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T and B leukemic cell lines exhibit different requirements for cell death: correlation between caspase activation, DFF40/DFF45 expression, DNA fragmentation and apoptosis in T cell lines but not in Burkitt's lymphoma

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The execution phase of apoptosis occurs through the activation and function of caspases which cleave key substrates that orchestrate the death process. Here, we have compared the sensitivity of various T and B cell lines to death receptor or staurosporine-induced apoptosis. First, we found a lack of correlation between death receptor expression and sensitivity to Fas or Trail. By contrast, a correlation between caspase activation, DNA fragmentation and cell death in T cell lines was evidenced. Among T cells, CEM underwent apoptosis in response to CH11 but were resistant to Trail in agreement with the absence of Trail receptors (DR4 and DR5) on their surface. The B cell line SKW 6.4 was sensitive to CH11 and staurosporine but resistant to Trail. As B cell lines expressed significant levels of DR4 and DR5, resistance to Trail in SKW 6.4 is likely due to the expression of the decoy receptor DcR1. Burkitt's lymphoma such as RPMI 8866 and Raji did not exhibit DNA fragmentation in response to CH11, Trail or staurosporine but showed long-term caspase-dependent loss of viability upon effector treatment. The B cell lines used in this study express very weak or undetectable levels of DFF40 and relatively high levels of DFF45. Interestingly, cytosolic extracts from RPMI 88.66 but not other B lymphoma exhibit altered levels of cytochrome c-dependent caspase activation. Taken together, our results show that: (1) death receptor expression does not correlate with sensitivity to apoptosis; (2) the very low ratio of DFF40 *vs* DFF45 is unlikely to explain by itself the lack of DNA fragmentation observed in certain B cell lines; and (3) a defective cytochrome c-dependent caspase activation might account at least in part for the insensitivity of certain Burkitt's lymphoma (RPMI 88.66) to apoptosis. Thus it seems that resistance of Burkitt's lymphoma to apoptosis is not governed by a general mechanism, but is rather multifactorial and differs from one cell line to another.
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Introduction

Apoptosis is crucial for normal physiological mammalian development, and disregulation of this process is often associated with human pathologies and more particularly with tumorigenesis.[1,2] Thus, insensitivity to apoptosis is an important mechanism by which human hematopoietic malignancies may resist drug treatment. Little is known about the molecular events that are affected in apoptosis-sensitive *vs* resistant cell lines, even though Bcl2 overexpression, for example, protected B cell follicular lymphoma from cell death.[3–5] Thus, analyzing the molecular aspects of program cell death in apoptosis-sensitive *vs* resistant hematopoietic cell lines may contribute to the characterization of new proteins involved in the regulation of this process.

Apoptosis is characterized by both biochemical and morphological changes that include caspase activation, chromatin condensation and generally fragmentation of genomic DNA into 180–200 base pair units. The apoptotic process is regulated at two main phases, a specific initiation phase that is death stimuli-dependent and a common effector/degradation phase which is conserved whatever the cell type.[6,7] Apoptosis can be induced by a wide variety of stimuli including clustering of death receptors by their specific ligands. Thus, upon binding death receptors recruit adaptor proteins such as FADD[8,9] that can interact with initiator caspases. Binding of caspase 8[10,11] to the death receptor leads to its autoactivation and allows the apoptotic signal to be transduced. The signal initiated at the death receptor level then induces effector caspase activation such as caspase 3[12–14] and 7[15] which are involved in DNA fragmentation. DNA fragmentation is mediated by DFF40 or CAD (caspase-activated DNAse) which behaves as a complex consisting of DFF40 and DFF45 also called ICAD (inhibitor of caspase-activated DNAse).[16] DFF45 is a substrate for both caspase 3 and 7 that is specifically cleaved in cells undergoing apoptosis.[17] Cleavage of ICAD by caspase 3 releases active CAD that translocates to the nucleus to induce chromatin condensation and DNA fragmentation. Very recently, endonuclease G has also been identified as a nuclease involved in the internucleosomal degradation of DNA during apoptosis. Endonuclease G is released from the mitochondrion and activated during apoptosis in a caspase-independent manner.[18]

In the present report we have analyzed the requirements for apoptosis in different B and T cell lines either sensitive or resistant to various forms of apoptosis. We compared CH11 or Trail-mediated apoptosis to staurosporine-induced apoptosis by measuring cell viability, DNA fragmentation, caspase expression and activation and DFF40 and DFF45 levels. We show that T and B cell lines exhibit different requirements for apoptosis. More particularly, we observed a correlation between caspase activation and DNA fragmentation in T cell lines but not B lymphomas. The sensitivity or resistance of these cell lines to apoptosis in terms of death receptor expression, caspase expression and activation, DFF40/DFF45 expression and DNA fragmentation is discussed.

Materials and methods

Reagents

Anti-Caspase 7, 8 Dc-R1, DR4 and DR5 goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (Becton Dickinson, Heidelberg, Germany). Anti-caspase 3 monoclonal antibody (IgG2a) was obtained from Transduction

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Laboratory (Becton Dickinson, Heidelberg, Germany). Anti-DFF45 (ICAD), anti-DFF40 (CAD) rabbit polyclonal were obtained respectively from Calbiochem (Meudon, France) and Alexis (Lausen, Switzerland). ZB4 anti-Fas monoclonal antibody (IgG1) was purchased from Calbiochem. Ac-DEVD-pNA and Ac-DEVD-CHO were purchased from Calbiochem. Anti-Fas monclonal antibody (IgM, CH11) was from Upstate Biotechnology and recombinant human Trail from Euromedex (Mundolsheim, France).

Cells

Jurkat, CEM and HPBall (T cell leukemia) were maintained in RPMI 1640 medium supplemented with 5% SVF, 100 μg/ml penicillin and 100 μg/ml streptomycin. The Burkitt's lymphoma Raji and RPMI 8866 and the EBV-positive B-lymphoblastoid cell line SKW 6.4 were maintained in RPMI 1640 medium supplemented with 10% SVF, 50 μM β-mercaptoethanol, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37°C as previously described.[19–21]

DNA fragmentation

Cells (10^6/ml) were cultured in RPMI 1640 medium in 12-well sterile dishes with 100 ng/ml of anti-Fas IgM (CH11), 100 ng/ml recombinant Trail or 1 μM staurosporine for different times at 37°C. Cells were then collected and lysed with 200 μl of lysis buffer A (10 mM Tris pH 7.5, 5 mM EDTA and 0.2% Triton X-100). Lysates were treated for 30 min with 100 μg/ml RNAse and then incubated for 30 min with 100 μg/ml proteinase K as previously described.[22,23] Cellular DNA was ethanol precipitated, dried and resuspended in Tris-EDTA buffer (10 mM Tris pH 7.5 and 5 mM EDTA). DNA was analyzed by electrophoresis on 1.2% agarose gels after staining with ethidium bromide.

Quantitative analysis of cell viability

Cells (10^6/ml) in quadruplicate, were incubated in 100 μl of RPMI 1640 medium containing 10% SVF in 96-well plates with various concentrations of CH11, Trail or staurosporine. After 24 or 48 h, cell survival was assessed by the XTT dye reduction assay (Roche, France) which measures mitochondrial respiratory function.[24] Cells were incubated for 2 h with the dye and cell viability measured at 490 nm. Cell viability is expressed as a percentage of that of untreated cells.

Facs analysis

Cells (10^6/ml) were incubated with saturating concentrations of the anti-Fas mAb (ZB4; Pharmingen), anti-DR4, anti-DR5 or anti-DcR1 antibodies (goat polyclonal; Santa Cruz Biotechnology) for 30 min at 4°C, followed by a biotin-conjugated anti-mouse or anti-goat secondary antibody (1/200, 30 min 4°C) and by streptavidin–phycoerythrin (1/200, 30 min 4°C).[25,26] Analyses were performed by flow cytometry on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Polymerase chain reaction

Two μg of RNA were reverse-transcribed and PCR reaction in 50 μl were performed according to the standard protocol with 2 μl of first-strand cDNA template, 10 μmol/l primers, 1 mmol/l dNTPs and 1 U Taq polymerase as previously described.[19] The amplification program was 94°C for 2 min, 60°C for 90 s (30 cycles). The following primer sequences were used for detection of DR4 and DR5: DR4 (633 bp fragment) 5′-GTG TCC ACA AAG AAT CAG GC-3′ sense, 5′-GCA TGT CTC TCT TCC ATC CT-3′ antisense), DR5 (400 bp fragment) 5′-AGG ACT ATA GCA CTC ACT TGG-3′ sense, 5′-TTG TGA GCT TCT GTC CAC AC-3′ antisense). Amplification products were separated on 1.2% agarose gels.

Western blot analysis

Western blot analysis has been described in detail elsewhere.[27,28] Briefly, after treatment, cells (4×10^6) were washed in PBS, resuspended in lysis buffer B (50 mM Hepes, pH 7.4, 150 mM NaCl, 20 mM EDTA, 100 mM NaF, 1 mM PMSF, 1 μM pepstatin, 20 μg/ml aprotinin, 1% NP-40), incubated for 30 min at 4°C, and finally centrifuged at 15000 *g* for 15 min. 150 μg of protein per condition were separated on 11% polyacrylamide gels and blotted on to a PVDF membrane (Immobilon, Millipore, France). After blocking non-specific binding sites, the membrane was incubated for 2 h at room temperature with anti-human caspase 3 antibody, anti-caspase 7 or 8 antibodies. The membranes were washed three times with TNA-1% NP-40 (Tris 50 mM, NaCl, 150 mM, pH 7.5) incubated further with horseradish peroxidase-conjugated rabbit anti-mouse or anti-goat antibody for 60 min at room temperature. Immunoblots were revealed using the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Saclay, France) by autoradiography.

Determination of caspase activity

4×10^6 cells were treated with 100 ng/ml CH11, Trail or 1 μM staurosporine for 7 h, washed once in PBS and lysed on ice in 100 μl lysis buffer (Hepes 50 mM, pH 7.4; NaCl 150 mM, EDTA 20 mM; 0.2% Triton X-100; 1 mM PMSF, 1 mM pepstatin, 20 μg/ml aprotinin). An equal volume of 2 × reaction buffer complemented with 400 μM Ac-DEVD-pNA and 10 mM dithiothreitol was then added to 50 μl of cell lysates (protein concentration 1 mg/ml) in 96-well plates and incubated at 37°C for different times. Caspase activity was measured by the release of pNA at 405 nm in either the presence or absence of 10 μM Ac-DEVD-CHO, an irreversible inhibitor of caspases as previously described.[29] Specific caspase activity represents the Ac-DEVD-CHO inhibitable activity and is expressed as nmoles of substrate hydrolysed per min and per mg of proteins.

Cell-free assays

Cytochrome c/dATP-dependent caspase activation in cytosolic extracts was assessed in a cell free system as described by Wolf *et al*.[30] Briefly, reactions were initiated by the addition of cytochrome c plus 1 mM dATP. After 30 min at 37°C, Ac-DEVD-pNA was added to samples and caspase activity assessed by the release of pNA at different times as described previously.[28]

Results

Sensitivity of different T and B cell lines to death receptor and staurosporine-mediated apoptosis

To evaluate the sensitivity of different T and B cell lines to various proapoptotic stimuli, the effects of two death receptor ligands, CH11 and Trail and that of the protein kinase inhibitor staurosporine (STP) were assessed by the XTT dye reduction assay. Figure 1a and b shows the percentage of viable cells after 24 and 48 h of treatment. Two T cell lines, Jurkat (Jd) and HPBALL were found to be highly sensitive to the three effectors. CEM cells were efficiently killed by both CH11 and STP but were resistant to Trail (Figure 1a and b). This was also the case for the EBV-positive B cell line SKW 6.4. The B cell line RPMI 8866 was sensitive to STP but resistant to CH11 and Trail. Finally, the B cell line Raji was resistant to CH11 and Trail but exhibited a weak sensitivity to STP after 48 h. As XTT represents a useful method to assess quantitatively cell viability but cannot distinguish between growth arrest, apoptosis or necrosis, we next analyzed DNA fragmentation in the different cell lines. DNA fragmentation was assessed after 7 or 24 h of treatment in the presence of various proapoptotic stimuli. Concerning the three T cell lines, we observed a good correlation between the results obtained by XTT and DNA fragmentation (Figures 1 and 2), demonstrating that variation in cell viability in these cells reflected apoptosis. After a 7 h treatment, no DNA fragmentation was detectable in any of the B cell lines tested (Figure 2a). Interestingly, CH11 and STP but not Trail were found to induce internucleosomal DNA degradation in SKW 6.4 after 24 h, whereas RPMI 8866 and Raji failed to undergo DNA fragmentation in response to any of these three effectors (Figure 2b). The lack of effect of STP on DNA fragmentation in Raji and RPMI 8866 cells did not match well with the significant inhibition of cell viability observed at 48 h (Figure 1a and b). These results attested to a great heterogeneity to apoptosis of these different cell lines. Globally, B cell lines at the noticeable exception of SKW 6.4 were resistant to all the proapoptotic stimuli in term of DNA fragmentation.

Death receptor expression in the different T and B cell lines

To explain the differences in sensitivity to CH11 and Trail-induced apoptosis we first analyzed death receptor expression in each of these cell lines by flow cytometry. Fas expression

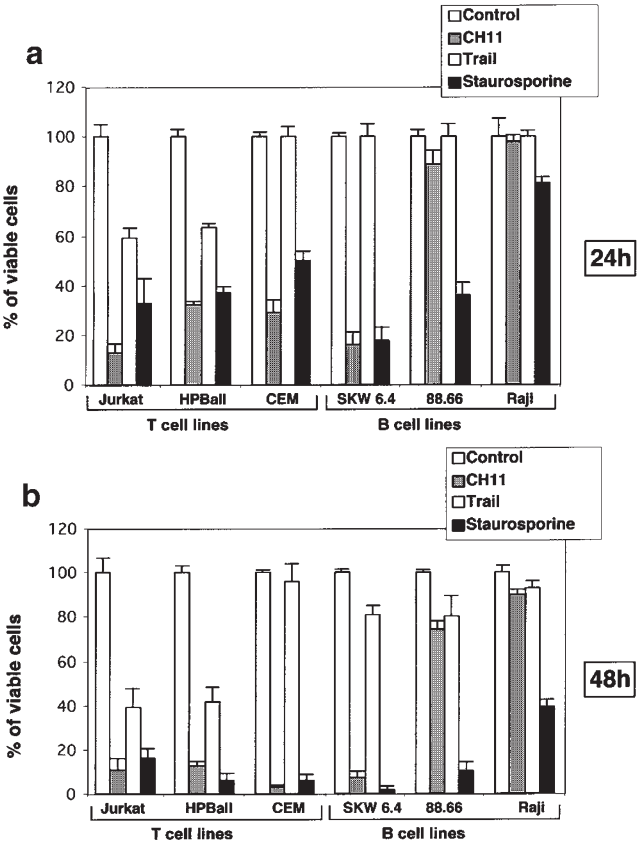

Figure 1 Effect of Fas, Trail and staurosporine on viability of various cell lines. Cells 10^6/ml in quadruplicate were incubated in 100 μl of RPMI 1640 medium containing 5% FCS for 24 (a) or 48 h (b) in 96-well plates with either 20 ng/ml anti-Fas antibody (CH11) or Trail or 1 μM staurosporine. Cell survival was assessed by the XTT dye reduction assay which measures mitochondrial respiratory function as described in the Materials and methods section.

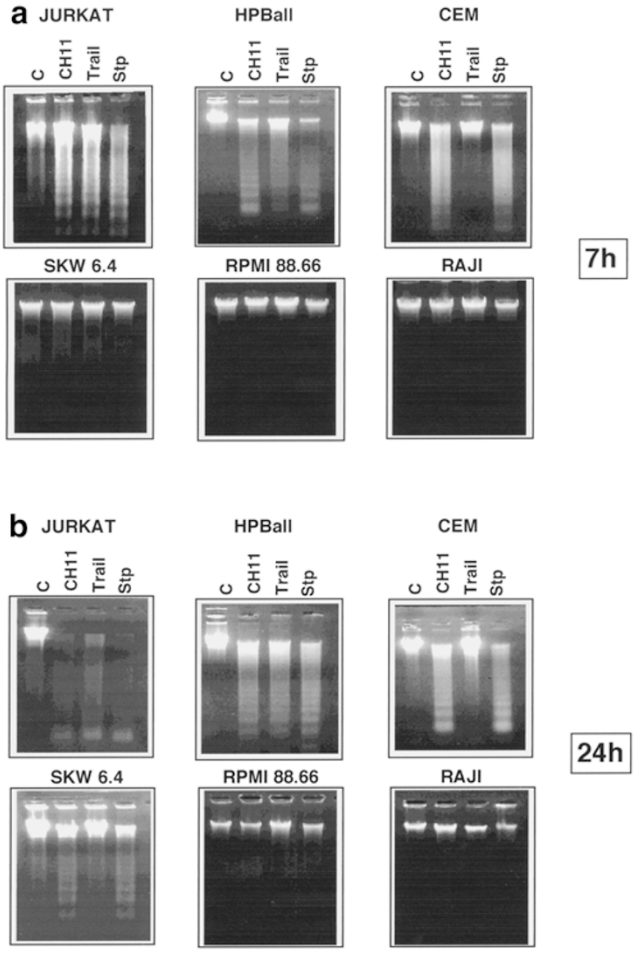

Figure 2 DNA fragmentation in T and B cell lines. Internucleosomal DNA degradation was visualized after electrophoresis on 1.2% agarose gel in the presence of ethidium bromide. T and B cell lines were incubated for 7 (a) or 24 h (b) with either 100 ng/ml anti-Fas antibody (CH11) or Trail or 1 μM staurosporine, before preparation of DNA.

was assessed with the ZB4 monoclonal antibody, and the presence of functional and decoy Trail receptors was detected with anti-DR4, anti-DR5 and anti-DcR1 polyclonal antibodies, respectively. Fas receptor was present to various extents on the surface of all the cell lines tested with mean fluorescence values varying from 20 in Jurkat cells to 97 in the B cell line RPMI 8866 (Table 1). Interestingly, B cell lines were found to express significantly more Fas reactivity than T cell lines but except for SKW 6.4 were resistant to Fas-induced DNA fragmentation (Table 1 and Figure 2). DR4, DR5 and DcR1 were expressed at very low levels on the surface of T cell lines as assessed by Facs although Jurkat and HPBaLL were sensitive to Trail. CEM cells were devoid of DR4, DR5 and DcR1, an observation in agreement with the lack of sensitivity of this particular cell line to Trail-mediated apoptosis (Table 1 and Figure 2). Facs analysis of B cell lines revealed a weak but significant expression of DR4 and DR5 in the three cell lines, but the latter was virtually absent from the surface of Raji cells. Finally, expression of the decoy receptor DcR1 was detected on all the B cell lines tested (Table 1). These results are somewhat surprising since cells which expressed the lower amount of DR4 and DR5 receptors (Jurkat and HPBall) exhibited the highest sensitivity to Trail as compared to B cells, although in the latter case the higher expression of the decoy receptor DcR1 could explain, at least in part, their insensitivity to Trail induced-apoptosis. Moreover, very low expression of DR4 and DR5 receptors in the context of a total lack of decoy receptor such as in T cell lines may account for the sensitivity of these cells to Trail. We therefore analyzed DR4 and DR5 expression in these cell lines by RT-PCR (Figure 3). Two couples of oligonucleotides amplifying either a 633 base pair fragment of DR4 or a 400 base pair fragment of DR5 were synthetized. The results of a representative PCR experiment using cDNA prepared from the six cell lines is shown in Figure 3. DR4 and DR5 transcripts were detected in B cell lines, and to a lower extent in Jurkat and HPBall cells but were absent from CEM cells. Actin transcripts were present at identical levels in all the cell lines tested (Figure 3). Thus, lack of Trail receptor expression in CEM is likely to explain the insensitivity of these cells to Trail-induced apoptosis. Finally, the high sensitivity of our PCR technique allows us to detect Trail receptor trancripts in cell lines that showed very low levels of receptors by Facs (Jurkat and HPBall). It should be pointed out that two DR5 transcripts were detected in all the cell lines tested (Figure 3). The higher molecular band corresponded to the size expected for DR5. Finally, both bands were sequenced and the lower one was shown to share a 81 base pair deletion in frame in the extracellular domain of DR5 (not shown).

Table 1 Death receptor expression in T and B cell lines

	Death receptor expression (AU)				
	Control	*α FAS*	*α DR4*	*α DR5*	*α DCR1*
Jurkat	2.5	21 (+)	5.4 (+/–)	5.3 (+/–)	5 (+/–)
HPBall	2.7	55 (++)	5 (+/–)	5 (+/–)	5 (+/–)
CEM	2.2	28 (+)	2.4 (–)	2.1 (–)	3 (–)
SKW 6.4	2.5	95 (+++)	10 (+)	10 (+)	20 (+)
RPMI 88.66	2	97 (+++)	19 (+)	9 (+)	22 (+)
Raji	2.2	71 (+++)	13 (+)	5 (+/–)	15 (+)

Cells (10^6/ml) were first incubated with either an anti-Fas monoclonal antibody (ZB4, IgG1) or anti-DR4, anti-DR5 or anti-DcR1 polyclonal antibodies, followed by biotinylated goat anti-mouse antibody or biotinylated rabbit anti-goat antibody, respectively, and finally phycoerythrin–strepatavidin. Stained cells were analyzed with a flow cytometer FACScan (Becton Dickinson). Results are expressed in arbitrary units as the mean fluorescence intensity. (–) no expression, (–/+) very low expression, (+) low expression, (++) medium expression (+++) high expression.

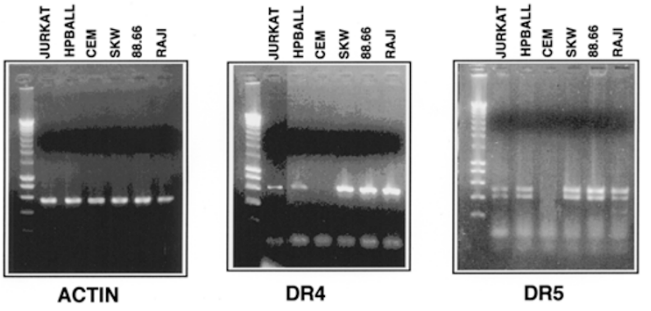

Figure 3 RT-PCR amplification of DR4 and DR5 mRNAs. RT-PCR amplification of DR4 and DR5 fragments from T and B cell lines. Amplification method was described in the Materials and methods section. Actin was used as a positive control in the experiment. Note the presence of two amplified fragments for DR5.

Caspase activation in T and B cell lines undergoing apoptosis

To better characterize the response of T and B cell lines to apoptosis, we performed a methodical analysis of the events involved during the onset of apoptosis. Noticeably, as caspase activation is most of the time linked to initiation and activation phases of the apoptotic program, we visualized by Western blotting the state of activation of caspases 3, 7 and 8 in the different cell lines following a 7 h treatment with CH11, Trail or STP. As depicted in Figure 4, cleavage of procaspases 3 and 7, reflecting an activation of these proteinases was evidenced in all the T cell lines and also in SKW 6.4 cells treated with CH11 or STP. In CEM and SKW 6.4 cells we failed to detect any activation of caspase 3 and 7 upon Trail treatment (Figure 4a and b). Procaspase 8 activation was evidenced in Jurkat and HPBall whatever the proapoptotic stimuli used and faintly detected in CH11-treated B cell lines. By contrast, we were unable to detect procaspase 8 activation in B cell lines and in CEM cells upon Trail treatment in agreement with the resistance of the latter cell line to this death ligand. It is worth mentioning that a weak caspase 3 activation also occurs in RPMI 8666 and Raji cells. In parallel, we also measured global caspase activity in cellular extracts prepared from activated or unstimulated cell lines after 7 h of stimulation (Figure 5). Thus, hydrolysis of Ac-DEVD-pNA by cellular lysates prepared from the different cell lines was followed as a function of time. Concerning T leukemias, there was again a correlation between caspase activity and the different biochemical criteria of apoptosis such as viability, DNA fragmentation and caspase processing (Figures 1, 2, 4 and 5). Interestingly, in B cell lines, despite a total resistance of RPMI 8866 and Raji cells to DNA fragmentation, we were able to detect significant caspase activity (Figures 2, 4 and 5).

Altered DFF40 expression in B cell lymphoma

The lack of DNA fragmentation observed in several B cell lines, despite significant caspase activity was reminiscent of

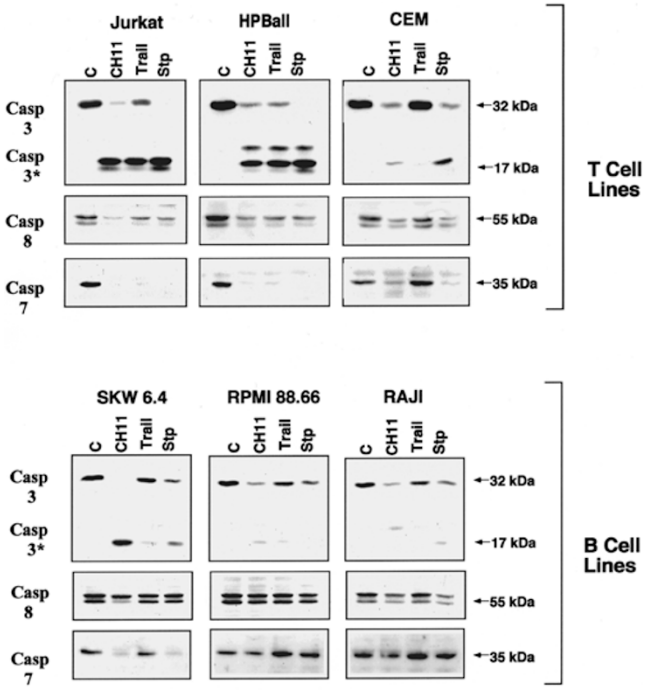

Figure 4 Caspase activition in T and B cell lines. T and B cell lines (4×10^6) were incubated for 7 h at 37°C in either the absence or presence of 100 ng/ml CH11 or Trail or 1 μM staurosporine. Cells were then lyzed and proteins separated by electrophoresis on 11% polyacrylamide gels. Proteins were then blotted on to PVDF membranes which were incubated with either anti-caspase 3, anti-caspase 7 or anti-caspase 8 antibodies. Arrows indicates the position of the zymogens for these different caspases. The cleaved 17 kDa caspase 3 subunit is also indicated by an arrow.

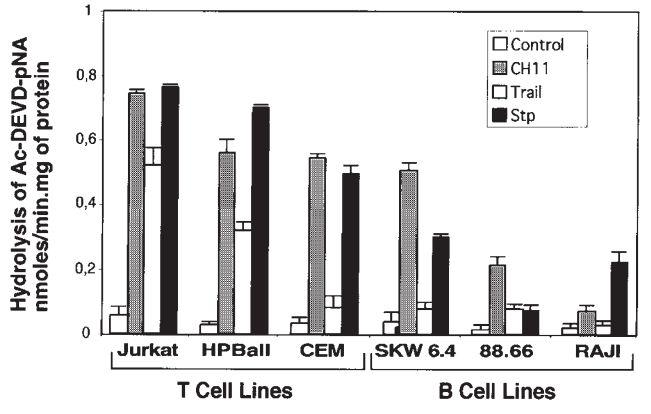

Figure 5 Caspase activation in T and B cell lines. Caspase activity was assessed on cell lysates prepared from cells incubated for 7 h with either 100 ng/ml CH11 or Trail or 1 μM staurosporine using 0.2 mM Ac-DEVD-pNA as substrate. Ac-DEVD-pNA hydrolysis was determined in quadruplicate at different times in the presence or absence of 10 μM Ac-DEVD-CHO in order to measure specific caspase activity. Results are expressed as nanomoles of substrates hydrolyzed per min and per milligram of protein and represents the mean of three different determinations.

previous observations indicating that apoptosis may occur in the absence of DNA fragmentation. Indeed, caspase 3 which is involved in DNA fragmentation is readily activated in B cell lines such as SKW 6.4 following treatment with Fas or staurosporine (Figures 3 and 5). In mice, DNA fragmentation is mediated by CAD which is normally complexed in the cytoplasm of non-apoptotic cells to its specific inhibitor ICAD. During induction of apoptosis ICAD is cleaved by caspase 3 and CAD is consecutively released from the complex and translocates to the nucleus where it can induce DNA fragmentation.[16,17] In humans, DFF40 and DFF45 are thought to be the ortologs of CAD and ICAD, respectively. We thus analyzed the expression of DFF40 and DFF45 in the different T and B cell lines (Figure 6). Interestingly, all the T cell lines tested expressed a large amount of DFF40 as compared to DFF45 while B cell lines showed a reciprocal expression of these two proteins (ie strong expression of DFF45 as compared to DFF40). However, in SKW 6.4 cells, the significant but late DNA fragmentation observed following treatment with Fas or staurosporine did not match with the very low level of DFF40 detected in these cells.

Defective cytochrome c-dependent caspase activation in 8866 cells

As global caspase activation is impaired in several Burkitt's lymphoma compared to T cell lines we analyzed the ability of cytosolic extracts from T and B cell lines to induce caspase

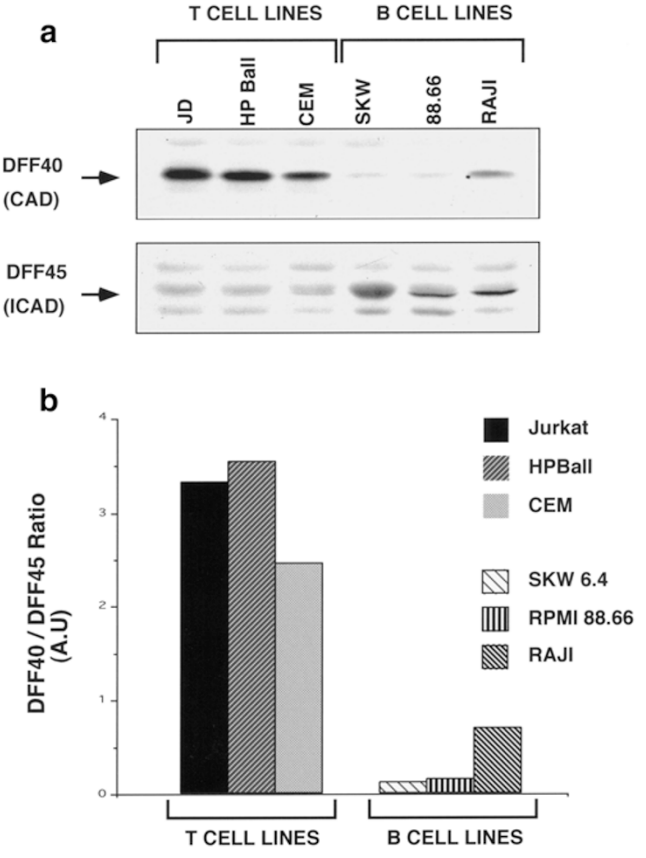

Figure 6 DFF40 and DFF45 expression in the different T and B cell lines. (a) DFF40 and DFF45 expression was determined in each cell line. Cells were lyzed and proteins separated by electrophoresis on 11% polyacrylamide gels. Proteins were then blotted to PVDF membranes which were incubated with either anti-DFF40 or anti-DFF45 antibodies. Arrows indicate the position of DFF40 and DFF45. (b) Quantitative determination of DFF40/DFF45 ratio in the different T and B cell lines. Note that the ratio DFF40/DFF45 is large in T as compared to B cell lines.

activation in a cell-free system. We initiated cell-free apoptosis by incubating cell extracts prepared from Jurkat, RPMI 8866, Raji and SKW 6.4 cell lines with cytochrome c plus dATP. Jurkat T cell and SKW 6.4 B cells displayed high levels of caspase activity following Cyt c/dATP addition as assessed by Ac-DEVD-pNA hydrolysis while RPMI 8866 cell extracts had very low levels of caspase activity (Figure 7a). Interestingly, addition of dATP alone in cytosolic extract from SKW 6.4 B cells is sufficient to induce a high level of caspase activity independently of Cyt c suggesting that Cyt c is already present in SKW 6.4 B cell extracts.

We next examined the expression of different protagonists involved in the regulation of apoptosis in T and B cell lines to determine whether variations in their expression might account for the different Cytc/dATP-dependent caspase activation observed in a cell-free system. As shown in Figure 7b, APAF-1, caspase 9 and caspase 3 (Figure 4), were expressed at approximatively identical levels in Jurkat T cell and the different B cell lines tested. Since we did not detect differences in APAF-1 and caspase 9 expression decrease in APAF-1 and/or caspase activity must account for the lack of caspase activation observed with RPMI 88.66 cells.

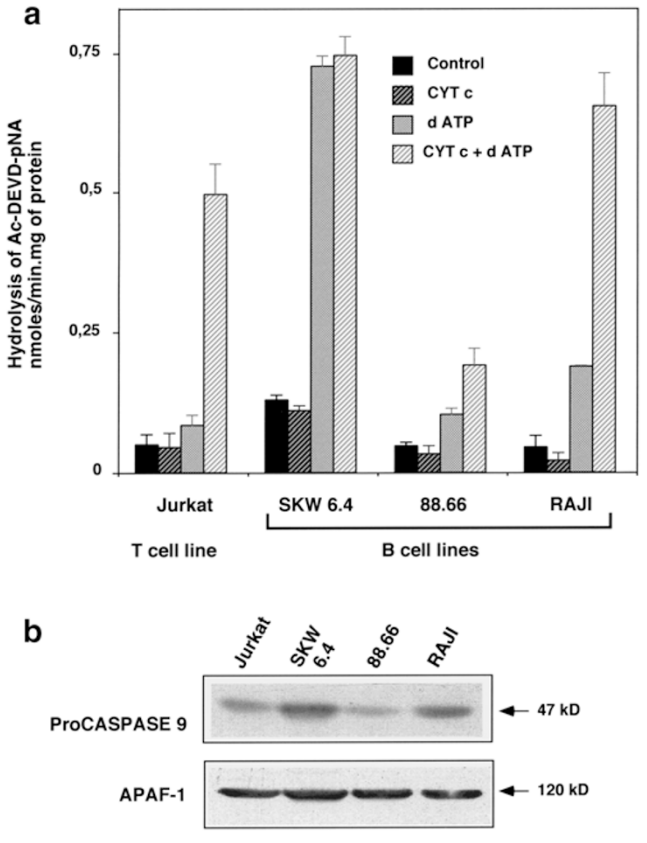

Figure 7 Altered Cyt c-dependent caspase activation in Burkitt's lymphoma. Cytosolic extracts were prepared from T and B cell lines (Jurkat, 8866, SKW6.4 and Raji cell lines). (a) Extracts were treated with cytochrome c (20 μM plus dATP 1 mM) for 30 min at 37°C and then assayed for caspase activity as described in Figure 4 against the caspase colorimetric substrate Ac-DEVD-pNA. Results are the mean ± s.e.m. for three different preparations. (b) Proteins present in cell extracts were separated by electrophoresis on 11% polyacrylamide gels. Proteins were then blotted to PVDF membranes which were incubated with either anti-caspase 9 or anti-APAF1 antibodies.

Discussion

In the present study, we have analyzed the requirements for apoptosis in different T and B cell lines. Three acute lymphoblastic leukemias, Jurkat, CEM and HPBall, two Burkitt's EBV-positive lymphomas, Raji and RPMI 8866, and a B lymphoblastoid cell line SKW 6.4 were analyzed for different biochemical hallmarks of apoptosis such as cell viability, DNA fragmentation, death receptor expression, caspase level and DFF40/DFF45 expression. Death receptor induced-cell death was induced by the Fas agonist CH11 or Trail and was compared to staurosporine-mediated apoptosis. All the cell lines tested showed evidence of cell death upon staurosporine treatment as assessed by the XTT dye reduction assay even though the B cell line Raji was less sensitive to STP treatment than other B cell lines. Nonetheless, insensitivity of B cell lines to Fas and Trail-induced apoptosis was found to be unrelated to the expression of EBV since Raji (EBV+) and Ramos (EBV–), for example, behaved similarly (not shown). Differences were observed between the requirements of T and B cell lines for death receptor-induced apoptosis. Indeed, the three T cell lines were found to be sensitive to CH11-mediated DNA fragmentation while interestingly loss of cell viability was not correlated to DNA fragmentation in Raji and RPMI 88.66 B cells.

Analysis of Fas expression on each cell line revealed a lack of correlation between Fas expression and sensitivity to apoptosis. Indeed, cells expressing the higher level of Fas expression, Raji and RPMI 8866, exhibited a total resistance to Fas-mediated apoptosis. Our results are at variance with those of Shain *et al*,[31] which show that sensitivity to CD95-mediated cell death correlates with Fas expression in RPMI 8226. The same B cell lines were also insensitive to Trail as was the T cell line CEM. The resistance of the CEM cell line can be accounted for by a lack of DR4 and DR5 expression as assessed by Facs and PCR analysis. However, this is clearly not the case for Raji and RPMI 8866, which were found to be resistant to Trail despite DR4 and DR5 expression. It is, nonetheless, possible that the relatively high level of DcR1 expression in these cell lines may counteract Trail effect. Nevertheless, insensitivity of certain Burkitt's lymphomas to death receptor-induced apoptosis has also been attributed to other mechanisms such as altered FLICE *vs* FLIP ratio.[32] Along the same lines it has been recently reported that resistance to Fas-mediated apoptosis in a non-Hodgkin's lymphoma B cell line results from a high constitutive expression of c-FLIP.[33] However, we have not been able to detect significant differences in FLICE and FLIP expression in these different B cell lines as compared to T cell lines (not shown). In T cell lines, we observed a correlation between caspase activation, global caspase activity, DNA fragmentation and finally cell death, whatever the stimuli used to induce apoptosis. By contrast, the results obtained with B cell lines are more difficult to interpret. Although a weak increase in global caspase activity was detectable in Raji and RPMI 8866 following CH11, Trail or STP stimulation, we failed to detect DNA fragmentation in these cells. After 48 h of treatment, STP and to a lesser extent Trail and CH11 were shown to induce cell death in both cell lines as assessed by the XTT assay but without any evidence of internucleosomal degradation of DNA. It is thus also conceivable that in B cell lines the low level of caspase activation may serve cellular processes other than apoptosis, such as differentiation, activation or regulation of the cell cycle.[34–36] It has been recently reported that certain B cell lymphomas can undergo a cell death process resembling necrosis.[37] Clearly, the cell death process we observed in B cell lines is different

from necrosis since (1) we failed to detect any random degradation of DNA as it is the case in necrosis and (2) we did observe some caspase activation following treatment of B cell lines with different proapoptotic stimuli. These results indicate that in these cells a defect may exist both at the level of and downstream of caspase activation. These observations are in good agreement with recent reports by Wolf *et al*,[30] who reported a defect in caspase activation in ovarian cancer cell lines due to diminished or absent APAF-1 activity and by Kawabata *et al*,[38] who showed the absence of DNA fragmentation in Raji B cells. Interestingly, they also show the presence and the cleavage of DFF45 in Raji B cells as well as in apoptosis-sensitive Daudi cells. Thus, we analyzed DFF40 and DFF45 expression in our different B and T cell lines. We confirmed the presence of DFF45 in all the B cell lines tested but above all demonstrated very low levels of DFF40 in the same cells. Finally, the ratio of DFF40 *vs* DFF45 was very clearly in favor of DFF40 in T cell lines. The situation was the opposite in B cell lines where DFF40 was virtually undetectable by Western blot. However, the very low ratio of DFF40 *vs* DFF45 is unlikely to explain by itself the lack of DNA fragmentation observed in certain B cell lines. Finally, we evidenced a defective Cytc-dependent caspase activation in 8666 cells which might account, at least in part, for the insensitivity of this particular B cell line to apoptosis. Thus it seems that resistance of Burkitt's lymphoma to apoptosis is not governed by a general mechanism, but is rather multifactorial and differs from one cell line to another.

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