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Characterization of Apt- cell lines exhibiting cross-resistance to glucocorticoid- and Fas-mediated apoptosis

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Abstract

Apoptosis induction by staurosporine, ceramide, and Fas stimulation was investigated in the mouse thymoma cell line W7.2 and a panel of dexamethasone (dex)-resistant W7.2 mutant cell lines, Apt3.8, Apt4.8 and Apt5.8, and a Bcl-2 transfected W7.2 cell line (Wbcl2). While W7.2 cells were found to be sensitive to these apoptosis inducers, the Apt- mutants and Wbcl2 cells were shown to be resistant to some or all of the treatments. Specifically, all three Apt- mutants and Wbcl2 cells were found to be resistant to ceramide and Fas-mediated apoptosis, whereas, Apt4.8 and Apt5.8 were sensitive to staurosporine-induced apoptosis under conditions in which Apt3.8 and Wbcl2 cells were resistant. Measurements of caspase activity and cytochrome c release in cytosolic extracts of dex and staurosporine-treated cells indicated that the recessive Apt- mutations effect steps upstream of mitochondrial dysfunction. Steady-state RNA levels of apoptosis-associated gene transcripts showed that the observed differential resistance of the Apt- cell lines could not be explained by altered expression of numerous Bcl-2 or Fas related genes. Transient transfection of human Fas gene coding sequences into the Apt- mutants and Wbcl2 cells did not induce apoptosis, even though these same cell lines were sensitive to ectopic expression of the FADD and caspase 8 genes. Taken together, these data provide genetic evidence for the existence of shared components in the dex- and Fas-mediated apoptotic pathways in W7.2 cells.

Keywords: apoptosis; thymocytes; glucocorticoids; Fas; Bcl-2; staurosporine

Abbreviations: Apt-, apoptosis-defective; C2, N-acetyl-D-sphingosine; dex, dexamethasone; DMEM, Dulbecco's Modified Eagles Media; GAPDH, glyceraldehydephosphate dehydrogenase; GFP, green fluorescent protein; GR, glucocorticoid receptor; PI, propidium iodide; RPA, RNase Protection Assay; STS, staurosporine; W7.2, WEH17.2

Introduction

The signal transduction pathways involved in apoptotic induction are both diverse and complex, and include membrane receptor activated signaling cascades,[1] mediators of cellular stress signals,[2] and gene transcription.[3–5] It has been proposed that mitochondrial dysfunction and subsequent release of mitochondrial components represent the cellular 'point of no return' in the apoptotic pathway.[6] Alternative hypotheses invoke the activation of cysteine proteases, known as caspases, as the commitment step in the conserved apoptotic pathway.[7] Either way, control of some critical step in most apoptotic pathways has been shown to involve the interplay between various apoptosis regulatory proteins.[8,9] The well-characterized Bcl-2 family of structurally-related proteins is one class of apoptosis regulatory protein.[10] Bcl-2 related proteins have been shown to exert their regulatory function upstream of events leading to release of cytochrome c from mitochondria.[11,12] The execution phase of apoptosis is broadly defined as a period in which effector caspases are activated by proteolytic cleavage resulting in selective degradation of target proteins.[13] Numerous caspase substrates have been identified, and while some have been shown to be critical for maintaining cellular homeostasis,[14–16] others include proteins that modulate mitochondrial dysfunction.[17,18]

Glucocorticoids are potent inducers of apoptosis in immature thymocytes,[19] and glucocorticoid treatment of human leukemias and lymphomas is an efficacious therapy that exploits the apoptotic-inducing function of this steroid.[20,21] Thymocytes also undergo apoptosis in response to activation of the Fas receptor, UV irradiation, ceramide analogues, staurosporine and growth factor deprivation.[22] While many of these apoptotic inducers promote cell death in a wide variety of cell types, glucocorticoids uniquely regulate apoptosis in only a limited subset of lymphoid and hematopoietic cells. In cells with low levels of Bcl-2, treatment of thymocytes or T cell lines with dexamethasone (dex) leads to loss of mitochondrial function, caspase activation and classic apoptotic cell morphology.[22] Studies in murine lymphoma cell lines and transgenic mice, have shown a requirement for glucocorticoid receptor (GR) transcriptional activation functions.[5,23,24] In contrast, results from dex-regulation studies of apoptosis in human T cell lines suggest that transcriptional inhibitory functions of GR are the most critical.[25,26] It is likely that both of these GR regulatory functions are required.

We are focusing our efforts on understanding GR-signaling events required for dex-induction of apoptosis in the murine thymoma cell line WEH17.2 (W7.2). One approach we have taken is to isolate and characterize a panel of dex-resistant cell lines that were derived from W7.2 cells following mutagenesis with ethyl methanesulfonate.[27] Somatic cell genetic analyses revealed that three of these dex-resistant cell lines, Apt3.8, Apt4.8 and Apt5.8, represent distinct complementation groups containing phenotypically recessive mutations in genes required for apoptosis induced by dex, γ irradiation and hydrogen peroxide.[27] To better understand early events in thymocyte apoptosis that may be shared between different signaling pathways, we have now investigated the sensitivity of these Apt- mutants to Fas activation, and treatment with ceramide and staurosporine. We found that Apt4.8 and Apt5.8, but not Apt3.8, were sensitive to staurosporine-induced apoptosis, whereas, alll three Apt- mutants were shown to be resistant to ceramide- and Fas-mediated apoptosis. Interpretations of these data are discussed in the context of current models of apoptosis signaling.

Results

Differential resistance of Apt- mutants to ceramide and staurosporine

We have previously shown that W7.2 cells undergo classic thymocyte apoptosis in response to dex-treatment as analyzed by electron microscopy,[27] DNA laddering[28] and trypan blue exclusion.[29] In the present study we used a quantitative apoptotic assay based on FACS analysis.[62] Figure 1 shows FACS analysis and fluorescent microscopy results following treatment of W7.2 cells with 100 nM dex for 24 h. Under these conditions, greater than 70% of the W7.2 cells treated with dex exhibit a condensed and propidium-iodide (PI)-positive apoptotic morphology as compared to $<10\%$ of the cells cultured in the absence of hormone. To determine if W7.2 cells were sensitive to other known apoptotic inducers, we used the same assays to examine cells following treatment with the ceramide analog N-acetyl-D-sphingosine (C2), and the kinase inhibitor staurosporine. As can be seen in Figure 1, W7.2 cells exhibit the same features of dex-induced apoptosis following treatment with 60 μM C2 and 30 nM staurosporine.

Based on these results, we next analyzed apoptosis in three dex-resistant W7.2-derived Apt- mutant cell lines,[27] and a W7.2 cell line, referred to here as Wbcl2, that ectopically expresses moderate levels of the human Bcl2 anti-apoptotic protein.[59] Figure 2 shows the dose response profiles of dex, C2 and staurosporine treatment of W7.2 and Wbcl2 cells. The data demonstrate that Wbcl2 cells exhibit an apoptosis-resistant phenotype following a 24 h treatment with 100 nM dex, 60 μM C2 and 100 nM staurosporine. These results are consistent with the known protective effects of Bcl2 in cultured cells treated with glucocorticoids,[27,30,31] ceramide analogs[32,33] and staurosporine.[34] The sensitivity of each Apt- cell line was then analyzed using the same concentrations of each inducer. We found that Apt3.8, Apt4.8 and Apt5.8 were all resistant

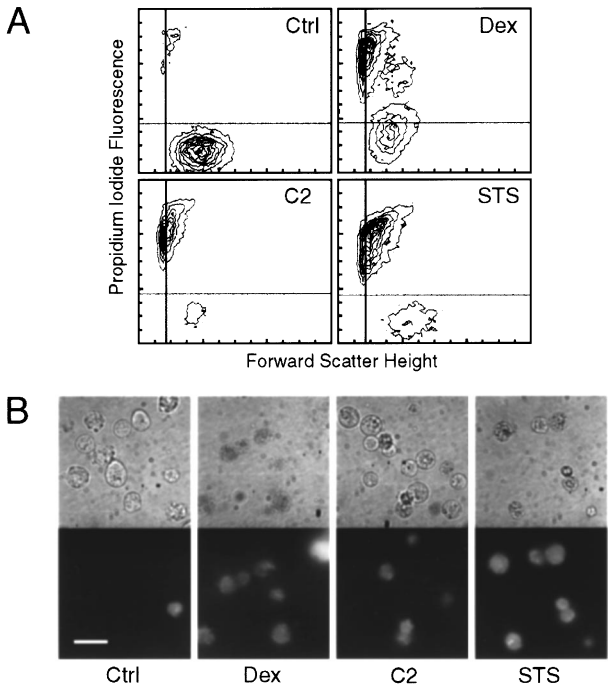

Figure 1 Induction of apoptosis in the thymocyte cell line W7.2. (**A**) Flow cytometry analysis of propidium iodine stained cells in media alone (Ctrl), 100 nM dexamethasone (dex), 60 μM N-Acetyl-D-sphingosine (C2), or 30 nM staurosporine (STS). (**B**) Phase contrast and fluorescent images of propidium iodine stained cells following treatments as in (**A**). The white magnification bar represents a length of 5 microns

to the apoptotic effects of C2. Apt4.8 and Apt5.8 exhibited a level of C2-resistance that was similar to Wbcl2, while Apt3.8 was found to be completely resistant to 60 μM C2.

When we treated the Apt- mutants with 100 nM staurosporine for 24 h, we found that Apt4.8 and Apt5.8 were relatively staurosporine-sensitive, exhibiting 64 and 54% apoptosis, respectively (Figure 2). However, Apt3.8 was as staurosporine-resistant as Wbcl2 and exhibited only 13% apoptosis under these same conditions. The staurosporine-resistant phenotype of Apt3.8 differentiates this cell line from Apt4.8 and Apt5.8, and suggests that the Apt3.8 mutation effects a component that may be common to a variety of apoptotic pathways.

In addition to the observed cross-resistance of the Apt cell lines to ceramide, and partial resistance to staurosporine, these same variant cell lines have previously been shown to be resistant to apoptotic-induction by cyclic AMP, γ irradiation and hydrogen peroxide.[27] Based on these results, we reasoned that lack of pro-apoptotic gene functions, for example, altered expression of Bax, Bad, or Bak genes,[35] could explain the cross-resistant phenotype. To examine this possibility, we used a multi-probe RNase protection assay (RPA) to determine the steady state level of RNAs expressed from seven Bcl2 family members in dex-treated cells (1 μM dex for 12 h).

The RPA results from a representative gel are shown in Figure 3. These data reveal that the steady-state levels of Bak, Bax and Bad RNA are equivalent in all five cell lines and not effected by dex treatment. Although no differences

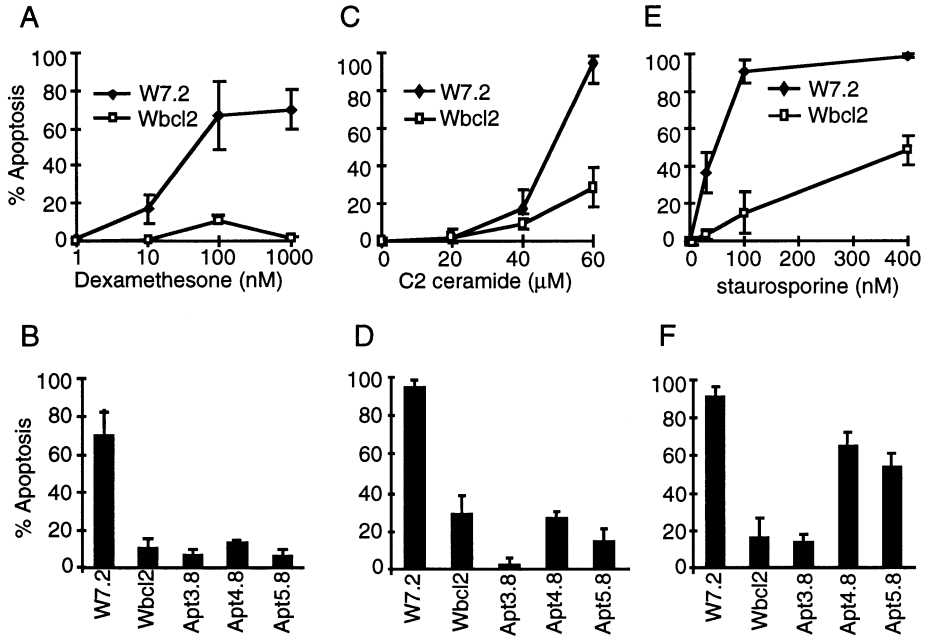

Figure 2 Apoptosis-deficient phenotype of W7.2 variant cell lines. (**A**, **C**, **E**) Dose-dependent induction of apoptosis by (**A**) dex, (**C**) C2 ceramide, or (**E**) staurosporine (STS) in W7.2 (closed diamonds) and Wbcl2 (open squares). Apoptosis was measured in 24 h cultures containing 1 nM to 1 μM dex by PI uptake and plotted as percent apoptosis compared to untreated control samples. (**B**, **D**, **F**) Mean level of apoptosis in the W7.2-derived cell lines treated with (**b**) 100 nM dex, (**D**) 60 μM C2 ceramide, or (**E**) 100 nM staurosporine (STS) for 24 h

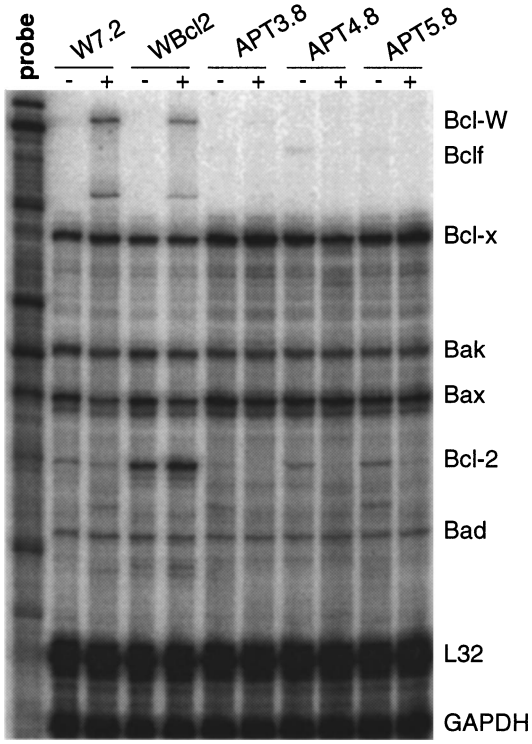

Figure 3 RNase Protection Assay of Bcl-2 related transcript levels. Expression of Bcl-2 related gene transcripts was compared between W7.2, Wbcl2, and the Apt- cell lines, in the absence (−) or presence of 1 μM dex for 12 h (+). L32 and GAPDH are loading controls

were found in the level of Bcl-x RNA across the cell lines, a dex-dependent decrease in Bcl-2 RNA levels was observed in W7.2 and the Apt- cells. Bclf transcripts were not reproducibly detected in any of the cell lines. Note that Wbcl2 cells do contain elevated levels of human Bcl-2 RNA as expected.[59] Interestingly, an increase in Bcl-w RNA was seen in response to dex treatment in W7.2 and Wbcl2 cells, however, expression of this anti-apoptotic gene was not observed in the Apt-lines.

Apt3.8 mutation is upstream of caspase activation pathway

Induction of apoptosis by staurosporine is associated with activation of multiple caspases.[34,36] To determine if the observed staurosporine-resistance of Apt3.8 cells is due to defects upstream of caspase activation, we measured caspase activity in extracts prepared from dex- and staurosporine-treated cells using an *in vitro* substrate cleavage assay. The results shown in Figure 4 were obtained with a cleavage assay using the baculovirus apoptosis inhibitor protein p35 as the radioactively-labeled target protein.[37,38] The baculovirus p35 protein is cleaved by caspases that recognize the tetrapeptide sequence, DEVD. Data in Figure 4A reveal that cell extracts prepared from dex-treated W7.2 cells contain caspase activity based on the expected *in vitro* cleavage of p35 protein into polypeptides of 25 and 10 Kd fragments. In contrast, cell extracts from dex-treated Wbcl2, Apt3.8, Apt4.8, and Apt5.8 cells, contained only very low levels of caspase activity (Figure 4A and Table

1). Staurosporine-dependent p35-cleaving activity was detected in cell extracts prepared from Apt4.8 and Apt5.8 cells, but not in extracts from Apt3.8 or Wbcl2 cells (Figure 4B). Table 1 summarizes the results from the p35 cleavage assays.

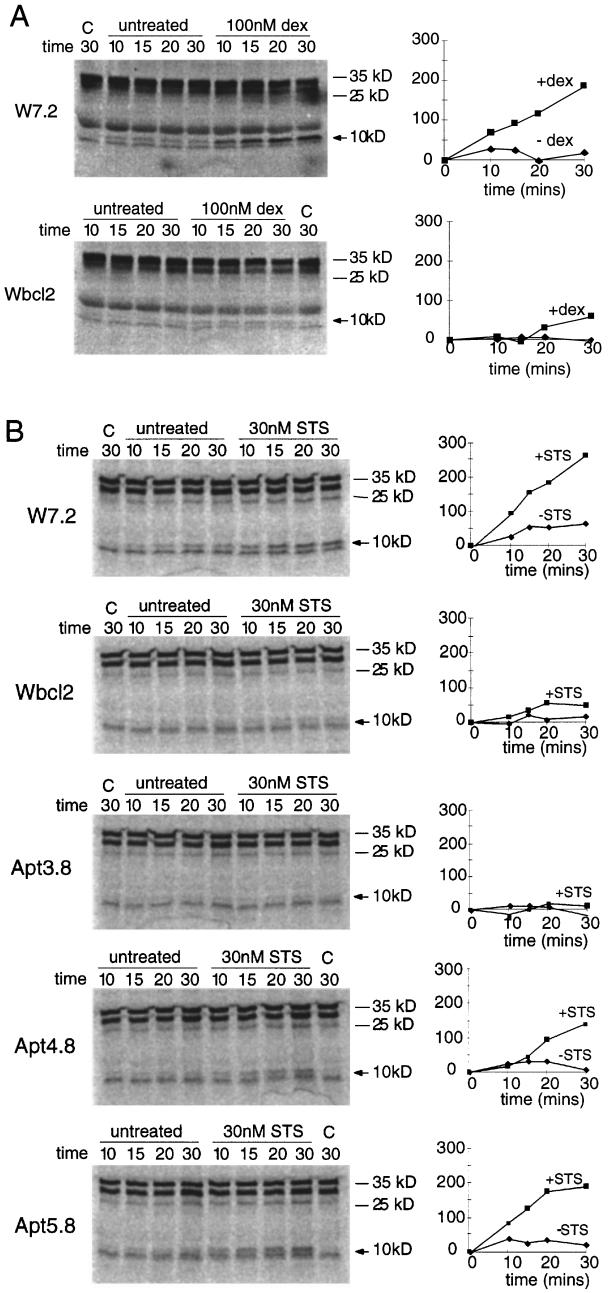

Figure 4 Caspase activation by dex and staurosporine (STS) in W7.2, Wbcl2, Apt3.8, Apt4.8 and Apt5.8 cells. ^{35}S-labeled p35 substrate was incubated with extracts made from untreated, dex-treated, or STS-treated cells, and the relative amount of 10 kDA product was quantitated and plotted as a function of reaction time. (**A**) Caspase activity in extracts from dex-treated W7.2 and Wbcl2 cells. (**B**) Caspase activity in extracts from staurosporine-treated cells. Additional protein bands present in every lane correspond to *in vitro* polypeptide products derived from translational initiation at internal methionine residues

The level of apoptosis in staurosporine- and dex-treated cells correlates with the amount of caspase activity in corresponding extracts. Specifically, Apt4.8 and Apt5.8, which were shown to be relatively sensitive to staurosporine (Figure 2), were also found to have high levels of caspase activity (Table 1). In contrast, Apt3.8 and Wbcl2 are both staurosporine-resistant and lack staurosporine-induced caspase activity. Similar results were obtained using a caspase activity assay based on cleavage of DEVD tetrapeptide fluorogenic substrate as measured by spectroscopy (M DeBoer and D Askew, unpublished data).

Based on the finding that cytochrome c release from mitochondria is associated with the commitment step in the apoptotic pathway,[6] and that STS treatment distinguishes between the Apt- phenotypes (Table 1), we examined cytosolic extracts from STS-treated cells to determine if the defect in Apt3.8 is upstream or downstream of cytochrome c release. The data in Figure 5 shows that while STS treatment induced high levels of cytochrome c release in STS-sensitive cells (W7.2, Apt4.8, Apt5.8), only a low level of cytochrome c was detected in the cytosol of STS-treated Apt3.8 cells.

Apt- cells are resistant to Fas-mediated killing

The role of ceramide signaling and sphingomyelinase activation in Fas-mediated apoptosis is controversial.[39–42] Moreover, the relationship between apoptosis-induction by treatment with ceramide analogs, and intracellular ceramide production resulting from Fas activation, is unclear.[43,41] Since the Apt- cells were found to be resistant to treatment with C2 ceramide, we wanted to determine if they were sensitive to Fas-mediated killing. Using an anti-Fas monoclonal antibody to activate the Fas pathway, we found that W7.2 cells underwent apoptosis following addition of 100 and 200 ng/ml anti-Fas mAb to the culture (Figure 6). In contrast, Wbcl2 cells were shown to be resistant to anti-Fas mAb at the highest doses, indicating that the W7.2 cell background supports the anti-apoptotic function of Bcl-2.[45] Treatment of Apt3.8, Apt4.8 and Apt5.8 with 200 ng/ml of anti-Fas antibody revealed that these Apt- mutants were resistant to Fas antibody-induced apoptosis (Figure 6). Fas-mediated killing in the Apt- cell lines was also measured by a co-culturing assay based on FasL-signaling and quantitative DNA fragmentation measurements.[46] The data shown in Figure 6C confirm that W7.2 cells are sensitive to Fas-induced apoptosis, whereas, Apt3.8, Apt4.8, Apt5.8 and Wbcl2 cells are markedly resistant.

Table 1 Summary of cross-resistance and caspase activation phenotypes

	% Apoptosis			Caspase activity		
Cell line	**Control**	**+dex**	**+STS**	**Control**	**+dex**	**+STS**
W7.2	13.8	70.3	90.3	0.7	5.3	6.8
Wbcl2	12.3	23.6	15.1	0.4	0.9	1.4
Apt3.8	15.3	20.6	13.2	0.3	1.0	1.0
Apt4.8	11.5	23.1	64.3	0.7	1.1	3.5
Apt5.8	17.5	23.0	53.7	0.8	0.6	6.7

PI uptake from Figure 2 is shown as the mean value for per cent apoptosis. The mean values for relative caspase activity (10 kDa product formed/min/μg protein) are based on p35 cleavage assays from Figure 4

The isolation of Fas-resistant cell lines in culture can sometimes be attributed to loss of Fas protein expression.[43,44] We therefore analyzed Fas protein levels using Western blot analysis. The data in Figure 7 demonstrate that Fas protein levels are equivalent in all five cell lines, suggesting that Fas-resistance in Apt3.8, Apt4.8 and Apt5.8, is not due to loss of Fas. Similar results were obtained using immunofluorescent flow cytometry to measure Fas antigen at the cell surface (data not shown). In addition, a multi-probe RNase protection assay was used to compare the steady-state RNA level of gene transcripts corresponding to known components of the Fas signaling pathway (Figure 8). Fas RNA levels were found to be similar amongst all the cell lines. Moreover, FLICE (Caspase-8), FasL, Fas, FADD, FAF, Fas2L, TNFRp55, TRADD, and RIP RNA levels were expressed at equivalent levels and not regulated by dex treatment. FAP RNA levels appear to decrease in Wbcl2 cells following dex treatment. The significance of this observation is not known.

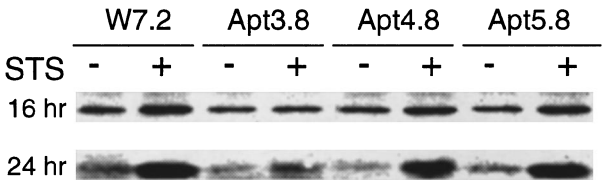

Figure 5 Staurosporine-induced cytochrome c release in cell extracts from W7.2 and Apt- cells. Cells were untreated (−) or treated (+) with 200 nM staurosporine (STS) for 16 or 24 h and cytosolic extracts were prepared as described.[64] Equal amounts of protein were loaded into each lane, electrophoresed, and immunoblotted with anti-cytochrome c antibody

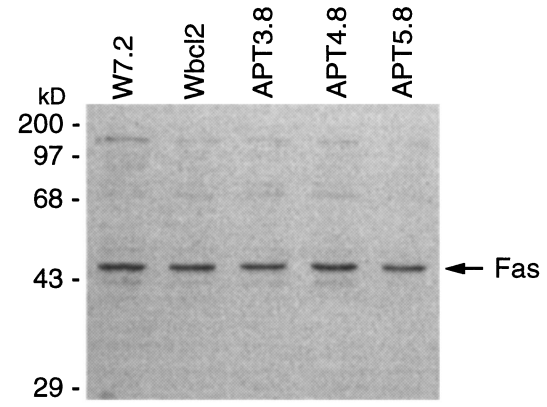

Figure 7 Fas protein expression is normal in Fas-resistant Wbcl2 and Apt- cell lines compared to W7.2 (parent). Western blot of total cell extracts (50 μg/lane) using anti-Fas mAb

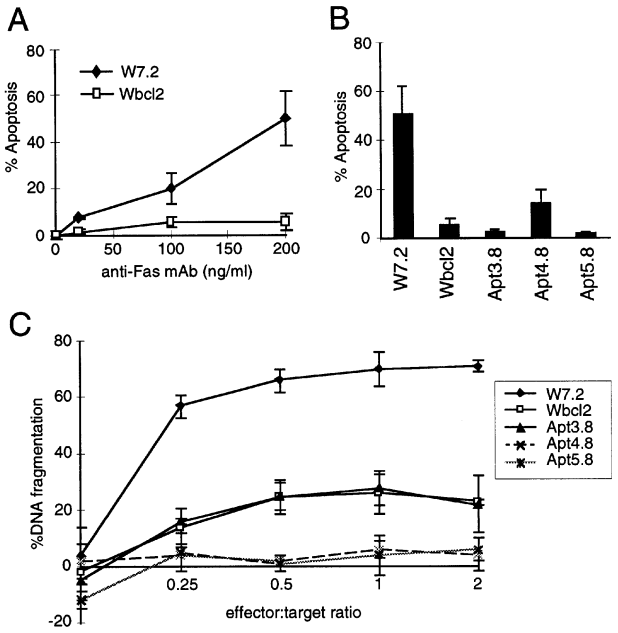

Figure 6 The Apt- and Wbcl2 cell lines are resistant to Fas-dependent apoptosis. (**A**) Dose-dependent induction of apoptosis by anti-Fas mAb in W7.2 (closed diamonds) and Wbcl2 (open squares). Apoptosis was measured in 24 h cultures containing 20 ng, 100 ng, and 200 ng/ml anti-Fas mAb by PI uptake and flow cytometry. (**B**) Comparison of apoptosis induction by 200 ng/ml soluble anti-Fas mAb for 24 h between W7.2, Wbcl2, and Apt- cell lines. (**C**) Induction of DNA fragmentation W7.2-derived target cells following exposure to FasL expressing activated A1.1 cells. Target cells for each experiment are listed in the key and the A1.1 effector : target cell ratios are indicated

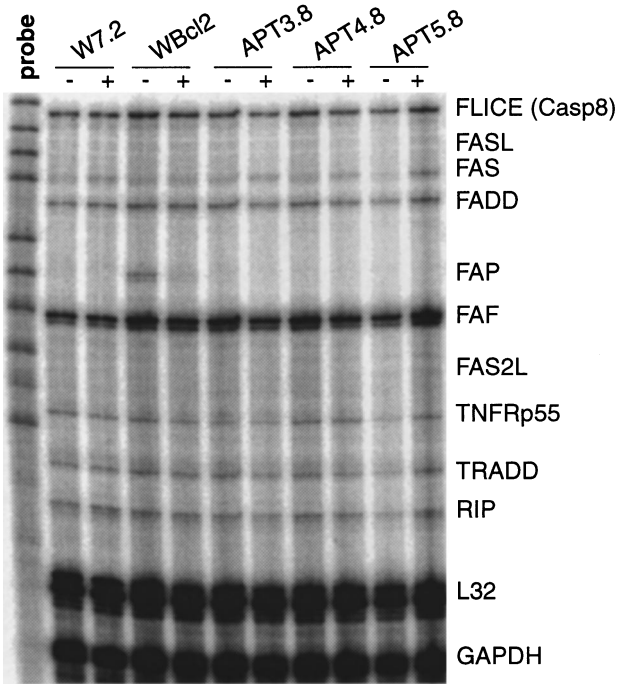

Figure 8 RNase Protection Assay of Fas and TNFR signaling gene transcript levels. Expression of Fas and TNFR signaling gene transcripts were compared between W7.2, Wbcl2, and the Apt- cell lines, in the absence (−) or presence of 1 μM dex for 12 h. L32 and GAPDH are loading controls

Functional analysis of Fas-signaling in the Apt- cells

Although Fas protein and RNA are expressed at normal levels in the Apt- cell lines (Figures 7 and 8, respectively), it was possible that the Fas-resistant phenotype could be due to mutations that affect Fas function. Therefore, we used a transient transfection assay based on co-expression of the green fluorescent protein (GFP) as a cell marker. This assay had previously been used to show that ectopic expression of the human Fas (hFas) gene in transfected murine cells causes induction of the apoptotic pathway.[47] For these experiments, we transfected the wt W7.2, Wbcl2 and Apt- cell lines with an hFas expression plasmid, and quantitated the number of GFP+ cells by FACS analysis. Results shown in Figure 9 reveal that W7.2 cells are Fas-sensitive in this assay since 70% fewer GFP+, PI− cells could be identified when the expression vector encoded hFas cDNA, as compared to the empty vector control. Consistent with the results obtained from Fas activation assays (Figure 6), Wbcl2 and the three Apt- mutants were found to be relatively resistant to ectopic expression of hFas. Interestingly, of the three Apt- cell lines, Apt4.8 is the most sensitive to Fas-mediated apoptosis under both assay conditions (compare Figures 6b and 9A).

We next used the transient GFP co-transfection assay to measure the sensitivity of the Apt- cell lines to ectopic FADD and caspase 8 expression, two signaling molecules known to be downstream of Fas.[1] The data in Figure 9B show that all five cell lines were equally sensitive to both FADD and caspase 8 expression in this assay. These results suggest that the cellular components needed for direct activation of caspase 3 by caspase 8, are functional in the Apt- cell lines. The sensitivity of Wbcl2 cells to FADD and caspase 8 expression in this assay reflects the utilization of a mitochondria-independent caspase 3 activation mechanism.[45]

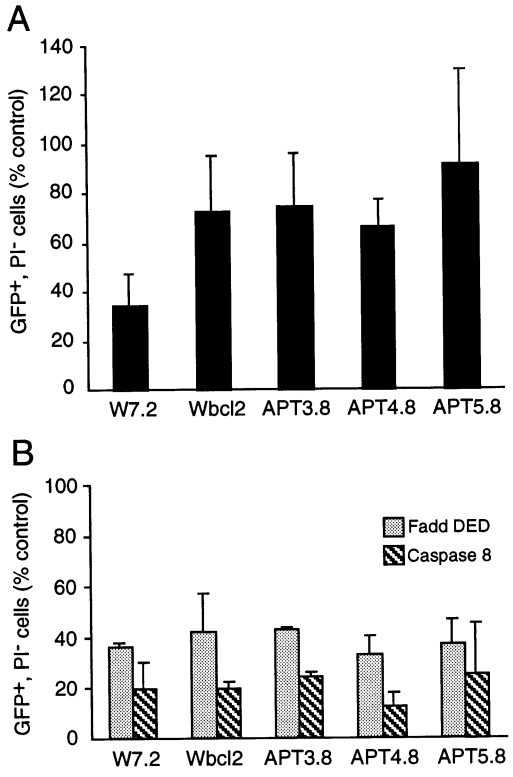

Figure 9 Apoptosis induced by ectopic expression of Fas signaling components. (**A**) Cell viability 24 h after co-transfection with a human Fas (hFas) expression vector (pDCPur.hFas) and green fluorescent protein (GFP) marker gene (pGFPemd). Per cent viable cells was measured by quantitation of non-apoptotic, GFP+propidium iodine-negative (PI−) cells in the hFas transfections compared to control transfections using pGFPemd +pDCNeo.βgal. (**B**) Apoptosis induction in Apt- cells by the Death Effector Domain fusion proteins GFP-FADD1-79 and GFP-FLICE1-120[65] was measured by quantitation of GFP+, PI− cells as in (**A**). Control transfections contained pEGFP-C1 only

Discussion

Mitochondrial dysfunction and caspase activation are two apoptotic events that have been identified as markers of irreversible cell death.[48] Based on studies aimed at clarifying the role of specific caspases in Fas-mediated apoptosis, it has been proposed that there are at least two Fas signaling pathways that predominate in different types of cells (Type I and Type II cells).[45] The majority of Fas-sensitive cells are Type I cells in which Fas activation leads to the FADD-mediated stimulation of caspase 8 autocleavage.[1] This initial activation step is rapidly followed by caspase 8 cleavage of the major effector caspases, such as caspase 3. In Type II cells, caspase 8 is thought to be limiting or inefficient,[45] resulting in a greater reliance on mitochondrial involvement through BID-mediated stimulation of cytochrome c release.[18,49] Once this occurs, pro-caspase 9 is cleaved as part of the apoptosome complex leading to caspase 3 cleavage and activation.[48]

One distinction between Type I and Type II cells is the ability of Bcl-2 to function as an anti-apoptotic protein based on the mitochondrial dependence of each pathway; Bcl-2 expression protects Type II, but not Type I, cells. Based on our Fas-activation results comparing W7.2 and Wbcl2 cells, we predict that the W7.2 cell line is most like the human T cell lines Jurkat and CEM, both of which are classified as Type II cells.[45] Most apoptotic pathways that do not involve the Fas/TNFR membrane receptors, for example, those stimulated by dex, ceramide, and staurosporine, are characterized by mitochondrial dysfunction leading to cytochrome c release and subsequent caspase 9 and caspase 3 activation.[50]

Although we do not yet know the molecular basis of the Apt- phenotypes of Apt3.8, Apt4.8 or Apt5.8, we have preliminarily ruled out mutations in a number of apoptosis-related gene candidates on the basis of RNA expression. Interestingly, low level induction of Bclw transcription was found in W7.2 and Wbcl2 cells, but not in the Apt- cell lines (Figure 3). This result may suggest that early events in the dex-induced pathway lead to elevation of Bcl-w gene expression, similar to what has been observed with the glutathione S-

transferase gene.[29] These early events may not be fully functional in the Apt- mutants. We also found that the RNA levels of numerous Fas-related signaling proteins, including caspase 8, were equivalent in all three Apt- cell lines (Figure 8). In addition, based on a recent report by Juo *et al*,[51] showing that a Fas-resistant Jurkat cell variant had a defect in caspase 8 expression, we used RPA to examine RNA levels of caspase 3, 6, 11, 12, 2, 7 and 1, however, no differences were found (data not shown). Moreover, dex-treatment had no effect on the expression level of any of the Bcl-2 (Figure 3), Fas (Figure 5), or caspase (data not shown) family member genes in the wild-type or Apt- W7.2 cells.

Staurosporine-induced apoptosis has been shown to induce cytochrome c release from the mitochondria[52] and caspase activation[53] through a mechanism that partially depends on Apaf-1, the mammalian CED-4 homologue.[54,55] Since the Apt4.8 and Apt5.8 mutants are staurosporine-sensitive, and release mitochondrial cytochrome c in response to staurosporine treatment, they would appear to each have a defect in early steps in the mitochondrial pathway. Based on our data, we propose that the early events defined by defects in Apt4.8 and Apt5.8 must be downstream of a convergence step required for dex-, ceramide- and Fas-mediated apoptosis. However, since Apt4.8 and Apt5.8 cells are staurosporine-sensitive, these Apt- mutations appear to effect apoptotic signaling events that are upstream of the staurosporine-initiated signal in the pathway. One possibility is that Apt4.8 and Apt5.8 lack proteins required for the initiation of mitochondrial dysfunction, for example, they could have defects in apoptotic BH3 domain containing signaling proteins similar to BID[56] or a homologue of the *C. elegans* EGL-1 gene.[57] Apt3.8, on the other hand, is staurosporine-resistant, as is the Wbcl2 cell line, and therefore may be defective in a protein required to modulate cytochrome c release in response to an upstream signal.

Initially, the observed cross-resistance of the Apt- mutants to dex and Fas was puzzling because most Fas-sensitive cells are of the Type I variety, and it was difficult to imagine how GR-regulated transcriptional events would be required in a rapid caspase 8→caspase 3 activation pathway. Recent identification of mitochondria-dependent Fas-activated pathway in the human T cell lines, Jurkat and CEM (Type II cells),[45] suggested that the Apt- mutations could disrupt mitochondria-associated events that occur downstream of BID cleavage in W7.2 cells. The observation that transiently transfected Apt- cells were killed by FADD and caspase 8 cDNA expression (Figure 9), could be explained by ectopic activation of the caspase 8→caspase 3 pathway, thus inducing apoptosis by circumventing the Apt- defects. Since Bcl-2 expression cannot block this caspase 8→caspase 3 pathway[58] this would explain the FADD and caspase 8 sensitivity of transfected Wbcl2 cells (Figure 9). An alternative explanation is that the high level expression of FADD and caspase 8 in transfected cells completely overwhelms the defect in Fas-activated Apt- cells.

Materials and Methods

Cell culture

W7.2, Wbcl2 (formerly called WHb.12),[59] Apt3.8, Apt4.8 and Apt5.8 were grown in DMEM supplemented with 10% charcoal-stripped, heat-inactivated calf bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C, 90% humidity, and 8% CO_2 as previously described.[60] Dexamethasone (Sigma), staurosporine (Sigma), N-acetyl-D-sphingosine (C2 ceramide) (Sigma), and anti-mouse Fas mAb (clone RMF8, Immunotech), were added directly to cultures at the concentrations described in the text.

Quantitation of Fas ligand-dependent apoptosis using a co-culturing method with anti-CD3-activated, Fas ligand-expressing A1.1 hybridoma cells was done as described.[61] Briefly, ^{3}H-thymidine-labeled target cells were combined with A1.1 cells and plated in untreated or anti-CD3-coated 96-well plates and incubated at 37°C for 8 h. Unfragmented, labeled high molecular weight DNA was collected by filtration through glass fiber filters (Scatron) and counted in a liquid scintillation counter (Pharmacia). Data are expressed as per cent DNA fragmentation: $100 \times (1 - \text{c.p.m. in experimental group per c.p.m. of untreated targets})$.

Propidium iodide staining

Cell cultures were inoculated at 2×10^5 cells/ml in 24-well plates, after 24 h, harvested, washed once with 15 ml PD buffer (13.7 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4 8.1 mM Na_2HPO_4) and then resuspended in 0.5 ml PD buffer. Propidium iodide (PI) was added to 5 μg/ml immediately before analyzing 10 000 cells/sample with a FACScan flow cytometer (Becton Dickinson) using the rhodamine channel to detect PI fluorescence. The number of viable and apoptotic cells were determined by plotting the flow cytometry data as forward scatter versus PI fluorescence as described.[62] Morphology of PI-stained cells was analyzed by phase contrast and fluorescent microscopy using a 100× oil-immersion lens.

Caspase cleavage assays

Cell extracts were prepared according to Chow *et al*.[63] Briefly, 10^8 cells were harvested, washed once in 50 mls PD buffer and resuspended in 250 μls S-buffer (50 mM NaCl, 40 mM β-glycophosphate, 10 mM HEPES pH 7.0, 5 mM EGTA, 2 mM $MgCl_2$, 20 μg/ml Leupeptin, 10 μg/ml Aprotinin, and 10 μg/ml Pepstatin A). Cells were lysed with four freeze–thaw cycles and centrifuged 15 mins at $20\,000 \times g$. The supernatant was centrifuged at $120\,000 \times g$ 30 mins and stored at −80°C. Protein extract concentration was determined using a BCA protein analysis kit (Bio-Rad).

^{35}S-methionine-labeled p35 substrate was generated by *in vitro* translation using T7 RNA polymerase and TNT reticulocyte lysate (Promega). The p35 cDNA (kindly provided by L Miller), was cloned into pBluescript SK$^+$ to create the plasmid template p35SK-T7. *In vitro* generated p35 protein was used directly in protease assay reactions without purification. For each set of timed reactions, the equivalent of 8 μg of cell extract protein, ^{35}S-methionine-labeled p35 substrate contained in 1 μl of TNT reaction mix and S-buffer to a total volume of 74 μl was incubated at 30°C. Aliquots (18 μl) were removed at 10, 15, 20 and 30 min. The cleavage reactions were stopped by adding an equal volume of 2× tricine sample buffer (0.1 M Tris, 24% glycerol, 8% SDS, 0.2 M DTT, 0.02% Coomassie blue G-250) and placing in a boiling water bath for 3 mins. p35 substrate and caspase products were separated on 10% tricine SDS polyacrylamide gels and the amount of radioactivity in individual bands was quantitated using a Molecular Dynamics phosphorimager and IPLab Gel software (Signal Analytics).

Cytochrome c release assay

Cytoplasmic cytochrome c was measured in cell extracts using the protocol of Wolf and Eastman.[64] Briefly, W7.2 or Apt- cells were treated with 200 nM STS for 16 or 24 h, harvested, and cytosolic cell extracts were prepared following cell lysis with streptolysin O (37°C for 60 min at 60 units/10^6 cells) in 100 μl of stabilization buffer (20 mM HEPES-KOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μg/ml leupeptin, 2 μg/ml aprotinin). The lysed cells were centrifuged at 16 000 $\times$ *g* for 30 min at 4°C and the protein concentration of the supernatant was determined. The cytosolic extracts (20 μg protein/lane) were loaded onto a 15% SDS-polyacrylamide gel, and the resolved proteins were electroblotted to polyvinylidene fluoride membranes as described.[64] The membranes were incubated with an anti-cytochrome *c* monoclonal antibody (Pharmingen), followed by chemiluminescence detection with a goat anti-mouse secondary antibody.

Transient transfection assay

Cells were transfected by electroporation in HBS at 10^7 cells/ml per electroporation chamber. For cotransfection assays, 15 μg of pGFPemd-c (Packard Instrument Co.) and pDC.neo,[27] pDCNeo.βgal, pDCPur.hFas or pCD8-FLICE[65] in a 1:3 molar ratio. In the GFP fusion assay, 15 μg of pEGFP.C1 (Clontech) or equal molar amount of pGFP-FADD 1-78[65] was used. After 24 h in 50% conditioned media, cells were analyzed by flow cytometry in the presence of propidium iodide as described above. Cellular expression of green fluorescent protein (GFP) was detected using a standard FITC filter set up.

Plasmid construction

Plasmid pDCNeo.βgal was generated by inserting the βgal gene coding sequence into the *Xho*I site, and a neomycin resistance gene expression cassette into the *Nru*I site, of pDC304 (Immunex). Plasmid pDCPur.hFas was generated by inserting the hFas gene coding sequence, contained in a *Not*I-*Eco*RV fragment of pBSSKII.hFas, into pDCPur digested with *Bgl*II, blunt-ended, and digested with *Not*I. Plasmid pDCPur contains the puromycin resistance gene expression cassette, an *Nde*I-*Bam*HI fragment of pPUR (Clontech), inserted into the *Nru*I site of pDC304.

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