Analysis of CD95 Threshold Signaling

TRIGGERING OF CD95 (FAS/APO-1) AT LOW CONCENTRATIONS PRIMARILY RESULTS IN SURVIVAL SIGNALING*

Inna N. Lavrik†, Alexander Golks‡1, Dagmar Riess‡, Martin Bentele‡, Roland Eils§, and Peter H. Krammer‡2

†From the 1Division of Immunogenetics, Tumorimmunology Program, and the 2Division of Theoretical Bioinformatics, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

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Recently we generated a mathematical model (Bentele, M., Lavrik, I., Ulrich, M., Stosser, S., Heermann, D. W., Kalthoff, H., Krammer, P. H., and Eils, R. (2004) J. Cell Biol. 166, 839 – 851) of signaling in CD95(Fas/APO-1)-mediated apoptosis. Mathematical modeling in combination with experimental data provided new insights into CD95-mediated apoptosis and allowed us to establish a threshold mechanism of life and death. Here, we further assessed the predictability of the model experimentally by a detailed analysis of the threshold behavior of CD95 signaling. Using the model predictions for the mechanism of the threshold behavior we found that the CD95 DISC (death-inducing signaling complex) is formed at the cell membrane upon stimulation with low concentrations of agonistic anti-APO-1 monoclonal antibodies; however, activation of procaspase-8 at the DISC is blocked due to high cellular FLICE-inhibitory protein recruitment into the DISC. Given that death signaling does not occur upon CD95 stimulation at low (threshold) anti-APO-1 concentrations, we also analyzed survival signaling, focusing on mitogen-activated protein kinase activation. Interestingly, we found that mitogen-activated protein kinase activation takes place under threshold conditions. These findings show that triggering of CD95 can signal both life or death, depending on the strength of the stimulus.

Apoptotic cell death is common in multicellular organisms. Apoptosis can be triggered by UV or γ-irradiation, chemotherapeutic drugs, and signaling from death receptors (2 – 6). CD95 is a member of the death receptor family, a subfamily of the tumor necrosis factor receptor superfamily (2 – 6). Cross-linking of CD95 with its natural ligand CD95L (CD178) (7) or with agonistic antibodies such as anti-APO-1 induces apoptosis. CD95-induced apoptosis involves two cleavage events: the release of cytochrome c from mitochondria. CD95-induced apoptosis is mediated by the activator caspase-8 (13). In this case, signaling requires an additional amplification loop that involves the cleavage by caspase-8 of the Bcl-2 family protein Bid to generate truncated (t) Bid and subsequent tBid-mediated release of cytochrome c from mitochondria. The release of cytochrome c from mitochondria results in apoptosome-mediated activation of the caspase cascade, followed by activation of procaspase-9, which in turn cleaves downstream, effector caspases (14).

Recently there have been a few reports demonstrating that CD95 is not only a potent apoptosis inducer but is also capable of activating multiple survival pathways (15 – 18). CD95 stimulation was shown to play a central role in neurite outgrowth and neuronal regeneration following injury (17). This effect was shown to involve the activation of the mitogen-activated protein (MAP) kinases ERK1/2 (17). In tumor cells resistant to CD95-induced apoptosis (e.g. MCF7-Fas-Bcl-xL), the triggering of CD95 was reported to result in activation of survival pathways involving NF-κB, ERK1/2, p38, c-Jun N-terminal kinase (JNK), and AKT (16). However, the exact molecular mechanism of survival signaling via CD95 remains unclear.

To gain a better understanding of CD95-induced apoptosis and to elucidate the decision-making steps of CD95 signaling, we focused on CD95-mediated survival signaling. We established a threshold mechanism of life and death. Here, we further assessed the predictability of the model experimentally by a detailed analysis of the threshold behavior of CD95 signaling. Using the model predictions for the mechanism of the threshold behavior we found that the CD95 DISC (death-inducing signaling complex) is formed at the cell membrane upon stimulation with low concentrations of agonistic anti-APO-1 monoclonal antibodies; however, activation of procaspase-8 at the DISC is blocked due to high cellular FLICE-inhibitory protein recruitment into the DISC. Given that death signaling does not occur upon CD95 stimulation at low (threshold) anti-APO-1 concentrations, we also analyzed survival signaling, focusing on mitogen-activated protein kinase activation. Interestingly, we found that mitogen-activated protein kinase activation takes place under threshold conditions. These findings show that triggering of CD95 can signal both life or death, depending on the strength of the stimulus.

The abbreviations used are: DISC, death-inducing signaling complex; c-FLIP, cellular FLICE-inhibitory protein; DED, death effector domain; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; Z-VAD-fmk, benzoylcarbonyl-VAD-fluoromethyl ketone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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† Present address: Novartis Pharma AG, CH-4002 Basel, Switzerland.

‡ To whom correspondence should be addressed. E-mail:krammer@dtkfz.de.

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we developed a mathematical model of this signaling pathway (1). To estimate the parameters of the model we performed quantitative measurements of CD95-induced apoptosis in human B lymphoblastoid SKW6.4 cells. SKW6.4 cells are Type 1 cells that are highly sensitive to CD95-mediated apoptosis. The model made a number of important predictions on the mechanisms of life/death decisions in CD95-induced apoptosis. According to these predictions CD95 signaling is characterized by a threshold behavior. The model predicted that the threshold is defined at the DISC and depends on c-FLIP concentrations at the DISC (10, 19).

In this study using the model predictions we address the question how initial events in CD95 signaling influence life/death decisions at the CD95. We show that the initial concentration of CD95L and the concentration of the inhibitor of caspase-8 c-FLIP at the DISC are the most important factors contributing to the cellular decisions for CD95-induced life and death.

EXPERIMENTAL PROCEDURES

Cell Lines—The B lymphoblastoid cell line SKW6.4 was maintained in RPMI 1640 (Invitrogen), 10 mM HEPES (Invitrogen), 50 μg/ml gentamycin (Invitrogen), 10% fetal calf serum (Invitrogen) in 5% CO2.

Antibodies and Reagents—Anti-FADD monoclonal antibodies (mouse IgG1) were purchased from BD Transduction Laboratories. Anti-CD95 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-caspase-8 monoclonal antibody C15 (mouse IgG2b) recognizes the p18 subunit of caspase-8. Anti-APO-1 is an agonistic monoclonal antibody (IgG3) recognizing an epitope on the extracellular part of CD95 (APO-1/Fas) (8). FI123C antibodies (IgG3) are isotype control antibodies for anti-APO-1 (20). The horseradish peroxidase-conjugated goat anti-mouse IgG1, -2a, and -2b were from Southern Biotechnology Associates. Z-DEVD-fmk was purchased from Merck. zIETD-afc and zDEVD-afc were from Molecular Probes (Leiden, Netherlands). All other chemicals used were of analytical grade and purchased from Merck or Sigma.

Flow Cytometry Analysis—The percentage of viable cells was determined by forward scatter/SSC using a FACScan cytometer (BD Biosciences). A minimum of 10,000 cells/sample was analyzed. Specific cell death was calculated as follows: (percentage of experimental cell death − percentage of spontaneous cell death)/(100 − percentage of spontaneous cell death) × 100.

Western Blots—For Western blot analysis the cells were lysed in buffer A (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), protease inhibitor mixture (Roche Applied Science) for 15 min on ice and centrifuged (15 min, 14,000 × g). Postnuclear supernatant equivalents of 0.5 × 10^6 cells or 25 μg of protein as determined by the BCA method (Pierce) were separated on 12% SDS-PAGE and blotted onto a nitrocellulose membrane (Amersham Biosciences). Western blots were quantified using the LumiImager TMF1 system and Lumi Analyst software Version 3.0 (Roche Applied Science). The measurements were always performed in the region of a linear relation between the amount of antigen and the signal strength. The light signal was measured in Boehringer light units and in relative amounts. The Boehringer light unit value calculated is the absolute integration value of the band that was evaluated. The S.D. was calculated from up to three independent experiments.

Caspase Activity Assays—Cytosolic lysates were incubated with 50 μM site-specific tetrapeptide substrates (zIETD-afc for caspase-8, zDEVD-afc for caspase-3) in caspase assay buffer B (50 mM HEPES, 100 mM NaCl, 10 mM dithiothreitol, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, pH 7.4) in a final volume of 200 μl. The release of the fluorogenic group AFC was determined after 1 h of incubation at 37 °C by a microplate fluorescence reader Wallach 1420 (PerkinElmer Life Sciences) at the excitation wavelength of 405 nm and emission wavelength of 535 nm.

DISC Analysis by Immunoprecipitation and Western Blotting—1 × 10^8 cells in a concentration of 10^6 cells/ml were treated with indicated concentration of anti-APO-1 (IgG3) for 10 min at 37 °C, washed twice in 1× phosphate-buffered saline (PBS), and subsequently lysed in buffer A (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), protease inhibitor mixture (Roche Applied Science), 1% Triton X-100 (Serva), and 10% glycerol) (stimulated condition) or lysed without treatment (unstimulated condition). The CD95 DISC was immunoprecipitated overnight with 2 μg of anti-APO-1 and Protein A-Sepharose. Beads were washed five times with 20 volumes of lysis buffer. The immunoprecipitates were analyzed on 12% polyacrylamide gel. Subsequently, the gels were transferred to Hybond nitrocellulose membrane (Amersham Biosciences), blocked with 5% nonfat dry milk in PBS/Tween (PBS plus 0.05% Tween 20) for 1 h, washed with PBS/Tween, and incubated with the primary antibodies in PBS/Tween at 4 °C overnight. Blots were developed with a chemiluminescence method following the manufacturer’s protocol (PerkinElmer).

Caspase Inhibition Experiments—Z-VAD-fmk (pan-specific caspase inhibitor) was added to the cells in the final concentration of 50 μM 30 min before the addition of anti-APO-1.

Proliferation Assays—For proliferation assays 1 × 10^5 cells were seeded into a 96-well titer plate and stimulated with either 1 ng/ml of anti-APO-1 or left untreated for up to 4 days. Proliferation was measured with a scintillation counter after tritiated thymidine ([3H]TdR) incorporation during the final 15–18 h of the culture.

RESULTS

CD95 Stimulation with Low Concentrations of Anti-APO-1 Antibodies Results neither in Cell Death nor in Caspase Activation—The analysis of CD95 signaling in human B lymphoblastoid SKW6.4 cells using mathematical modeling in combination with biochemical approaches led to the finding of a threshold behavior of CD95-induced apoptosis (1). We established the threshold concentration of anti-APO-1 antibodies that did not result in cell death in SKW6.4 cells to be 1 ng/ml (1). We performed a more detailed analysis of CD95-mediated cell death in SKW6.4 cells than in our previous studies (1) (Fig. 1). We varied the concentration of anti-APO-1 from 0.1 ng/ml to 1 μg/ml and measured cell death at different time points from 2 h to 4 days (Fig. 1A). Notably, we used the same concentration of
SKW6.4 cells (10^6 cells/ml) as in our previous studies (1). Upon stimulation with 1 ng/ml of anti-APO-1 antibodies (threshold concentration) we did not observe any cell death (Fig. 1A) nor did we see caspase activation using the fluorescent substrates of caspases zIETD-afc and zDEVD-afc (Fig. 1B).

To assure that the threshold concentration of the anti-CD95 stimulus does not lead to an initial low level of caspase activation, which would disappear at a later time point, we analyzed caspase cleavage by Western blot in the first hours after stimulation (Fig. 1C). SKW6.4 cells (10^6 cells/ml) were stimulated with 1 ng/ml (threshold concentration) or with 100 ng/ml of anti-APO-1 antibodies. No processing products of procaspase-8, -3, or poly(ADP-ribose) polymerase cleavage were observed upon stimulation with the threshold concentration of 1 ng/ml of anti-APO-1 (Fig. 1C). In contrast, upon stimulation with 100 ng/ml we observed cleavage of procaspase-8, procaspase-3, and poly(ADP-ribose) polymerase, indicating triggering of the CD95-induced apoptosis. Thus, we
reconfirmed the threshold concentration of anti-APO-1 (1 ng/ml) for SKW6.4 cells and further characterized the threshold behavior of these cells showing that caspases are not activated within the first hours after stimulation.

The CD95 DISC Is Formed at Threshold Concentrations in Lower Amounts and c-FLIP Inhibits Caspase-8 Activation at the DISC upon Threshold Stimulation—Next, we tested the threshold mechanism at the DISC. Our model has made the following predictions for the mechanism of threshold behavior of CD95 signaling: lower amounts of CD95 DISCs, high c-FLIP recruitment to the DISC due to the highest affinity of c-FLIP to the DISC compared with other DED proteins (21), and, consequently, inhibition of caspase-8 activation (1) (Fig. 2A).

Our model suggested that upon threshold stimulation the CD95 DISC is formed, albeit in low amounts (1). So far we had not analyzed CD95 DISC formation and composition under threshold concentrations of anti-APO-1 antibodies. To address this issue we performed CD95 immunoprecipitations upon stimulations with 1, 10, and 100 ng/ml of anti-APO-1 antibodies (Fig. 2B). We used the same concentration of SKW6.4 cells (10^6 cells/ml) to strictly follow the threshold conditions for these cells established in Fig. 1 and Ref. 1. After stimulation the CD95 DISC was immunoprecipitated by using only Protein A-Sepharose without the addition of anti-APO-1 antibodies (Fig. 2B). We considered as the amount of procaspase-8 activation from the lysates was calculated using quantitative Western blot. We considered as the amount of procaspase-8 in one lane the sum of the signal corresponding to the bands of procaspase-8 (p55/p53) and its cleavage product p43/p41. As the amount of c-FLIP in one lane we summarized the signals resulting from c-FLIP (p55), its cleavage product (p43), and c-FLIP (p43-c-FLIP).
model that the CD95 DISC is formed upon threshold concentrations of anti-APO-1, albeit in lower amounts (1).

Interestingly, in Fig. 2B we also observed that the amount of c-FLIP recruited to the DISC was higher for stimulation with 1 ng/ml than with 100 ng/ml. This result confirms the next prediction of the threshold mechanism.

Importantly, the processing of procaspase-8 at the DISC upon threshold concentrations also confirmed the model predictions (Fig. 2B). We observed that upon stimulation with 1 ng/ml procaspase-8 is processed only to p43/p41, indicating that processing of procaspase-8 is stopped after the first cleavage step. In contrast, for the stimulation with 100 ng/ml we also observed the generation of the p18 subunit, indicating that for the higher concentrations of anti-APO-1 the second cleavage step takes place and active caspase-8 is released into the cytosol to start the apoptotic signaling cascade.

To rule out that under threshold stimulation of 1 ng/ml we immunoprecipitate low amounts of DISCs, which are spontaneously formed in the cells without stimulation, we performed immunoprecipitations from unstimulated cells with 1 and 10 ng/ml of anti-APO-1 (Fig. 2C). Procaspase-8 and c-FLIP were not associated to CD95 under these conditions.

As the affinities of procaspase-8 and c-FLIP to the DISC are critical parameters for the decision making at the DISC, we compared the amounts of procaspase-8 and c-FLIP that were recruited to the DISC from corresponding lysates by quantitative Western blotting (Fig. 2D). For each molecule, e.g. procaspase-8 or c-FLIP, we measured their amounts at the DISC versus their amounts in the lysates.

To prevent proteolitical processing of procaspase-8 to p26/p24, which is not detectable by anti-caspase-8 antibody directed against the C terminus of procaspase-8, we added pan-caspase inhibitor Z-VAD-fmk. Under these conditions we observed that the amount of c-FLIP at the DISC was eight times higher than the amount of c-FLIP in the lysates. The amount of procaspase-8 at the DISC was five times higher than the amount of procaspase-8 in the lysates. Thus, the affinity of c-FLIP to the DISC was roughly twice as high compared with that of procaspase-8. Thus, we showed that c-FLIP has a higher affinity to the DISC than procaspase-8.

We could not compare the ratio of procaspase-8 to c-FLIP at the DISC due to the fact that we were using different antibodies. The question of stoichiometry of the DISC is a matter for future studies. Thus, we have confirmed our model predictions and show that at threshold stimulation c-FLIP is up-regulated at the DISC due to the high affinity of c-FLIP to the DISC, leading to the inhibition of caspase-8 activation and further downstream apoptotic events.

**Triggering of CD95 with Low Amounts of a CD95 Stimulus Does Not Result in Apoptosis but in MAP Kinase Activation**—Next we analyzed whether stimulation of CD95 with threshold amounts of anti-APO-1 antibodies triggers reported survival pathways (15–18). We observed that stimu-
CD95 Triggers Survival at Low Concentrations

**DISCUSSION**

In this study using the model predictions we address the question how initial events in CD95 signaling influence life/death decisions at the CD95. We showed that the initial concentration of CD95L and the concentration of the inhibitor of caspases c-FLIP are the most important factors contributing to the cellular decisions for CD95-induced life and death. We demonstrated that upon threshold stimulation 1) the CD95
DISC is formed, albeit in low amounts; 2) c-FLIP is quickly recruited to the DISC due to its high affinity to the DISC; and 3) c-FLIP inhibits caspase-8 activation by blocking the second step of procaspase-8 processing at the DISC, resulting only in the formation of p43/p41 subunits.

We performed our study using only SKW6.4 cells and anti-APO-1 antibodies as we quantitatively characterized CD95-induced apoptosis in SKW6.4 cells in our previous work and experimentally determined the threshold concentration of anti-APO-1 antibodies in SKW6.4 cells (1). This quantitative characterization allowed us to proceed directly to more detailed DISC analysis in the present study.

The central molecule defining the threshold behavior in CD95-induced apoptosis is c-FLIP (1). c-FLIP has a dual function in CD95-mediated apoptosis. c-FLIP\_S/R is a devoted inhibitor of CD95-induced apoptosis, whereas c-FLIP\_L is described to play both pro- and anti-apoptotic roles (10, 19, 21, 22). When present in the cells in low concentrations, c-FLIP\_L accelerates procaspase-8 activation at the DISC (21). Upon high concentrations in the cells (e.g., overexpression conditions), c-FLIP\_L was reported to inhibit CD95-induced apoptosis (19, 21). The mechanism of c-FLIP\_L inhibition involves blocking the second step of procaspase-8 processing (19). We observed that at threshold concentrations of anti-APO-1 procaspase-8 processing at the DISC is also stopped after the first step with formation of p18 and p10. Thus, apoptosis is blocked and "life" signaling is predominant.

In addition to inhibition of the second cleavage step of procaspase-8 at the "threshold" DISC, we also observed inhibition of the first cleavage step as judged by reduced amounts of p43/p41 (Fig. 2B). This might be explained by up-regulation of c-FLIP\_R at the threshold DISC blocking the first cleavage step.

**FIGURE 5.** The model of signal transduction for the CD95 receptor in CD95-sensitive cell lines. Upon stimulation CD95 can trigger both survival and the death pathways. The signal transduction for the death pathway was characterized in detail and involves DISC formation with subsequent activation of procaspase-8 at the DISC (left side). Activation of procaspase-8 involves two cleavage steps, p43/p41 and p10 generation, which is followed by the second step with formation of p18 and p10. Active caspase-8 heterotetramer p10-p18 goes to the cytosol to propagate the apoptotic signal. When triggered with high concentrations of CD95 stimulus the death signaling is predominant. At threshold concentrations of CD95 stimuli (right side) procaspase-8 processing at the DISC is stopped after the first step (formation of p43/p41 and p10). Thus, apoptosis is blocked and "life" signaling is predominant.
To get more insights into the mechanism of inhibition of procaspase-8 at the DISC upon threshold stimulation we compared the affinities of c-FLIP to procaspase-8 at the DISC (Fig. 2D). We found that affinity of c-FLIP is indeed higher than that of procaspase-8. This allows quick recruitment of c-FLIP to the DISC upon threshold stimulation in accordance with model predictions.

Stimulation with low concentrations of anti-APO-1 antibodies can result in two scenarios. First, upon stimulation at the threshold concentration of anti-APO-1 caspase activity would be observed, however at a level low enough to not result in apoptosis. According to the second scenario caspase activation does not take place upon stimulation with threshold concentrations of anti-APO-1. The first scenario contradicts our mathematical modeling (1) as well as the experimental data (Fig. 1). The second scenario involves inhibition of caspase-8 activation at the DISC level. Furthermore, the second scenario was predicted by the model and confirmed by the original set of experimental data (1). The data presented in this study further confirm the model predictions, demonstrating in detail inhibition of caspase-8 activation at the DISC that consequently prevents the following apoptosis signaling cascade.

To establish the functional relevance of stimulation with low concentrations of a CD95 stimulus we analyzed survival signaling, e.g. MAP kinase activation. Interestingly, we demonstrated that MAP kinase activation is not stopped upon threshold stimulation when death is blocked. These results go along with the fact that MAP kinase activation is not blocked by the addition of the pan-caspase inhibitor Z-VAD-fmk. This indicates that the mechanism of survival signaling via CD95 does not involve death components of the CD95 signaling pathway and caspase-8 activation. Transduction of the survival signal might occur via as yet uncharacterized proteins associated to the CD95. Our findings go along with the reports of survival signaling from CD95 in other cell types other than lymphocytes, e.g. primary neurons (15). In these cells the concentration of “death components” associated to the CD95 receptor might be lower than “survival components”, resulting in the preferential signaling of survival. Interestingly, our study also goes along with the findings that cells carrying heterozygous mutations in the CD95 DD are deficient in induction of apoptosis but can still efficiently activate the transcription factor NF-κB and the MAP kinases ERK1/2 and p38 (23).

Based on our studies we suggest the following model for CD95 signaling in CD95-sensitive cell lines (Fig. 5). At high doses of CD95 stimuli the CD95 DISC is formed and caspase-8 is activated, triggering the apoptotic cascade (Fig. 5, left). Survival signaling takes place in parallel; however, apparently it is not strong enough to prevent the cells from dying. At threshold concentrations c-FLIP molecules block caspase-8 activation at the DISC in accordance with the above described mechanism and survival signaling becomes the major event (Fig. 5, right).

Understanding of the switch mechanisms between life and death of cells plays an important role in therapeutic intervention strategies in diseases that are associated with apoptosis. Thus, the detailed molecular mechanism of signal transduction for survival signaling from CD95 needs to be addressed further in future studies. It should also be noted that this study once again shows that mathematical modeling in combination with biochemical analysis is a powerful tool that allows a detailed characterization of complex signaling pathways.

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