Differential Modulation of Apoptosis Sensitivity in CD95 Type I and Type II Cells*

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We have recently identified two different pathways of CD95-mediated apoptosis (Scaffidi, C., Fulda, S., Srinivasan, A., Feng, L., Friesen, C., Tomaselli, K. J., Debatin, K.-M., Krammer, P. H., and Peter, M. E. (1998) EMBO J. 17, 1675–1687). CD95-mediated apoptosis in type I cells is initiated by large amounts of active caspase-8 formed at the death-inducing signaling complex (DISC) followed by direct cleavage of caspase-3. In contrast, in type II cells very little DISC and small amounts of active caspase-8 sufficient to induce the apoptotic activity of mitochondria are formed causing a profound activation of both caspase-8 and caspase-3. Only in type II cells can apoptosis be blocked by overexpressed Bcl-2 or Bel-xL. We now show that a number of apoptosis-inhibiting or -inducing stimuli only affect apoptosis in type II cells, indicating that they act on the mitochondrial branch of the CD95 pathway. These stimuli include the activation of protein kinase C, which inhibits CD95-mediated apoptosis resulting in a delayed cleavage of BID, and the induction of apoptosis by the ceramide analog C2-ceramide. In addition, we have identified the CD95 high expressing cell line BoeR as a CD95 apoptosis-resistant type II cell that can be sensitized by treatment with cycloheximide without affecting formation of the DISC. This also places the effects of cycloheximide in the mitochondrial branch of the type II CD95 pathway. In contrast, c-FLIP was found to block CD95-mediated apoptosis in both type I and type II cells, because it acts directly at the DISC of both types of cells.

CD95 (APO-1/Fas) is a member of the death receptor family (1). Triggering of this receptor results in the formation of the death-inducing signaling complex (DISC)‡, a complex of signaling proteins recruited by the activated CD95 instantly after the addition of agonistic anti-CD95 antibodies or the CD95 ligand (2). Formation of the DISC comprising the adapter molecule FADD/MORT1 (3, 4) and caspase-8 (5, 6) results in the release of active caspase-8 at the DISC and cleavage of various intracellular death substrates (7, 8). Both DISC proteins, FADD and caspase-8, have now been demonstrated to be essential components of the CD95 signaling machinery (9–12).

We have recently identified two different CD95 apoptosis signaling cell types (13). Type I cells require activation of caspase-8 at the DISC closely followed by activation of caspase-3. Blocking the release of apoptogenic factors (i.e. cytochrome c and apoptosis-inducing factor) from mitochondria by heterologous expression of Bcl-2 or Bcl-xL had no effect on caspase-8 or caspase-3 cleavage or on CD95 sensitivity of these cells. In type II cells DISC formation was strongly reduced despite similar expression levels of the DISC components CD95, FADD, and caspase-8. In these cells, caspase-8 and caspase-3 were primarily activated downstream of mitochondria, and their activation was blocked by the overexpression of Bcl-2. We now demonstrate that a number of treatments that have been reported to either inhibit or enhance apoptosis can only act on type II cells. The following treatments were tested: 1) activation of protein kinase C by treating the cells with phorbol 12-myristate 13-acetate (PMA), which results in the inhibition of CD95-mediated apoptosis (14–17); 2) sensitization of CD95 apoptosis-resistant cells with cycloheximide (CHX) (18); and 3) induction of apoptosis by treating cells with C2-ceramide (19). In contrast, stable overexpression of the caspase-8-like molecule c-FLIP (20, 21) blocked CD95-mediated apoptosis by inhibiting the activation of caspase-8 directly at the DISC of both type I and type II cells.

EXPERIMENTAL PROCEDURES

Cell Lines—The B lymphoblastoid cell line SKW6.4, the pre-B-cell line BoeR (2), and the T cell lines H9 and CEM were maintained in RPMI 1640 (Life Technologies, Inc.), 10 mM Hepes (Life Technologies, Inc.), 50 μg/ml gentamycin (Life Technologies, Inc.), and 10% fetal calf serum (Life Technologies, Inc.) in 5% CO2. The T cell line Jurkat (clone J16) was maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) supplemented as described above. CEM cells expressing c-FLIP were cultured in supplemented RPMI 1640 medium containing 0.5 μg/ml puromycin (Sigma). Jurkat cells transfected with empty vector or Bcl-2 were cultured as described elsewhere (22).

Antibodies and Reagents—Monoclonal antibodies against FADD and caspase-3 were purchased from Transduction Laboratories (Lexington, Kentucky). The C15 mAb (mouse IgG2b) recognizes the p18 subunit of caspase-8 (23). The anti-c-FLIP mAb NF6 (mouse IgG1) was generated against glutathione S-transferase-Nc-FLIP as described (21), and anti-APO-1 (anti-CD95) is an agonistic monoclonal antibody IgG3, κ recognizing an epitope on the extracellular part of APO-1 (CD95/Fas) (24). The horseradish peroxidase-conjugated goat anti-rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxi-
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(960 µf, 220 V) using a Gene Pulser™ (Bio-Rad) with control vector (pEPsFLAG) or c-FLIP expression vector (pEPsFLAG-c-FLIP). Transfectants were selected in supplemented RPMI 1640 medium containing 0.5 µg/ml puromycin (Sigma). High expressing clones were identified by Western blot analysis using the anti-c-FLIP mAb NF6.

**DISC Analysis by Western Blotting**—The amount of DISC-associated FADD was determined as follows: 10⁷ cells were either treated with 2 µg/ml anti-APO-1 for 5 min at 37 °C and then lysed in lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, small peptide inhibitors (2), 1% Triton X-100 (Serva) and 10% glycerol) (stimulated condition) or lysed and then supplemented with anti-CD95 (unstimulated condition). The CD95 DISC was then precipitated for 2 h at 4 °C with protein A-Sepharose (Sigma). After immunoprecipitation the beads were washed 5 times with 1 ml of lysis buffer. For Western blotting, immunoprecipitates or cytosolic proteins equivalent to 10⁶ cells or 20 µg of protein were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech), blocked with 2% bovine serum albumin in PBS/Tween. After washing with PBS/Tween the blots were developed with horseradish peroxidase-conjugated secondary antibody diluted 1/20,000 in PBS 0.05% Tween 20 for at least 1 h, washed with PBS/Tween, and incubated with the primary antibody in PBS/Tween for 16 h at 4 °C. Blots were developed with the chemiluminescence method (ECL) following the manufacturer's protocol (Amersham Pharmacia Biotech). Protein concentrations of cellular lysates were determined by the BCA method (Pierce). Cleavage of BID was determined in the following way. Jurkat cells (0.5 million/ml) were pretreated with PMA (20 nM) or untreated for 30 min; they were then treated with anti-CD95 mAb (100 ng/ml, Upstate Biotechnology, clone CH11) for different periods of time. The lysates were prepared in radioimmunoprecipitation buffer and separated by a 14% SDS-polyacrylamide gel electrophoresis. The Western blot analysis was performed with an anti-human BID antibody (1:2000, a gift from Junying Yuan) and developed with ECL.

**Flow Cytometric Analysis of Mitochondrial Membrane Potential (ΔΨm)**—To measure ΔΨm, anti-CD95 (1 µg/ml)-treated or -untreated cells (5 × 10⁶) were incubated with 5 µg/ml JC-1 (5, 5′, 6′, 6′-tetrachloro-1, 1′, 3′, 3′-tetracyanobenzene) for 30 min at 37 °C (Molecular Probes, Inc., Eugene, OR). This cyanine dye accumulates in the mitochondrial matrix under the influence of the ΔΨm and forms J aggregates that have characteristic absorption and emission spectra. The JC-1 staining method is reported to provide more accurate estimates of ΔΨm than 3,3′-dihexyloxacarbocyanine iodide (25). After incubation for 20 min at room temperature in the dark, cells were washed once with PBS/Tween, cells suspensions were prepared for flow cytometry, and the 488-nm line of an argon ion laser was used for excitation. Orange and green emitted fluorescence were collected through 585/42 (FL2) and 530/30-nm (FL1) bandpass filters. Flow cytometry was performed on a FACS2 3-color flow cytometer and analyzed using LYSIS II software (Becton-Dickinson Immunocytochemistry Systems, Mountainview, CA). After gating out small sized (i.e. noncellular) debris, 20,000 events were collected for each analysis. Upon incubation of the cells with anti-CD95 the orange fluorescence (FL2) did not significantly change, whereas the green fluorescence (FL1), which corresponds to the monomer form of the dye, because of the reduction of ΔΨm increased with time. This increase was therefore taken as a measure for the loss of ΔΨm.

**Induction of Apoptosis and Cytotoxicity Assay**—5 × 10⁶ cells were incubated in 24-well plates (Costar, Cambridge, MA) with anti-CD95, PMA, C2- ceramide, or C2-dihydropyran-6-carboxylic acid in 1 ml of medium at 37 °C. C2-ceramide was used at concentrations of 20–100 µM. Quantification of DNA fragmentation as a specific measure of apoptosis was carried out by nuclear staining with propidium iodide essentially as described previously (26).

In Vitro Translation and in Vitro Cleavage Assay—Caspase-8/9a (23) was in vitro translated using a T7 polymerase-directed reticulocyte lysate system (TNT, Promega). In vitro cleavage assays were performed as follows. CD95 DISC was immunoprecipitated from 5 × 10⁷ cells as described above. Subsequently, the beads (containing the DISC) were incubated in 50 µl of reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% CAPS, 10 mM dithiothreitol, and 20% sucrose) for 24 h at

**RESULTS**

**Activation of PKC Inhibits Apoptosis only in Type II Cells**—CD95-mediated apoptosis can be inhibited by the activation of PKC (e.g. with PMA) (14–17). It is apparent that in most reports Jurkat T cells were used. To test whether this inhibition is generally found in all cells we compared the four cell lines in which the CD95 signaling pathway was recently characterized in detail (13). Of these cells only the type II cells CEM and Jurkat were protected by PMA (Fig. 1, A and B). CD95-mediated apoptosis in the type I cells SKW6.4 and H9 was not inhibited by the addition of PMA, indicating that the CD95 apoptosis pathway of type I cells was not connected to PKC activation (Fig. 1, C and D). Recently, a molecular link between DISC-activated caspase-8 and the mitochondria was described.
(27–29). The BH3 domain containing Bcl-2 family protein BID was shown to be a direct target for caspase-8. Cleavage of p22bid by caspase-8 results in the formation of a truncated p15bid protein that directly affects mitochondria and seems to be responsible for the release of cytochrome c. To narrow down the entry point of PKC activation in the pathway we tested whether the cleavage of BID would be affected by treating the cells with PMA (Fig. 1E). Activation of PKC clearly reduced the formation of p15bid formed at 60 and 120 min, suggesting that PKC phosphorylates BID itself or a target that affects the processing of BID.

Only Type II Cells Are Sensitive to C2- ceramide—The role of ceramide in CD95-mediated apoptosis remains controversial (reviewed in Ref. 30). However, it is well established that the addition of the ceramide analog C2-ceramide induces apoptosis in many cell types, whereas the ceramide analog C2-dihydroceramide has no such activity (19). Recently, it has been shown that C2-ceramide acts by causing the formation of reactive oxygen intermediates and may even affect mitochondrial components directly (31–33). Therefore, we tested whether type I and II cells were equally sensitive to C2-ceramide treatment. The results shown in Fig. 2A demonstrate that only the two type II cells were sensitive to C2-ceramide-induced apoptosis. Because C2-ceramide represents a CD95-independent pathway of apoptosis induction and may affect mitochondria directly this finding underscores the independence of type I cell apoptosis from mitochondrial apoptogenic activities. Apoptosis induced by C2-ceramide required activation of caspases, because in Jurkat cells it could be inhibited by pretreating the cells with the broad spectrum caspase inhibitor zVAD-fmk (Fig. 2B). In addition, Jurkat T cells overexpressing Bcl-2, which blocks mitochondrial apoptogenic activities (13), were also resistant to C2-ceramide-induced apoptosis (Fig. 2C), supporting the notion that mitochondria are required for the apoptosis-inducing activity of C2-ceramide. It has been shown that treatment of cells with apoptosis-inducing concentrations of C2-ceramide results in the activation of caspase-3 (17, 34). We therefore tested activation of caspase-3 in our four model cell lines upon the addition of C2-ceramide. Only in the two type II cell lines was caspase-3 processed (Fig. 2D). Because apoptosis in this system could be inhibited by Bcl-2, which prevents the release of apoptogenic factors by mitochondria (Fig. 2C), activation of caspase-3 may occur downstream of mitochondria as previously shown for type II cells (13). Our data support the view that C2-ceramide acts at the level of mitochondria and furthermore suggest that type I cells that are resistant to C2-ceramide lack a component of the mitochondrial apoptosis pathway.

Modulation of the Apoptosis Sensitivity in a Type II Cell Line by Treating it with CHX—We previously described a pre-B cell line, BoeK, that expressed large amounts of cell surface CD95 (Fig. 3A) yet was resistant to CD95-mediated apoptosis (Fig. 3B) (2). Because BoeK seemed to have a defect in the formation of the DISC, we concluded that this apparent defect was responsible for the resistance phenotype. However, when we treated BoeK cells with CHX they were as CD95 sensitive as other highly sensitive cell lines expressing similar amounts of surface CD95 (Fig. 3, A and B). Only CHX-treated BoeK cells responded to anti-CD95 treatment with a drop in ΔΨm, indicating that CHX acted upstream of mitochondria (Fig. 3C). Because BoeK cells also expressed similar levels of the essential signaling molecules FADD (Fig. 4A) and caspase-8 (23), we tested whether CHX acted at the level of the CD95 receptor by modulating formation of the DISC. To test this, association of FADD with activated CD95 was taken as a measure for proper formation of the DISC (Fig. 4B). As expected in the type I prototype cell line SKW6.4, large amounts of FADD were recruited to the activated CD95 receptor (Fig. 4B, lane 2). In contrast, in the prototype type II cell line Jurkat, this association was hard to detect and required a much longer exposure (Fig. 4B, lane 10). Similarly, in the BoeK cells very little but significant amounts of FADD were found bound to activated CD95 receptors (Fig. 4B, lane 6) identifying BoeK as type II cells. Interestingly, sensitizing these cells by incubation with CHX did not substantially change the formation of the DISC, indicating that in this apoptosis pathway CHX acts downstream of the CD95 receptor. We have previously shown that the only proteolytic activity associated with the DISC is cleavage of p15 (7). To test whether the DISC in BoeK cells had the capacity to convert procaspase-8 into active caspase-8 subunits, we performed an in vitro caspase-8 cleavage assay (7) with the DISC isolated from BoeK cells (Fig. 4C). In this assay the DISC
FIG. 3. BoeR cells are CD95 apoptosis-resistant type II cells. A, surface staining of Jurkat (type II), SKW6.4 (type I), and BoeR cells for CD95. Cells were stained with anti-CD95 followed by phycoerythrin-conjugated goat anti-mouse antibody (gray curve) or with secondary antibody alone (white curve). B, sensitivity of cell lines for CD95-induced apoptosis. Jurkat (●), SKW6.4 (●), and BoeR cells in the absence (●) or presence (○) of 10 μg/ml CHX were treated with increasing amounts of anti-CD95, and DNA degradation was determined after 20 h. C, BoeR cells were treated with 1 μg/ml anti-CD95 for different periods of time in the absence (●) or presence (○) of 10 μg/ml CHX. \( \Delta \Psi_m \) was determined as described under "Experimental Procedures." Numbers were corrected for background values obtained with 10 μg/ml CHX alone (−20% decrease of \( \Delta \Psi_m \), after 6 h).

of unlabeled cells is immunoprecipitated, and 

\[ ^{35}S \text{-labeled caspase-8/a} \]

is added. After incubation for 24 h at 4 °C, caspase-8/a is processed at the DISC into active fragments. As a control the DISC of the type I cells SKW6.4 was prepared (Fig. 4C, lanes 1–4). The intermediate cleavage fragment p43 and the prodomain p26 remained in part bound to the DISC (Fig. 4C, lane 2), whereas the active subunits p18 and p12, including a cleavage intermediate were found only in the supernatant (Fig. 4C, lane 4). By Western blotting, procaspase-8, as part of the DISC, was hardly detectable in the type II cells BoeR (data not shown). However, the caspase-8 enzymatic activity present was sufficient to process caspase-8 producing small amounts of active caspase-8 at the receptor level (Fig. 4C, lanes 5–12) demonstrating that also the DISC of type II cells was functionally active. Such small amounts were shown to be sufficient to generate a signal that affects mitochondria in type II cells (13). Again this DISC-associated activity was not enhanced by pretreating cells with CHX (Fig. 4C, lanes 7, 8, 11, and 12). All data taken together, we conclude that BoeR cells are type II cells in which an anti-apoptotic program is in place that can be tuned down by inhibition of protein biosynthesis. CHX therefore acts in the pathway of type II cells presumably upstream of the mitochondrion but downstream of the DISC.

FIG. 4. BoeR cells form very little but functionally active DISC that is unaffected in its activity by CHX. A, Western blot analysis of FADD in cellular lysates of 10^6 cells. B, SKW6.4 and BoeR cells were untreated or treated with 10 μg/ml CHX for 1 h, and Jurkat cells were either left untreated (−) or treated (+) with anti-CD95 for 5 min. Subsequently, CD95 was immunoprecipitated, and the amount of associated FADD was determined by Western blot analysis. Blots were exposed to the x-ray film for 5 min (long). A short exposure of only 10 s (short) is shown for the SKW6.4 cells. C, CD95 was immunoprecipitated from either untreated (−) or anti-CD95 treated (+) SKW6.4 or BoeR cells. BoeR cells were either left untreated or treated with 10 μg/ml CHX (+ CHX) for 6 h. Immunoprecipitates were washed four times and incubated with in vitro translated \(^{35}S\)-labeled caspase-8/a. After 24 h the supernatant (sup) was separated from beads. Beads were washed three times, and both beads and supernatants were loaded on an 15% SDS-polyacrylamide gel. The positions of caspase-8/a (CASP-8/a) and its fragments p43, p26 (the prodomain), p18, and p12 are indicated.

the receptor was too low to be detected by Western blot yet high enough to trigger the release of apoptogenic factors by mitochondria (13). To test whether this assumption is correct we generated CEM cells stably expressing c-FLIP (Fig. 5A). Apoptosis in these transfectants triggered through CD95 was significantly inhibited confirming that in both type I (21) and type II (Fig. 5B) cell apoptosis can be inhibited by c-FLIP. To determine the location of the c-FLIP inhibition in the CD95 pathway, \( \Delta \Psi_m \) was determined upon triggering CD95 in the type II CEM cells expressing c-FLIP (Fig. 5C). Our results suggest that c-FLIP was able to block CD95-mediated apoptosis and prevent the apoptogenic activity of mitochondria as manifested by an unaltered \( \Delta \Psi_m \), also in the type II cells. To finally prove that c-FLIP blocked the activity of the DISC itself, we isolated the DISC from CEM cells stably expressing c-FLIP and tested its activity to process in vitro translated caspase-8 (Fig. 5D). As expected the DISC of the type I cells SKW6.4 was active in processing caspase-8. We now show that in type II cells Jurkat, CEM, and BoeR the DISC also contains such an activity. However, this activity was blocked when the DISC was prepared from CEM cells expressing c-FLIP, confirming that in both type I and type II cells the CD95 apoptosis cascade is initiated by caspase-8 and that c-FLIP can inhibit this form of apoptosis in both cell types.
The CD95 signaling pathway is characterized by a sequential activation of a number of caspases (35). The death signal is initiated at the receptor level by the activation of caspase-8 in the DISC (7). We have recently described the existence of two different CD95 apoptosis signaling pathways in different cell types (13). Type II cells depend on the apoptogenic activity of mitochondria, whereas type I cells are independent of this function. In the latter cell type the apoptosis signal is mainly transduced by caspases. The distinction between the two cell types was supported by the fact that only in type II cells would overexpression of Bcl-2 or Bcl-xL, two proteins that inhibit the apoptogenic activity of mitochondria, inhibit CD95-mediated apoptosis.

In addition to members of the Bcl-2 family a number of other pathways have been reported to interfere with CD95 signaling in recent years. This raises the question as to how general these effects are. Issues exist as to whether the protection is cell or tissue specific and at what stage within the emerging signaling pathway they interfere. Remarkably, most recent reports describing such effects were performed in type II cells, consistent with the view that type I cells activate an apoptosis signaling pathway that solely depends on caspases. It seems that only cells whose apoptosis depends on the apoptogenic activity of mitochondria are sensitive to PKC-mediated effects. It has been argued that PKA treatment of Jurkat cells results in the generation of superoxide anions likely produced by mitochondria that may be responsible for the inhibition of CD95-mediated apoptosis (15). However, we found evidence that PKA acts upstream of mitochondria. Recently, it has been shown that BID, a BH3 domain-containing member of the Bcl-2 family, is specifically cleaved by caspase-8 (27–29). Processed p15<sub>BID</sub> translocates to mitochondria where it is an integral membrane protein required for cytochrome c release. We observed that the cleavage of BID by caspase-8 is reduced when PKC is activated by PKA. This provides an example for a PKC target that resides in between DISC and mitochondria.

Ceramide has previously been suggested to be involved in the CD95 pathway (46). However, recent data indicate that it may not play a crucial role in the transmission of the CD95 death signal (47–50). In addition, Herr et al. (51) demonstrated that treatment of CEM and Jurkat cells with low concentrations of C<sub>2</sub>-ceramide (up to 10 μM) resulted in up-regulation of the CD95 ligand killing cells by autocrine suicide or by fratricide. This would place the action of C<sub>2</sub>-ceramide upstream of the CD95 receptor. However, at higher C<sub>2</sub>-ceramide concentra-

**DISCUSSION**

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The DISC. C2-ceramide may act on the level of mitochondria resulting in interfering with the pathway upstream of mitochondria but downstream of only the type II cells Jurkat and CEM were sensitive to C2-ceramide. Indeed C2-ceramide was reported to directly affect mitochondria, playing a role in this form of apoptosis induction (52–54).

Mitochondrial involvement in the induction of apoptosis. In retrospect most of the reports on the apoptosis-inducing effects of ceramide analogs on lymphoid cells were obtained by using type II cells such as Jurkat, HL-60, or CEM (17, 34, 37, 51, 55–61). Incidentally, another lipid that was shown to counteract the effects of ceramide and to inhibit CD95-mediated apoptosis, sphingosine-1 phosphate, was also shown to be active in Jurkat T cells (17, 62).

Two publications on the function of ceramide in apoptosis signaling demonstrated that C2-ceramide inhibits the anti-apoptotic protein kinase B (Akt) (63, 64). This would place the action of ceramide upstream of mitochondria at the protein kinase B checkpoint (65). Consistent with this we did not see a direct effect when C2-ceramide was added to isolated mitochondria to induce DNA fragmentation of isolated nuclei (data not shown), a technique we have previously used to quantify the apoptogenic activity of mitochondria (13). A cytoplasmic component, perhaps protein kinase B, seems to be required that may then affect mitochondria. However, our data suggest that in type I cells a component downstream of the mitochondria may be missing that would allow the processing of caspase-3.

In contrast to the execution of death receptor-mediated apoptosis, which depends on preformed apoptosis signaling molecules, maintaining a state of resistance to apoptosis often requires de novo protein biosynthesis. Treatment with CHX will therefore often sensitize apoptosis resistant cells. This was shown for apoptosis-resistant human peripheral T cells (18). We have previously described the pre-B cell line BoeR, which is resistant to CD95-mediated apoptosis despite very high expression levels of CD95, FADD, and caspase-8. BoeR, however, can be sensitized by CHX. We found that this sensitization does not involve altered recruitment or activation of caspase-8 at the DISC. Instead, CHX affected a step downstream of the DISC but upstream of mitochondria. The data suggest that the postulated protein synthesis-dependent apoptosis resistance factor may only affect type II cells.

Type II cells show reduced DISC formation. Mitochondria in these cells may function as amplifiers activating caspase-9, caspase-3, and caspase-8 (Fig. 6). In type II cells activation of these caspases can be blocked by Bcl-2, and apoptosis sensitivity is strongly reduced. Likewise, PMA treatment reduces apoptosis sensitivity only in type II cells. Only type II cells are sensitive to C2-ceramide-induced apoptosis. Our data suggest that only cellular modulators that target the DISC will inhibit the CD95 pathway in both cell types. We have recently shown that the stable expression of c-FLIP in the type I cell BJAB blocks CD95-mediated apoptosis (21). We now confirm that in the type II cell line CEM, stably expressed c-FLIP also blocks CD95-mediated apoptosis by blocking caspase-8 processing at the DISC, consistent with the view that in both cell types CD95 signaling is initiated at the DISC by activation of caspase-8. Our data that c-FLIP only acts at the DISC level of both type I and type II cells are consistent with a recent report demonstrating that c-FLIP, when overexpressed in Jurkat cells, blocks CD95-mediated apoptosis but not apoptosis induced by reagents that activate caspases downstream of the DISC (66).

Our data indicate that the CD95 pathway in type I and II cells can be distinguished by overexpression of Bcl-2. In addition, a number of other reagents affect CD95-mediated apoptosis in type II cells could be assigned to interfere with the responses of our prototype cell lines (Fig. 6). Recently, a third CD95 apoptosis signaling type was proposed (67). Type I L929 cells triggered through CD95 and treated with caspase inhibi-
tors underwent a slow form of cell death, which has typical properties of necrosis such as the production of reactive oxygen radicals. This form of necrosis was designated type III cell death. Our data clearly suggest that strategies to interfere with caspases may not block cell death completely. So far the only cellular molecule that was shown to inhibit all death receptor-mediated apoptosis in all cells is c-FLIP. This molecule, however, needs to be expressed at levels high enough to block activation of caspase-8 at the DISC.

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