Hao, J.J. and Zhan, X. (2003) Biochem. J. 371, 485–493.
[18] Algeciras-Schimnich, A., Pietras, E., Barnhart, B.C., Legembre, P., Vijayan, S., Holbeck, S.L. and Peter, M.E., submitted.
[19] Kulms, D., Dussmann, H., Poppelmann, B., Stander, S., Schwarz, A. and Schwarz, T. (2002) Cell Death Differ. 9, 598–608.