

Volume 12, issue 1, March 2001 | Articles originaux

A Jurkat T cell variant resistant to death receptor-induced apoptosis. Correlation with heat shock protein 27 and 70 levels.

Abstract

Ligation of Fas induces an apoptotic program in Jurkat cells (Jd). We describe a Jurkat T cell variant (Jr) which shows total resistance to Fas-mediated apoptosis but which exhibits sensitivity to non-death-receptor pro-apoptotic stimuli such as staurosporine. Resistance to Fas-induced apoptosis in Jr cells is correlated with high expression of Hsps. A prior heat-shock increases Hsp27 and 70

expression and protects Jd and Jr cells from Fas- and staurosporine-induced apoptosis. Staurosporine, but not the anti-Fas antibody CH11, abrogates constitutive Hsp70 expression at 37° C and staurosporine also inhibit Hsp27 expression in Jd and Jr cells at 42° C. These data suggest that constitutive expression of Hsp27 inhibits Fas-mediated apoptosis, but only induced expression of Hsp70 can protect T cells from staurosporine-induced apoptosis. Thus, Hsp27 could play a role in the regulation of death receptor-mediated apoptosis, while Hsp70 could regulate mitochondrial-dependent cell death.

Full text

INTRODUCTION

Apoptosis or programmed cell death (PCD) is a fundamental and indispensable process during normal cell development, tissue homeostasis and regulation of immune responses [1]. PCD is quite distinct from necrosis and is characterized by both biochemical and morphological modifications including reduction in cell volume, cell blebbing, nuclear condensation and finally internucleosomal degradation of DNA. A wide variety of stimuli have been shown to induce apoptosis in different cell types. For example, triggering activation of death receptor of the tumor necrosis factor receptor family (TNF-R), treatment of cells with UV radiation, reactive oxygen species (ROS), ceramides, heat shock, drugs such as staurosporine, etoposide, cycloheximide or growth factor deprivation. Apoptosis is closely associated with the activation of a highly conserved and growing family of cysteine proteinases, now classified as caspases [2], that share substantial homologies with the product of the ced-3 gene required for normal development in *Cænorabditis elegans* [3]. To date, fourteen caspases have been identified in mammalian cells, which play a role either in PCD or in the maturation of procytokines such as IL-1beta [4]. Caspases are synthetized as inactive precursor molecules and are converted to active proteinases by proteolytic cleavage specifically after aspartic residues. At present, more than fifty cellular substrates of caspases have been identified that can either activate death effector molecules or inactivate proteins mainly involved in the maintenance of cellular integrity. In mature T lymphocytes and most leukemic T cell lines, apoptosis can be induced by triggering the Fas receptor (Fas/CD95/Apo-1) which plays a crucial role in the regulation of the peripheral immune response [5]. CD95 is a member of the TNF-R superfamily that induces apoptosis upon binding to its specific ligand (Fas-L) [6, 7]. The activation of Fas

required aggregation induced by Fas-L or agonist antibodies such as CH11. Fas shares an intracellular death domain (DD) that is essential for apoptosis [8]. The DD of CD95 recruits other DD-containing proteins, such as FADD [9] and RIP [10]. FADD in turn binds to FLICE/MACH1 [11, 12], also known as procaspase 8. Binding of Fas-L to its receptor triggers trimerization that induces a conformational change both necessary and sufficient to cause autoactivation of procaspase 8. Caspase 8 then transmits the activation signal to the downstream apoptotic machinery. Besides caspases, several other molecules have been shown to participate in the regulation of apoptosis, in particular the pro- and anti-apoptotic members of the Bcl-2 family, the proto-oncogenes, Myc, Fos and Jun, acidic sphingomyelinase, NF-kappaB, the tyrosine kinase and phosphatase, p59Fyn and FAP-1, and the heat shock proteins (Hsp27, Hsp60 and Hsp70). Stress or heat shock proteins constitute a large family of proteins that provide enhanced resistance to numerous cellular stresses such as elevated temperatures and reactive oxygen species (ROS). Hsps are thought to act both as molecular chaperones [13, 14] and as sensors for the level of intracellular ROS, probably by inducing an increase in cell glutathione concentration. Overexpression of Hsp27 and Hsp70 or induced expression of Hsp70 have been shown to promote cytoprotection and to rescue cells from Fas and staurosporine-induced apoptosis [15, 16]. Moreover, down-regulation or disruption of Hsp27 and Hsp70 favours apoptosis [17]. However, little is known about how the Hsps protect cells. We have examined here the role of Hsp27 and Hsp70 in Fas and staurosporine-induced apoptosis in the human leukemic Jurkat T cell line and in a Fas-insensitive variant expressing constitutive Hsp27 and increased level of Hsp70.

METHODS

Cells

Jurkat T cells used in this study (clone Jd) and (clone Jr, Wursburg Jurkat T cells) have been described elsewhere [18, 19].

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, Euromedex), 1 muM staurosporine (Sigma), 50 muM etoposide (Sigma), 20 muM cycloheximide (Sigma), 1 mM H_2O_2 (Sigma) or

50 muM C_2-ceramide (Sigma) for 18 hours at 37° C as indicated. The cells were collected and lyzed with 200 mul of lysis buffer A (10 mM Tris pH 7.5, 1 mM EDTA and 0.2% Triton X-100). Lysates were treated for 60 min with 100 mug/ml RNase and then incubated for 60 min with 100 mug/ml proteinase K. Cellular DNA was ethanol-precipitated, dried and resuspended in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 7.5). DNA fragmentation was analysed as previously described [20, 21].

Quantitative analysis of apoptosis by the XTT dye reduction assay

Cells (10^5) in quadruplicate, were incubated in 100 mul of RPMI 1640 medium containing 5% FCS in 96-well plates with various concentrations of the different effectors. After 18 hours, cell survival was assessed using the XTT dye-reduction assay (Boerhinger, Manheim) which measures mitochondrial respiratory function. Cells were incubated for 2 hours with the dye and cell viability measured at 490 nm. Cell viability is expressed as a percentage of that of untreated cells.

Western Blot analysis

Western blotting conditions have been described in detail elsewhere [22, 23]. Briefly, after treatment, cells (4×10^6) were washed in PBS, lyzed in lysis buffer B (50 mM Hepes, pH 7.4, 150 mM NaCl, 20 mM EDTA, 100 muM NaF, 10 mM Na_3VO_4, 1 mM PMSF, 1 muM pepstatin, 20 mug/ml aprotinin, 1% NP40) incubated for 30 min at 4° C and finally centrifuged at 15,000 g for 15 min: 150 mug of protein were separated on an 11% polacrylamide gel and blotted onto a PVDF membrane (Immobilon, Millipore). After blocking non-specific binding sites, the membrane was incubated for 2 hours at room temperature with anti-human procaspase 3 antibody (Transduction Laboratory), anti-human PARP, FADD, Toso, Bcl-2, Bax, procaspase 2, 7, 8, 10, Hsp27 (C-20), Hsp60 (K-19) and Hsp70 (K-20 non-crossreactive with Hsc70) antibodies (Santa Cruz). The membranes were washed three times with TNA, 1% NP40 (Tris 50 mM, NaCl 150 mM, pH 7.5), and incubated further with horseradish peroxidase conjugated goat anti-mouse or rabbit anti-goat antibodies (Dako) for 60 min at room temperature. Immunoblots were revealed using the enhanced chemiluminescence detection kit (Amersham) by autoradiography.

Heat stress

To induce heat shock proteins, cells were treated during 2 hours at 42° C. Cells were them incubated at 37° C with 100 ng/ml of CH11 (Euromedex) or 1 muM staurosporine (Sigma) for 4 hours. Effects of heat induction on Hsp expression and apoptosis were visualized by SDS-PAGE and Western blotting with specific anti-Hsp27 or anti-Hsp70 antibodies and by DNA fragmentation, respectively.

Flow cytometry/FACS analysis

Cell (10^6) were successively incubated with 2 mug/ml of anti-Fas (clone ZB4, Euromedex), (Fab')2 fragments of rabbit anti-mouse conjugated to biotin (1/200, Dako) and with streptavidin conjugated to phycoerythrin (1/200, Dako) for 30 min at 4° C in 100 mul of PBS, 0.1% BSA, 0.01% NaN_3. After a final wash in PBS, cell were resuspended in 500 mul of PBS, 1% formaldehyde and analyzed by dual colour immunofluorocytometry (Facs Star Plus, Becton Dickinson).

Caspase activity measurement

Each assay (in triplicate) was performed with 50 mug of protein prepared from control cells or cells stimulated for 4 hours with 100 ng/ml CH11 (Euromedex). Cellular extracts were then incubated in a 96-well plate, with 0.2 mM of Ac-YVAD-pNA or Ac-DEVD-pNA for various times at 37° C. The caspase activity was measured at 410 nm in the presence or absence of 1 muM of Ac-DEVD-CHO. The specific caspase activity was expressed in nmoles of paranitroaniline released per min and per mg of protein.

RESULTS

Jr cells are resistant to Fas-induced apoptosis

Previously, we have shown that a variant of the leukemic Jurkat cell line (Jr) was resistant to Fas-induced apoptosis [19]. Resistance of Jr to Fas-induced apoptosis is further illustrated in Figure 1. To quantitatively measure the sensitivity of both cell lines to apoptosis, survival was assessed by the 3-4, [5-dimethylthiazol-2-yl-] 2-5 diphenyl tetrazolium bromide (XTT) dye reduction assay after 18 h of incubation in the presence of various concentrations of CH11. CH11 induced the death of more than 80% of Jd cells at 10 ng/ml, almost 100% of Jd cells at 100 ng/ml, but did not affect cell viability in Jr cell cultures whatever the concentrations used (Figure 1A). Similarly, CH11 also induced Ac-DEVD-ase

activity in Jd cells that was totally abolished by Ac-DEVD-CHO, a potent and wide spectrum caspase inhibitor (Figure 1B left panel), while no Ac-DEVD-ase activity was detected in Fas-stimulated Jr cells (Figure 1B). Under the same conditions, we were unable to detect any Ac-YVAD-ase activity in either cell variant upon Fas stimulation (Figure 1B, right panel). Insensitivity to Fas in Jr cells could be explained by a lack of expression of the Fas receptor and/or by post-receptor mechanisms. We first compared by immunofluorocytometry the level of expression of Fas on the surface of Jd and Jr cells. Fas expression was not significantly different in either cell clone (Figure 1C). In fact, we found that many T cell lines were sensitive to Fas treatment while exhibiting lower levels of Fas than Jr cells (data not shown). Interestingly, we also observed that, as opposed to Jd cells, Jr cells were insensitive to Trail killing (not shown). Taken together, these observations strongly suggest that resistance of Jr to Fas-mediated apoptosis is due to post-receptor mechanisms. Although in many cell lines, resistance to Fas-mediated cell death can be overcome by inhibitors of protein synthesis, this is not the case in Jr cells since addition of low concentrations of cycloheximide did not sensitize them to Fas-mediated apoptosis.

Jr cells are sensitive to staurosporine and ceramide-induced apoptosis

We next examined whether Jr cells could initiate an apoptotic program when stimulated with pro-apoptotic stimuli other than CH11. Jd and Jr cells were incubated for 18 hours in the presence or absence of CH11, staurosporine, etoposide, cycloheximide, hydrogen peroxide or C2 ceramide before determination of cell viability using the XTT assay. Jd cells were found to be sensitive, although to various extents, to all the effectors tested (Figure 2A). While unaffected by CH11, cycloheximide or H_2O_2 treatment, Jr cells were efficiently killed by staurosporine and C2-ceramide . Consistently, all stimuli induced internucleosomal degradation in Jd cells at 6 hours but only staurosporine and C2-ceramide caused DNA fragmentation in Jr cells (Figure 2B). We also followed, by Western Blotting, the disappearance of procaspase 3, which represents a reliable index of its activation. Cleavage of procaspase 3 and its substrate poly-ADP-ribose polymerase (PARP) was assessed after a 6 hours exposure of Jd and Jr cells to different pro-apoptotic stimuli (Figure 2C). It should be noted that although PARP was efficiently cleaved in Jd cells exposed to C2 ceramide, the 25 kDa fragment was not detected. In fact, in C2 ceramide-treated cells, PARP was cleaved to a 55 kDa fragment which has been previously associated with necrosis. In Jd cells, all the effectors tested induced the

cleavage of procaspase 3 and PARP, while only staurosporine and C2 ceramide were effective in Jr cells.

Caspase expression and activation in Jd and Jr cells

The experiments described above demonstrate that under certain circumstances, Jr cells could initiate an apoptotic program indicating that the executive machinery of apoptosis is functional in these cells. Since Jr cells are totally insensitive to Fas killing, we first hypothesized that expression of proteins of the DISC may be altered in these cells. Because large prodomain caspases such as procaspases 8 and 10 coupled Fas receptor to the executive machinery of apoptosis, we analyzed by Western Blot, the expression and activation not only of these caspases, but also that of procaspases 2, 3 and 7 in both cell types. For this purpose Jd and Jr cells were stimulated for various times in the presence or absence of 100 ng/ml CH11. The level of expression of procaspases 2, 3, 7, 8 and 10 was found to be equivalent in Jr and Jd cells (Figure 3A). Incubation of Jd cells with CH11 resulted in the disappearance of procaspases 3, 7 and, to a lesser extent, procaspases 8 and 10. Conversely, we failed to detect any cleavage and activation of these caspases in Jr cells. Thus, although expressing normal level of caspases, Jr cells appeared to be totally insensitive to Fas. This observation is compatible with a defect located upstream of the caspases. Therefore, we measured the level of expression of the adaptor FADD which is essential for Fas-induced apoptosis. No difference in FADD expression was observed in the Jd *versus* Jr clone (Figure 3B). In addition, the level of expression of Bcl-2, Bax and Toso [24] was not significantly different in either cell clone.

Role of Hsp27 and 70 in the protection against apoptosis

Hsp proteins, more particularly Hsp27 and Hsp70, have been shown to promote cytoprotection and to rescue cells from Fas- and staurosporine-induced apoptosis [15]. We hypothesized that overexpression of Hsps in Jr cells could explain resistance to Fas-induced PCD in our cellular model. Thus, we investigated, by Western blotting, the expression of Hsp27, 60 and 70 in Jd and Jr cells. Hsp60 is constitutively expressed in mammalian cells, and a recent study has demonstrated that this Hsp was present in a complex with procaspases 3 and Hsp10 [25]. Western blot analysis of cellular extracts from Jd and Jr cells revealed a very high constitutive expression of Hsp27 in Jr cells and

the absence of this Hsp in Jd cells (Figure 4A). In addition, expression of Hsp70 was 3 times higher in Jr cells than in Jd cells. Conversely, we failed to detect any difference in the level of expression of Hsp60 between the two cell lines (Figure 4A).

While totally resistant to Fas-induced apoptosis, Jr cells can undergo programmed cell death following staurosporine treatment (Figure 2). We therefore, analyzed in more detail the sensitivity of Jd and Jr clones to Fas and staurosporine following a prior 2 hours incubation at high temperature (42° C). DNA fragmentation was then assessed after a further 4 hours treatment at 37° C in the presence or absence of either CH11 or staurosporine. Jd cells exposed to CH11 or staurosporine at 37° C exhibited characteristic DNA ladders, reflecting internucleosomal DNA degradation, a hallmark of apoptosis (Figure 4B), whereas in identical conditions, ladders were detected in staurosporine-treated Jr cells. Pretreatment of Jd or Jr cells at 42° C abolished Fas- and staurosporine-induced DNA fragmentation (Figure 4B). We also determined global caspase activity using Ac-DEVD-pNA as substrate, in cellular extracts prepared from cells pretreated or not at 42° C and then stimulated for 4 hours with either CH11 or staurosporine. Sensitivity of Jd and Jr cells to Fas and staurosporine was greatly correlated with the increase in caspase activity as shown on Figure 4C. Pretreatment of Jd cells at 42° C inhibited Fas and staurosporine-induced caspase activity by 95%. A drastic inhibition of caspase activity was also found in staurosporine-treated Jr cells, an observation in good agreement with the lack of DNA fragmentation observed in Figure 4B. Finally, as expected, we failed to detect any Ac-DEVD-ase activity in Fas-stimulated Jr cells (Figure 4C). Western blot analysis of Hsp expression revealed a significant increase in the level of both Hsp27 and Hsp70 in heat-stressed Jd cells (Figure 4D). Interestingly, staurosporine but not CH11, was found to drastically inhibit Hsp70 expression in Jd and Jr cells at 37° C but not at 42° C. Moreover, staurosporine also inhibited Hsp27 expression by Jd and Jr cells at 42° C (Figure 4D). Thus, in Jr cells, high constitutive expression of Hsp27 correlates well with the resistance to Fas-mediated apoptosis observed at 37° C. On the other hand, maintenance of a high level of Hsp70 appears to be necessary for protection against staurosporine-induced cell death following a prior heat-shock at 42° C.

DISCUSSION

Although a wide variety of signals are capable of inducing apoptosis in many cell lines, it always occurs by a highly conserved mechanism involving molecules that either favour or repress biochemical pathways required for cell suicide. Many factors may affect sensitivity of cells to these signals, including the state of differentiation and the cellular environment. Stress conditions are one of the situation where cells can undergo an apoptotic program. Hsp molecules (Hsp27 and Hsp70) seem to determine cellular resistance to a variety of stress factors in a given cell. In this line, cells which contains elevated levels of Hsps, either by overexpression or synthesised in response to prior stress conditions such as hyperthermia, are less sensitive to stress and are saved from apoptotic stimuli. The precise mechanism by which Hsps represses cell death are far from being fully understood, but there are some indications that Hsps can prevent PCD by affecting SAPK/JNK activation and/or inhibiting the executive phase of apoptosis [16, 26, 27]. In this study, we analyzed PCD in two variants of the human leukemic Jurkat T cell line (Jd and Jr). Parental Jd cells were found to be sensitive to a large variety of pro-apoptotic signals including staurosporine, etoposide, hydrogen peroxide, cycloheximide or C2-ceramide, but exhibited a particularly high sensitivity to Fas- and Trail receptor-induced apoptosis (Figure 2) ([19] and not shown). While efficiently killed by staurosporine and C2-ceramide, Jr cells exhibited a complete resistance to Fas- or Trail-mediated apoptosis (Figure 1 and 2, and data not shown). Accordingly, caspase 3 was clearly detectable in Jd cells whatever the pro-apoptotic stimuli tested, but was observed only in staurosporine or C2-ceramide-treated Jr cells (Figure 2). This clearly indicates that the executioner caspases are functional in Jr cells. Moreover, Western blot analysis of the main caspases involved in the regulation of apoptosis shows that they are present at similar levels in both cell clones. Nevertheless, it appears that differences in caspase expression in each cell line is unlikely to account for the variations observed in the sensitivity of Jd and Jr cells to apoptosis. However, we cannot totally rule out the possibility that the activity of initiatory caspases such as caspase 8 or 10 is impaired in Jr cells, which would be also consistent with the resistance of these cells to death receptor-induced apoptosis. Other possible mechanisms to explain the resistance of Jr cells could be variations in the expression of pro-apoptotic molecules such as the adaptor FADD, the pro-apoptotic members of the Bcl-2 family (Bax, Bad and Bid...) or proteins that promote cell survival such as Bcl-2 or Bcl-X_L. Our results however, show that the expression of FADD, Bcl-2, Bax, and Toso were not significantly affected in Jr *versus* Jd cells. Moreover, no modification in the expression of any of these proteins was detected following CH11 treatment. Thus, apart from a discrete mutation in the sequence of one of

these proteins leading to a loss of function, we can exclude the possibility that resistance of Jr cells to Fas-induced apoptosis may reflect an alteration in the expression of one of these molecules. Interestingly, while testing for the presence of proteins whose expression is modified in Jr cells *versus* Jd cells, we identified Hsp proteins as candidates possibly involved in the resistance of Jr cells to Fas-mediated PCD. Indeed, Jr cells exhibited high constitutive Hsp27 levels. Furthermore, we found that Hsp70 expression was at least 3 times higher in Jr than in Jd cells. Moreover, there was a good correlation between Hsp expression and resistance to PCD, more particularly in Jd cells following hyperthermia. Resistance to apoptosis was also accompanied by a drastic decrease in global caspase activity. Our data showing a protective role of constitutive and inducible Hsps towards apoptosis are in agreement with other studies in the literature [15, 28, 29]. Furthermore, the observation that Jr cells are resistant to death receptor-induced apoptosis but sensitive to staurosporine suggests that constitutive expression of Hsps may more specifically impair apoptosis at the death receptor level.

The targets for Hsp inhibition of apoptosis are not entirely known at present, but it is well established that Hsps can act as molecular chaperones or as sensors for ROS concentrations, two functions which could participate in their protective effect on apoptosis [13]. Interestingly, cellular stresses have been shown to activate protein kinase B (Akt) and a direct association of Akt and Hsp27 has also been demonstrated, but only when cells were treated with stress-inducing agents [30]. Akt is also known to prevent apoptosis [31, 32], but the precise relationship between activation of Akt and its interaction with Hsp27 is not clear. However, an effect of Hsp27 at the level of Akt could explain at least partially, the resistance of Jr cells to Fas-mediated apoptosis. On the other hand, Hsp70 has been proposed to act either directly at the death-inducing signaling complex level (DISC) [33], or later in the death signaling pathway, more probably downstream of caspase 3 [34]. Hsp27 has been also shown to inhibit cytochrome c-dependent activation of procaspase 9 [35]. However, it is unlikely that inhibition of procaspase-9 by Hsp27 contributes to the resistance of Jr cells to Fas-induced apoptosis since caspase 9 appears dispensable for Fas-mediated apoptosis [36, 37]. Furthermore, it has been also shown that Hsp27 inhibits Src kinase activity in cardiomyocytes [38]. In this line, we have previously reported that the Src kinase p59Fyn was cleaved and relocalized during Fas-mediated apoptosis in Jd but not Jr cells [19]. Thus, an interesting hypothesis to explain the insensitivity of Jr cells to Fas-mediated apoptosis

would be that high constitutive expression of Hsp27 in these cells contributes to repress p59Fyn activity, thus inhibiting apoptosis.

Elevated levels of Hsp70 or Hsp27 can also protect cells from TNF-induced cell death [34, 39], and targeted disruption of Hsp70 in the male germ line of transgenic mice results in apoptosis [17]. In addition, overexpression of Hsp27 has been previously shown to inhibit Fas- and staurosporine-induced cell death [15]. Thus the high constitutive level of both Hsp27 and Hsp70 in Jr cells is likely to represent one of the causes of the resistance of these cells to Fas-mediated apoptosis. Accordingly, several recent studies have shown that Hsp70 and 90 inhibit activation of procaspase-9 and apoptosis by direct interaction with Apaf-1, while Hsp27 blocks cell death by interacting with cytochrome c [40-42].

CONCLUSION

In conclusion, although we cannot rule out the possibility that other defects in Jr cells may account for the resistance to apoptosis, the data reported here show that high constitutive expression of Hsps in Jr cells could significantly contribute to their resistance to death receptor-induced apoptosis. Experiments are currently underway to determine precisely the level of Hsp action, but recent data from our group indicate that the high constitutive expression of Hsp27 found in Jr cells may be due to post-translational mechanisms, since we found an equal amount of Hsp27 mRNA in Jd as compared to Jr cells (not shown). In this line, it is worth noting that Hsp antiapoptotic function also has been shown to be regulated by phosphorylation and oligomerization [43-44]. This observation may explain why introducing an anti-sense Hsp27 cDNA failed to restore death receptor-induced apoptosis in Fas-resistant Jr cells (not shown).

Acknowledgements. This work was supported by INSERM and The Ligue Nationale contre le Cancer: Equipe Labellisée.

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Figures

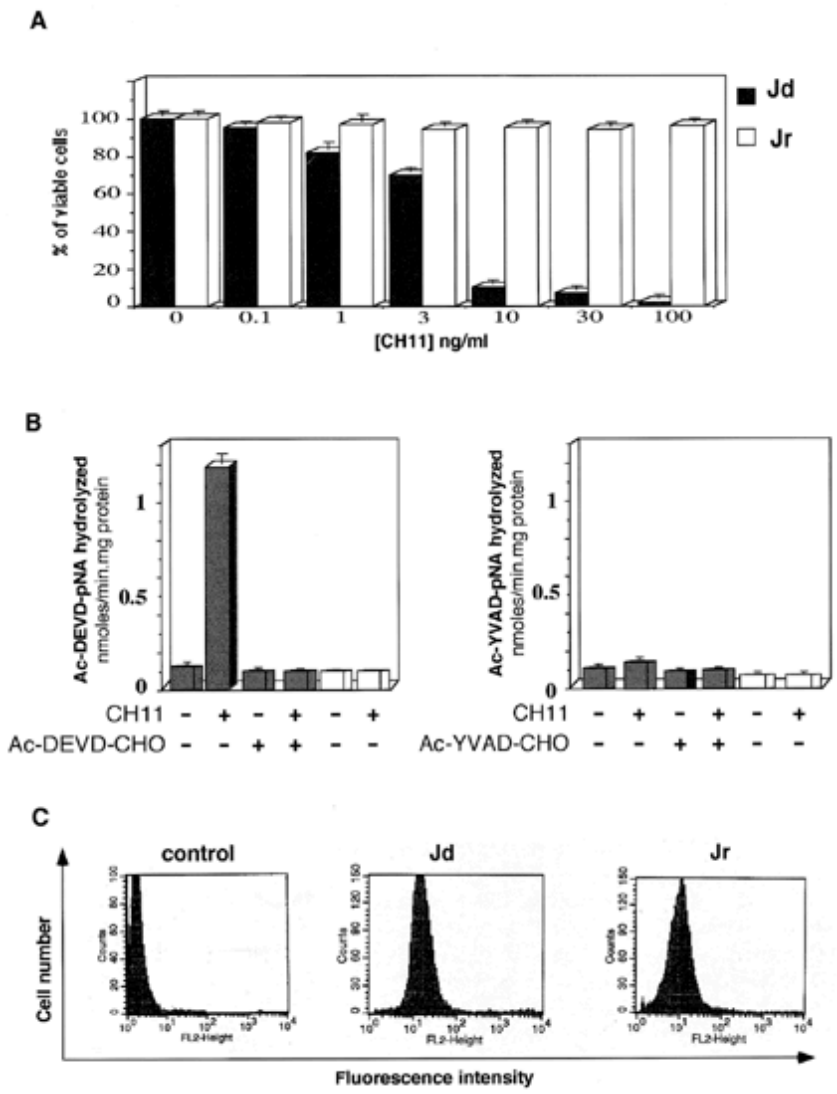


Figure 1

Sensitivity of Jd and Jr T cell clones to Fas-induced apoptosis.

A: effect of different concentrations of the anti-Fas mAb CH11 on Jd and Jr cell viability after 18 hours of treatment: viability was determined by the XTT assay as described in the Materials and Methods section. **B:** Determination of caspase enzymatic activity in Jd and Jr cells: cells were treated with or without 100 ng/ml CH11 for 6 hours. Caspase activity was assessed by the hydrolysis of Ac-DEVD-pNA or Ac-YVAD-pNA. Results are expressed as nmoles of substrate hydrolyzed per min and per mg of protein. **C:** immunofluorocytometric analysis of Fas expression. Cells were incubated with 2 mug/ml of the

anti-Fas mAb, ZB4, (Fab')2 fragments of rabbit anti-mouse conjugated to biotin and with streptavidin conjugated to phycoerythrin and analyzed by dual colour immunocytofluorimetry.

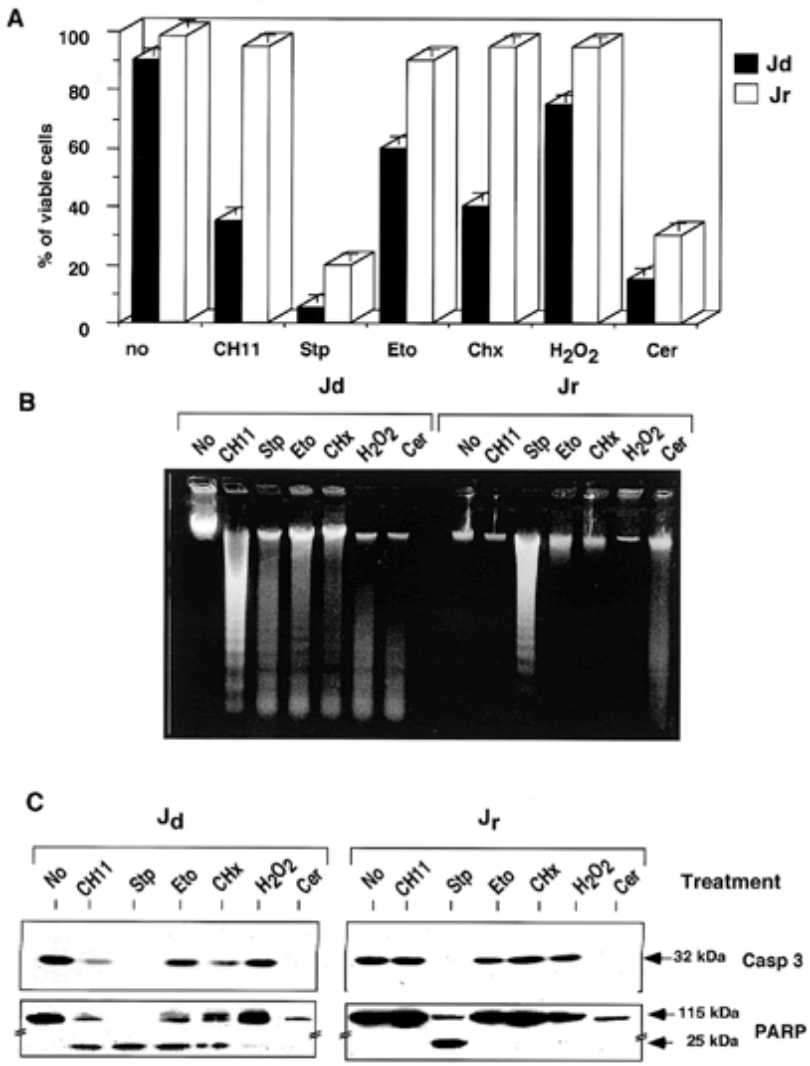


Figure 2

Effect of different proapoptotic stimuli on Jd and Jr cells apoptosis.

A: effect of different pro-apoptotic stimuli on Jd and Jr cell viability. Cells were incubated for 18 hours with 100 ng/ml CH11, 1 muM staurosporine, 50 muM etoposide, 20

muM cycloheximide, 1 mM H_2O_2 or 50 mug/ml C2-ceramide before determination of cell viability by the XTT assay. **B:** internucleosomal DNA fragmentation was visualized by agarose gel electrophoresis on cells after 6 hours of incubation with the different effectors.
C: cells were incubated for 6 hours with the different pro-apoptotic stimuli and then lyzed, and proteins were separated by electrophoresis on 11% polyacrylamide gels. Proteins were blotted to PVDF membranes which were further incubated with either anti-caspase 3 or anti-PARP antibodies. Arrows indicate the migration of procaspase 3, p115 PARP and p25 PARP.

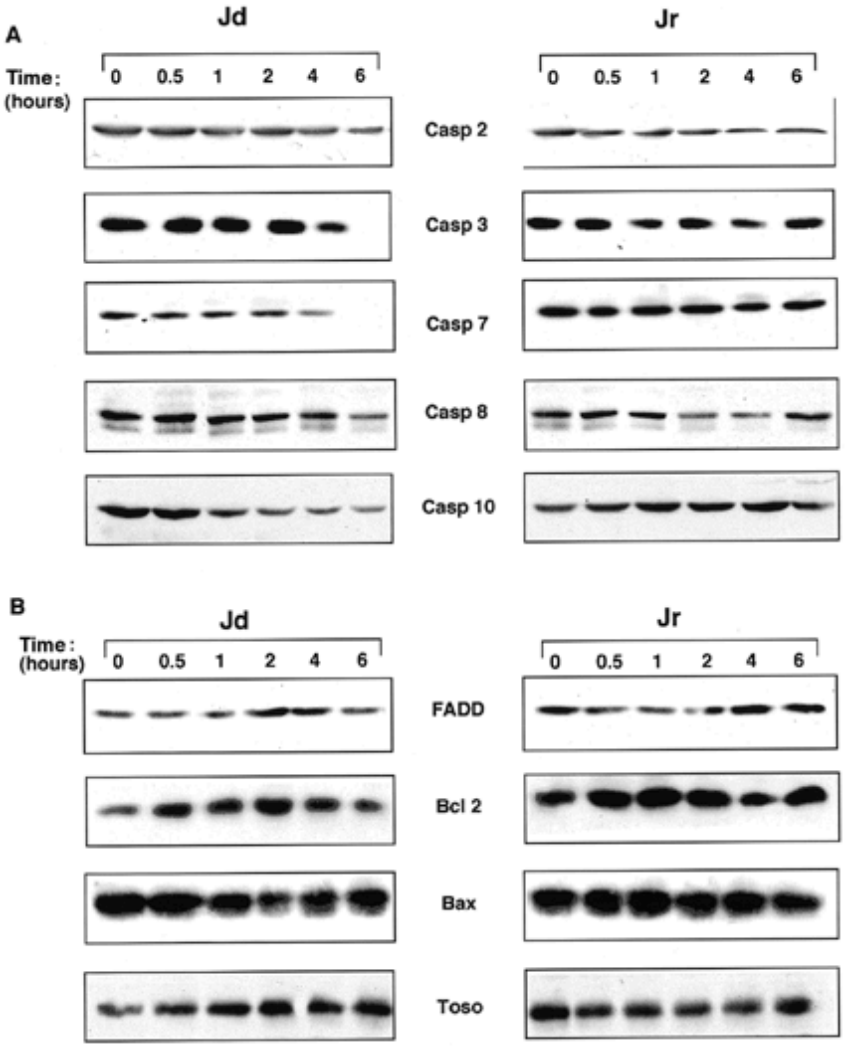


Figure 3

Expression of different pro- or anti-apoptotic proteins in Jd and Jr cells.

A: cells were incubated for various times (0 to 6 hours) with 100 ng/ml of CH11. They were then lyzed and proteins separated on 11% polyacrylamide gels. Proteins were blotted on PVDF membranes which were incubated with anti-procaspase 2, anti-procaspase 3, anti-procaspase 7, anti-procaspase 8 or anti-procaspase 10 antibodies. **B:** experimental conditions were those described in **(A)**. Proteins were blotted on PVDF membranes which were incubated with anti-FADD, anti-Bcl-2, anti-Bax or anti-Toso antibodies.

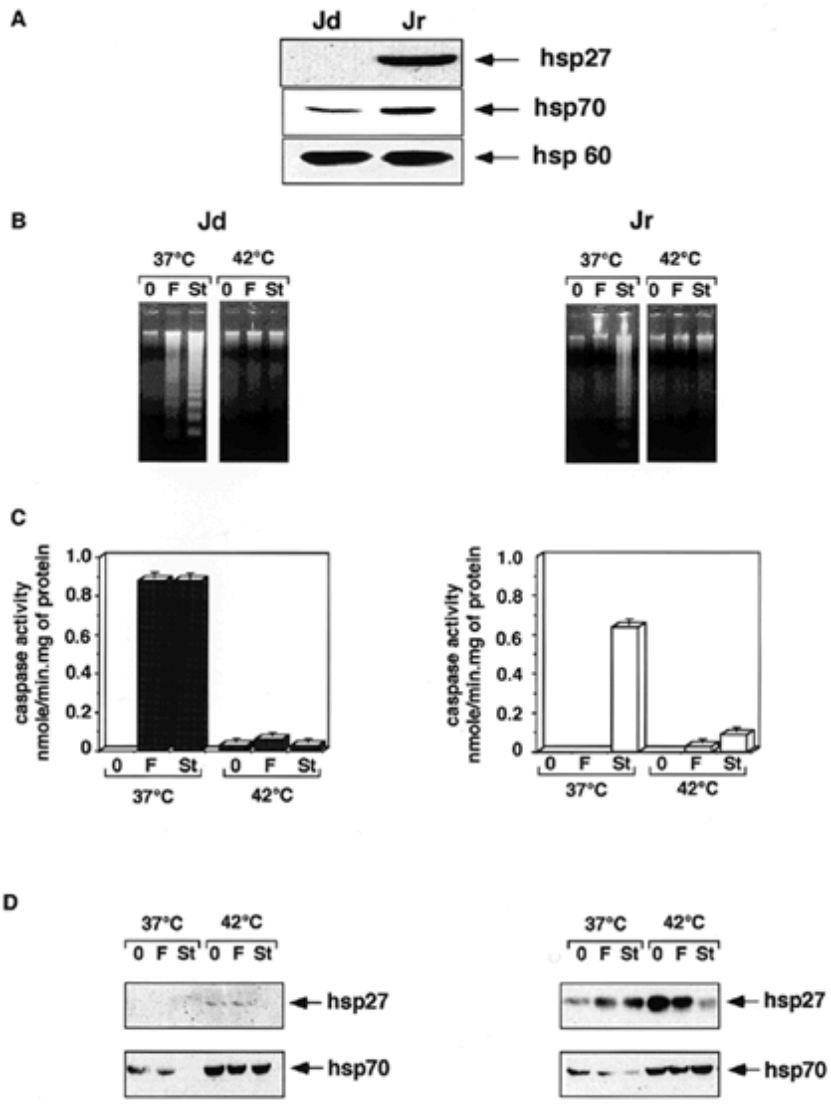


Figure 4

Expression of heat shock proteins in Jd and Jr cells and role in the protection against apoptosis.

A: Jd or Jr cells were lyzed and proteins separated on 11% polyacrylamide gels. Proteins were blotted on PVDF membranes which were then incubated with anti-Hsp27, anti-Hsp60 or anti-Hsp70 antibodies.
B: internucleosomal DNA fragmentation in heat-stressed Jd and Jr cells. Cells were preincubated for 2 hours at 37° C or 42° C before additon of 100 ng/ml of CH11 or 1 muM staurosporine for 4 hours at 37° C. DNA fragmentation was then assessed as described in Figure 2. **C:** determination of caspase enzymatic activity in heat-stressed Jd and Jr cells: cells were treated as described in **(B)**. Caspase activity was assessed by the hydrolysis of Ac-DEVD-pNA. Results are expressed as nmoles of substrate hydrolyzed per min and per mg of proteins.
D: cells were incubated as in **(B)**, lyzed and proteins separated by electrophoresis on 11% polyacrylamide gels. Proteins were then blotted to PVDF membranes which were incubated with either anti-Hsp27 or anti-Hsp70 antibodies.

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IL-10, recombinant, rat, cytokines.

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