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Letter to the Editor

Differences between CD95 type I and type II cells detected with the CD95 ligand

Dear Editor,

CD95 (APO-1/Fas) is a member of the death receptor family. Members of this family are characterized by the presence of the death domain (DD) in their cytoplasmic tails. Upon triggering, CD95 assembles a structure of molecules which we have called the death-inducing signaling complex (DISC).[1] Oligomerized CD95 binds the adapter FADD/Mort1 that recruits the cysteine protease caspase-8 initiating the activation of a caspase cascade.[2,3] We have recently identified two different pathways of CD95-mediated apoptosis.[4] CD95-mediated apoptosis in type I cells is initiated by large amounts of active caspase-8 formed at the DISC followed by direct cleavage of caspase-3. In contrast, in type II cells little DISC is formed and small amounts of active caspase-8 stimulate the apoptogenic activity of mitochondria causing a profound activation of both caspase-8 and caspase-3. Activation of mitochondria was shown to be partially dependent on the BH3-domain-containing Bcl-2 family member BID.[5,6,7] BID is cleaved by caspase-8 *in vivo* and its cleavage fragment p15 then acquires an activity similar to the proapototic Bcl-2 family member Bax. Since type II cells depend on the mitochondrial branch of the pathway apoptosis in these cells can be blocked by overexpressed Bcl-2 or Bcl-x_L. We have recently demonstrated that type I and type II cells also differ in other properties, e.g. sensitivity to apoptosis induction by C_2-ceramide.[8]

We have initially proposed the 'two pathway' model by comparing a number of cell lines and by using the agonistic anti-CD95 antibody anti-APO-1. The different signaling pathways used by different tissues *in vivo* may be the result of an adaptation to the type of ligand encountered. Using the anti-CD95 antibody may therefore not reflect the physiological situation. We have tested whether type I and type II cells also respond differently when stimulated with the CD95 ligand (CD95L). First, we tested whether type II cells stimulated with CD95L would also be resistant to apoptosis by overexpression of Bcl-2 or Bcl-x_L. To this end highly active leucine zipper tagged CD95L (LZ-CD95L)[9] was compared to anti-APO-1 when added to CEM cells transfected with Bcl-x_L (Figure 1A,B). The results demonstrate that cells respond similarly when stimulated with anti-CD95 or CD95L. The two other Bcl-2/Bcl-x_L overexpressing cell lines recently characterized by using anti-APO-1 where also tested with the LZ-CD95L (Figure 1C,D).[4] Again the results were essentially identical to the results obtained with the anti-APO-1 mAb. The type II cell line Jurkat was partially inhibited by overexpression of Bcl-2 (Figure 1C) whereas the type I cell line SKW6 overexpressing Bcl-2 (note: the expression level of Bcl-2 is about ten times higher than in the Jurkat cells) was unaffected (Figure 1D).

We have used soluble CD95L in these experiments since in the human system it is not known where the membrane bound form of CD95L plays a role. Furthermore, it was shown that peripheral T cells dying by activation induced cell death through the CD95/CD95L system use, at least in part, soluble CD95L.[10]

We also found differences between type I and type II cells on the level of the DISC. When the anti-CD95 antibody was used for stimulation very little FADD could be coimmunoprecipitated with CD95 in type II cells despite similar amounts of CD95 immunoprecipitated and similar expression levels of FADD.[4] Again this finding could have been due to different properties of anti-CD95 *versus* CD95L. We therefore compared the amount of CD95 and FADD immunoprecipitated from the four prototype cell lines when stimulated with LZ-CD95L (Figure 1E,F). In this experiment cells were stimulated with LZ-CD95L and after lysis the stimulated CD95 receptor was immunoprecipitated with anti-CD95. In all cells comparable amounts of CD95 was immunoprecipitated (Figure 1E). Without stimulation no FADD was associated (Figure 1F, lanes 1, 3, 5, 7). Only in stimulated type I cells significant amounts of FADD were immunoprecipitated (Figure 1F, lanes 2, 4) whereas in type II cells the amount of FADD associated with the stimulated receptor was very small (Figure 1F, lanes 6, 8). This situation is similar to the results obtained with the anti-APO-1 antibody and confirm that formation of the DISC in type II cells is indeed impaired. It is not clear what regulates differential association of FADD with CD95 in type I and type II cells.

Although we have identified the two different CD95 apoptosis signaling pathways by comparing tumor cell lines accumulating evidence suggests that the type I/type II distinction also exists in normal cells *in vivo*. A number of transgenic mice expressing Bcl-2 or Bcl-x_L in different tissues already suggested that the role of mitochondria in the CD95 signaling pathway may be dependent on the tissue. Peripheral T cells have been shown to be unaffected by overexpression of Bcl-2.[11] They can therefore be classified as type I. In contrast hepatocytes expressing Bcl-2 as a transgene have been shown to be significantly resistant towards CD95 mediated apoptosis.[12,13] We had therefore predicted that hepatocytes are type II cells.[4] Recent data on BID deficient mice have confirmed that BID, and therefore the type II pathway, is indeed important in hepatocytes.[14] A number of other knock out data on the role of Apaf-1, caspase-3 and caspase-9 also suggested that depending on the tissue cells utilize apoptosis pathways that are more or less dependent on mitochondrial activity (for review see[15]). Our data are in line with these findings. Future experiments will be directed to determine why and under which circumstances cells use one or the other CD95 signaling pathway.

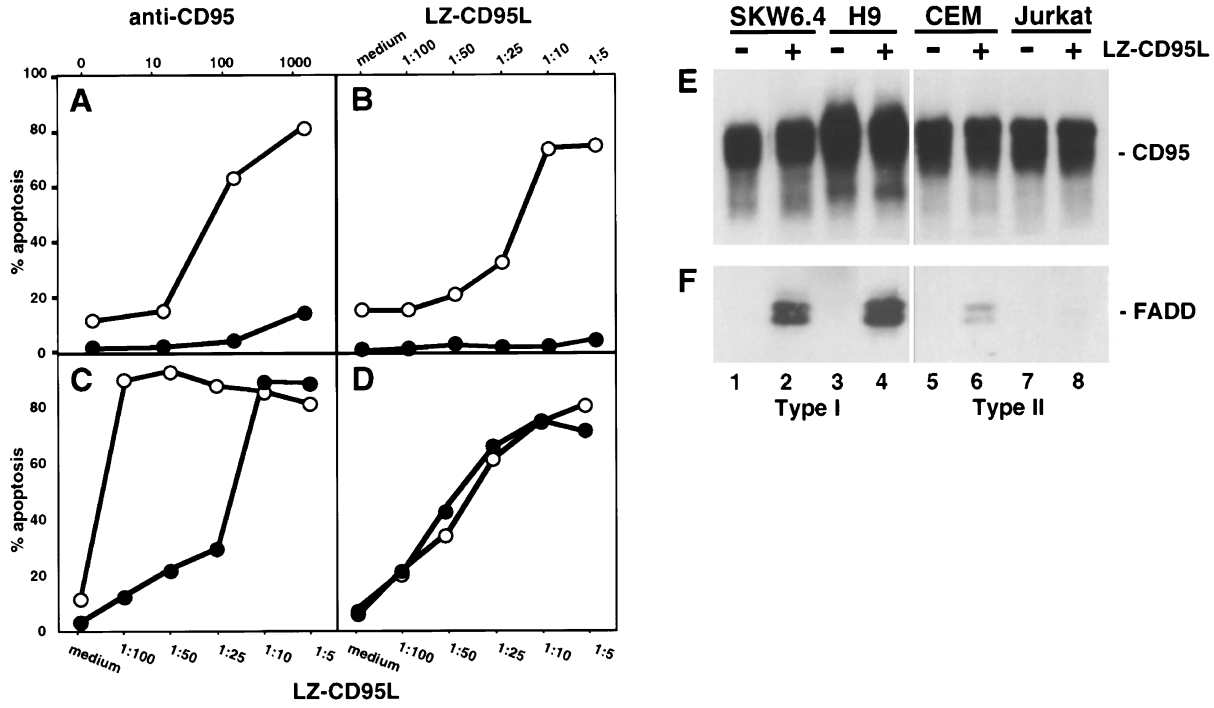

Figure 1 Type I and type II cell lines overexpressing Bcl-2/Bcl-x_L differ in their apoptosis sensitivity when stimulated with either anti-CD95 or CD95 ligand. (**A – D**) Type II cells overexpressing Bcl-2 or Bcl-x_L are rendered resistant to apoptosis induced by leucine zipper tagged CD95 ligand (LZ-CD95L). Cells were cultured as described[4] and incubated for 18 h with the indicated amounts of anti-CD95 or LZ-CD95L, respectively.[4,9] LZ-CD95L was produced as cell culture supernatant of CV1-EBNA cells transiently transfected with an expression construct for LZ-CD95L as decribed.[9] Indicated dilutions of this supernatant were used [note: a 1 : 100 dilution results in half maximal apoptosis of $5 \bullet 10^5$ untransfected Jurkat cells (clone J16)]. Subsequently apoptosis was quantified by staining nuclei with propidium iodide as described.[4] (**A,B**) CEM neo and CEM Bcl-x_L, (**C**) Jurkat neo and Jurkat Bcl-2, (**D**) SKW6 neo and SKW6 Bcl2. (○——○) control transfectants, (●——●) Bcl-2 or Bcl-x_L transfectants, respectively. (**E,F**) Reduced recruitment of FADD to the DISC of type II cell lines as compared to the type I cell lines upon stimulation with LZ-CD95L. Cells were left untreated (−) or stimulated with 1 ml LZ-CD95L supernatant (+) for 5 min at 37°C before lysis and immunoprecipiation with anti-CD95 as described.[4] Subsequently immunoprecipitates were resolved by 12% SDS – PAGE, transferred to nitrocellulose and immunoblotted with (**E**) anti-CD95 (C-20, Santa Cruz, CA) and (**F**) anti-FADD (Transduction Laboratories, Lexington, Kentucky), respectively[4]

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