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Differential Regulation of Cell Cycle-Related Proteins by CD95 Engagement in Thymocytes and T Cell Leukemic Cell Line, Jurkat

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Abstract CD95 engagement results in apoptosis in thymocytes and in the Jurkat human leukemic T cell line. Biochemical analyses in CD95-engaged thymocytes and Jurkat cells revealed dysregulation of the G1/S cell cycle control point. Cyclin E was upregulated upon CD95 engagement, suggesting G1-to-S progression, but there was no upregulation of cyclin A. Instead, cyclin E was degraded by caspases. In addition, c-myc that normally acts on S-phase progression through the activation of cdc25A appeared to be involved in the inhibition of S-phase progression after CD95 ligation. This implies that G1 → S progression and apoptosis are intimately linked in cells undergoing CD95 ligation. Furthermore, our data suggest that CD95-induced apoptosis occurs at the G1/S phase transition. We therefore suggest that CD95 engagement not only triggers death signals but also affects the G1/S checkpoint. J. Cell. Biochem. 80:328–338, 2001. © 2001 Wiley-Liss, Inc.

Key words: apoptosis; cell cycle proteins; G1 checkpoint; thymocytes; cyclins; caspases

Apoptosis is the process in which cells commit suicide, and it is characterized by distinctive morphological and biochemical changes [Ashkenas and Werb, 1996; King and Cidlowski, 1998; Zhou et al., 1998]. Apoptosis may occur as a result of cytotoxic stresses, engagement of specific death receptors including the tumor necrosis factor receptor-1 (TNFR1), and treatment with chemicals including some chemotherapeutic agents [D'Adamio et al., 1997; Ashkenazi and Dixit, 1998; King and Cidlowski, 1998].

CD95 (Apo-1 or Fas) is a member of the TNFR1 family, and is expressed on various cell types including thymocytes [Nagata, 1997], activated T cells [Crispe, 1994; Green and Scott, 1994], T cell leukemic lines [Zhou et al., 1998], and hepatocytes [Huang et al., 1994]. CD95–CD95L (CD95 ligand) interaction results in apoptosis through well-studied mechanisms. This process is known to be involved in immune tolerance, the regulation of immune responses and the killing of virally infected cells [Crispe, 1994; Green et al., 1994]. When CD95 interacts with CD95L, CD95 is trimerized, and caspase-8 is subsequently activated through interaction with FADD (Fas Associated Death Domain) [Chinnaiyan et al., 1995]. Activated caspase-8 causes the activation of caspase-3, leading to the cleavage of multiple vital substrates and apoptotic cell death [Srinivasula et al., 1996]. CD95 itself has also been reported to be involved in other functions, including a costimulatory effect that potentiates the response to T cell receptor (TCR) signals in human cells [Alderson et al., 1993]. This implies that the CD95 signal might be related to cell cycle progression.

The apoptotic machinery has a complicated and poorly understood relationship with cell cycle control. In T cells, activation is essential to induce full susceptibility to CD95-induced apoptosis, yet apoptosis signals from CD95 preferentially kill thymocytes or activated T cells that are in the G0/G1 phases of the cell

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cycle, but spare cells in S phase [Dao et al., 1997]. While it was recently reported that the TCR-mediated, antigen-induced cell death occurs from a late G1 phase in mature T cells [Lissy et al., 1998], Boehme and Leonardo [1993] reported that S phase cells are more susceptible to various apoptotic signals. These apparent contradictions leave the relationship between CD95 signals and the cell cycle very obscure.

Although the cell cycle's involvement in apoptosis is not fully understood, it is likely that there is a tight linkage between death signals, including CD95 signals, and cell cycle regulation [King et al., 1998]. In fact, it has been reported that various proteins, which are involved in cell cycle control, also regulate susceptibility to apoptosis. These include p53, the retinoblastoma protein family (p110Rb, p107, and p130), cdc25, max, c-myc, and E2F-1, [Mazel et al., 1993; Field et al., 1996; Linette et al., 1996; Vairo et al., 1996; Yamasaki et al., 1996]. This led us to investigate if CD95 signals act on the G1/S cell cycle checkpoint.

CD4 + CD8 + thymocytes, which comprise more than 90% of total thymocytes, are noncycling and are known to be sensitive to many apoptotic stimuli including CD95-induced death. Also, a T cell leukemic cell line, Jurkat, is sensitive to CD95-mediated apoptosis, as well as activation-induced cell death (AICD) [Lissy et al., 1998; Zhou et al., 1998]. We report here that ligation of CD95 on thymocytes and Jurkat cells resulted in the modulation of cell cycle proteins, such as cyclin D1, cyclin D3, cyclin E, p107, p130, $p27^{Kip-1}$, c-myc and cdc25A, resulting in biochemical changes characteristic of late G1 arrest.

METHODS

Mice

CBA mice were purchased from the Jackson laboratory (Bar Harbor, ME) and the Fas^{lpr-cg} mice were derived from Dr. Matsuzawa's colony (University of Tokyo, Japan). The animals were maintained and bred by the Yale Animal Resources Center, and used at 3 or 4 weeks of age.

Cells and Reagents

Jurkat cells were maintained in RPMI media (prepared in-house by the Yale Immunobiology media facility) containing 10% FCS, 2 mM L-glutamine, 50 μM β-ME, 10 mM HEPES and 100 U/ml each of penicillin and streptomycin. Polyclonal or monoclonal antibodies against a number of cell cycle molecules were purchased from Santa Cruz Biotechnology. They are anti-cyclin D1 (sc-717), anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-755), anti-cyclin E (sc-481), anti-cyclin A (sc-596), anti-E2F-1 (sc-193), anti-E2F-4 (sc-866), anti-$p27^{Kip-1}$ (sc-776), anti-CDK2 (sc-163), anti-p130 (sc-317), anti-p107 (sc-250), anti-c-myc (sc-764), and anti-cdc25A (sc-7157) antibodies and used as 1:500 dilutions. Anti-mouse and anti-rabbit Ig antisera coupled with horseradish peroxidase (HRP) were also from Santa Cruz Biotechnology. Anti-β-actin MAb was purchased from Sigma (St. Louis, MO).

Cell Culture and FACS Analysis

Thymocytes were isolated by mechanical disruption of fresh mouse thymi in a ground-glass tissue grinder and used ex vivo. Killing experiments were co-cultures between fresh thymocytes and control (pSRα72.N1 vector-transfected) or CD95L-transfected fibroblasts. The fibroblasts were NIH-3T3 cells, and were described previously [Dao et. al., 1997]. Both NIH-3T3 cell clones were maintained under constant selection using G418 at a concentration of 500 μg/ml, and plated without G418 only to establish semi-confluent monolayers the day before experiments. In order to test CD95 mediated apoptosis, thymocytes or Jurkat cells were suspended at 3×10^7 and 5×10^6 cells, respectively, in a final volume of 10 ml of RPMI culture medium. Thymocytes or Jurkat cells were layered over control or CD95L-expressing fibroblasts in 10 cm dishes (Falcon 35-3003, Becton-Dickinson, Franklin Lakes, NJ). At indicated time points, non-adherent thymocytes or Jurkat cells were harvested and then tested for apoptosis (PI staining) or lysed for Western blot analysis. To detect subdiploid cells, the cells were incubated with propidium iodide (PI) at 50 μg/ml (Sigma) for 30 min in 0.1% sodium citrate, 0.3% Nonidet P-40 and 50 μg/ml RNAse. PI stained cells were analyzed using a FACScan (Becton-Dickinson, Mountain View, CA) and data were analyzed using CELLQUEST software.

Immunoblotting Analysis

Thymocytes and Jurkat cells were centrifuged and the pellet was washed and lysed in

TABLE I. Cell Cycle Analysis upon CD95 Engagement in CBA and *lpr/cg* Mouse Thymocytes[a]

Thymocytes from		Treatment[b]			
		CBA		*lpr/cg*	
Exp.[c]	Phase[d]	pSR	CD95L	pSR	CD95L
1	<G1	1.31	26.14	0.63	0.52
	G0/G1	75.34	53.15	58.27	59.27
	S	6.08	13.36	4.41	4.55
	G2/M	17.39	7.47	29.15	27.42
2	<G1	21.21	46.06	2.28	5.12
	G0/G1	62.55	31.89	81.80	75.48
	S	8.27	12.50	7.54	8.51
	G2/M	7.21	9.94	8.37	11.04
3	<G1	2.31	34.07	2.14	1.70
	G0/G1	73.79	45.71	69.40	71.08
	S	6.73	11.99	7.02	6.84
	G2/M	17.33	8.43	18.58	17.53

[a]The percentages of thymocyes in each cell cycle are shown.
[b]Thymocytes were co-cultured with control (pSR) or CD95L expressing fibroblasts for 5 h.
[c]Three independent experiments were applied.
[d]PI staining was performed to detect the percentages of each phase in thymocytes. Apoptotic subdiploid cells were shown as <G1.

the Laemmli buffer containing a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Total cell extracts were normalized for protein concentration, and samples containing 30 μg of protein were loaded onto 6, 8, 10, 12.5 or 15% SDS-PAGE gels, separated by electrophoresis, and transferred to PVDF Immobilon membranes (Millipore, Bedford, MA) using an electrotransfer apparatus from Bio-Rad (Hercules, CA). Transferred membranes were incubated with primary antibodies recognizing each of the cell cycle related proteins for 1–3 h at room temperature, followed by extensive washing. Immune complexes were tagged using goat anti-rabbit or rabbit anti-mouse antibodies conjugated with HRP, then visualized using the Enhanced Chemi-Luminescent kit from Amersham Life Sciences (Buckinghamshire, UK).

RESULTS

CD95 Engagement Induced Apoptosis in Thymocytes but S Phase Cells were Protected from Apoptosis

In order to analyze the cell cycle of thymocytes undergoing CD95-mediated apoptosis, PI staining was performed after incubation with control or CD95L expressing 3T3 cells [Dao et al., 1997] for 5 h. The lpr-cg mice were used as control as they express mutant CD95 that cannot trigger the death signal. After 5 h incubation, 25–47% of cells exposed to CD95L-expressing 3T3 cells appeared to be apoptotic (i.e., subdiploid) (Table I) although there was a variation in spontaneous apoptosis in the absence of CD95L (Table I, Exp. 2). As expected, no apoptosis was detected in cells from lpr-cg mice. Interestingly, whereas there was a significant reduction of G1 phase cells from wild-type mice, CBA, the percentage of S phase cells was increased upon CD95 ligation (Table I). However, this was not the case in thymocytes from lpr-cg mice after the engagement with CD95L-expressing 3T3 cells (Table I). This finding raised the question whether these might be a modulation of the cell cycle in thymocytes during CD95-induced apoptosis. Alternatively, this could be simply an effect of the preferential apoptosis of G0/G1 phase cells, as we have described before [Dao et al., 1997; Hingorani et al., 2000].

Differential Expression of Cell Cycle-Related Molecules in Thymocytes by CD95 Engagement

As there were changes in the cell cycle profile from CD95-ligated thymocytes (Table I), im-

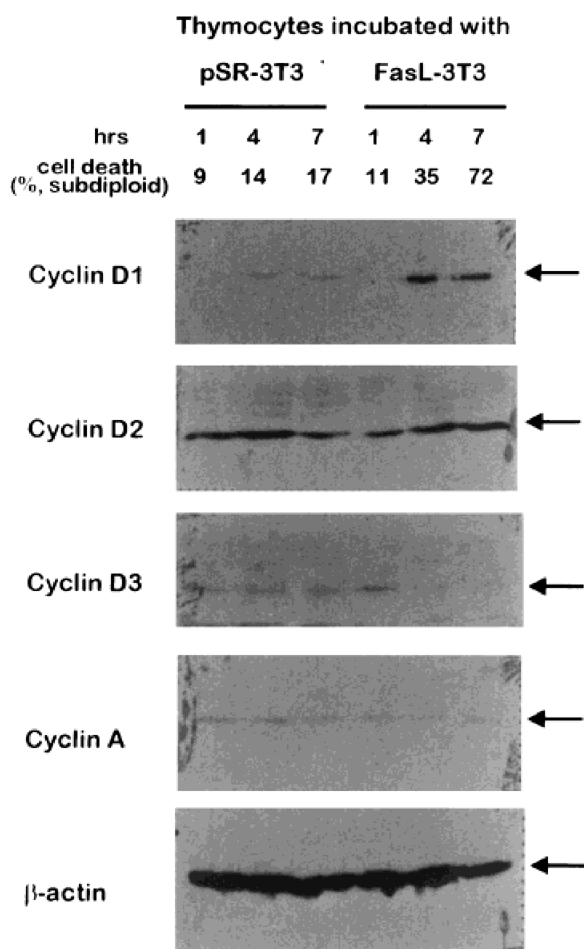

Fig. 1. Expression of cyclins upon CD95 ligation. Normal mouse thymocytes were co-cultured with either control (pSR-3T3) or CD95L-expressing (FasL-3T3) cells. At indicated time points, thymocytes were collected and the percentages of apoptosis were based on PI staining. 30 μg of protein extracts achieved from thymocytes were used in each lane. Each arrow indicates the expression of cyclin. Actin was probed as a loading control. The experiment shown is a representative of three that gave similar results.

munoblotting was performed to examine the expression patterns of various cell cycle-related proteins. Figure 1 shows that there were no significant changes in the expression of cyclin D2 and cyclin A. In the case of cyclin D1, there was a noticeable increase as a result of CD95 signaling (Fig. 1). Cyclin D1 protein levels were increased after 4 and 7 h of incubation with CD95L-expressing 3T3 cells and the increased level of cyclin D1 was maximized at the 4 h time point. In addition, cyclin D3 appeared to be slightly downregulated by CD95 ligation.

The p110Rb pocket proteins are phosphorylated as the cell cycle progresses from G1 to S phase [King and Cidlowski, 1998]. Among pocket proteins, in this study, the phosphorylation of p130 and p107 was examined. In accordance with the results shown in Table I and Figure 1, both p130 and p107 were seen to be phosphorylated (Fig. 2). After 1 h of incubation with CD95L-expressing 3T3 cells, the phosphorylation of those proteins was evident (Fig. 2, arrowheads). Like the p110Rb molecule [Tan and Wang, 1998], there are caspase sensitive sites in both p130 and p107, and these can produce 20 and 15 kD fragments, respectively, from the sequence analysis (sequences not shown). In fact, the cleaved products were produced by CD95 ligation in thymocytes (Fig. 2). As a result of the cleavage, the density of the p130 and p107 bands was reduced (Fig. 2).

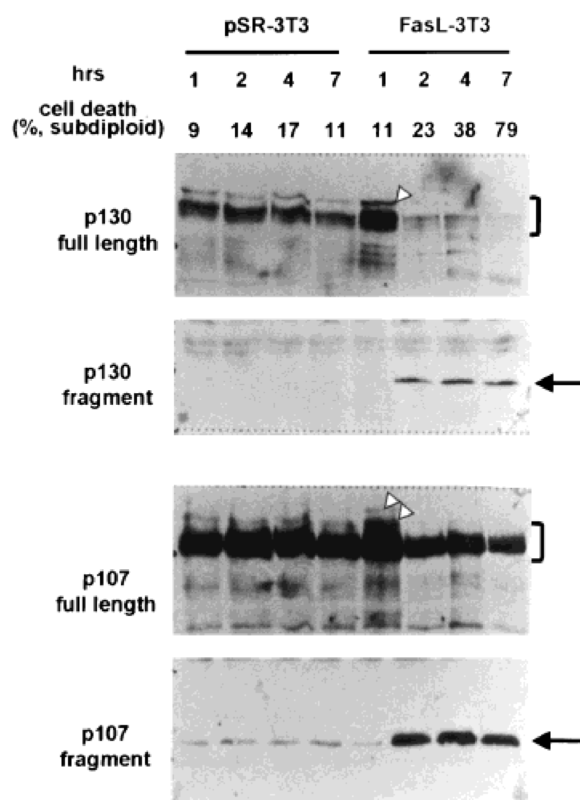

Fig. 2. Cleavage of the pocket proteins upon CD95 engagements. The pocket proteins, p130 and p107, were detected from thymocytes incubated with CD95L expressing or control fibroblasts as in Figure 1. 30 μg of protein extracts were used for Western blot analysis and both under- or hyperphosphorylated proteins were indicated as brackets. Especially, hyperphosphorylated proteins were indicated as arrowheads. Fragments derived from cleavage by caspase activity were also shown as arrows.

Upregulation and the Cleavage of Cyclin E in CD95-Mediated Thymocyte Death

Cyclin E is expressed in late G1 and degraded in S phase as cyclin A is made [King and Cidlowski, 1998]. As the G1 phase cells were thought to be the targets of CD95-induced apoptosis [Dao et al., 1997; Hingorani et al., 2000], cyclin E was checked in addition to cyclin D and cyclin A. Unlike cyclin A (Fig. 1), cyclin E was easily detected by Western blot analysis in thymocytes (Fig. 2). Intact cyclin E was upregulated by CD95 engagement (Fig. 3, arrows), with a concomitant appearance of cleaved products (Fig. 3, arrowheads) that could have been generated by the activity of caspases. In fact, there are three possible caspase-3 cleavage sites, with the sequence motif DxxD, in both human and mouse cyclins E (at positions 6, 169, and 366 in mouse, based on sequence accession number CAA53482 in GenBank). This was also the case in Jurkat cells following CD95 engagement (Fig. 7). Therefore, cyclin E is one of the substrates of caspase(s) activated by CD95 ligation in thymocytes and a T cell line.

Regulation of Other Cell Cycle Related Molecules in Thymocytes

Cell cycle progression is controlled by cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CKI) and transcription factors including the E2F family as well as cyclins. In order to understand the impact on cell cycle progression during CD95-mediated death, we examined the changes of cell cycle proteins by Western blotting. In the case of E2F-1, there was an increased amount of higher molecular weight form, which is thought to be the phosphorylated E2F-1, especially at the 4 h time point (Fig. 4 arrowhead). On the contrary, the total amounts of E2F-4 were slightly reduced during CD95-mediated death in thymocytes. It is well known that CDK2 acts in late G1 and S phases by binding to cyclin E and subsequently to cyclin A [Hunter and Pines, 1994]. It has been shown that p27^{Kip-1} is expressed in the G1 phase and quickly downregulated at the G1/S boundary [Shirane et al., 1999] following the upregulation of CDK2 [Gil-Gomez et al., 1998]. As shown in Figure 4, the expression of p27^{Kip-1} is downregulated after 2 h incubation with CD95L.

The Control of c-Myc Pathway by CD95 Engagement in Thymocytes

Apart from the cyclin/cdk and E2F pathway, it is clear that c-myc also plays an important role in cell cycle control [Jansen-Durr et al.,

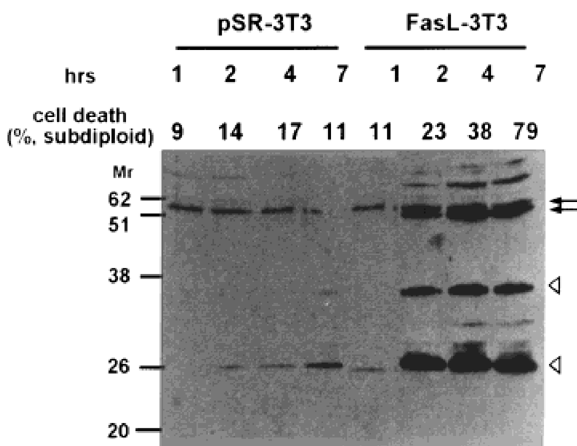

Fig. 3. The expression and cleavage of cyclin E. Cyclin E was checked at each time point after incubated with FasL-3T3 or control 3T3 (pSR-3T3) cells as in Figure 1. CD95 ligation induced the expression and the cleavage of cyclin E. Intact proteins are indicated as arrows and the arrowheads indicate the cleaved products of cyclin E.

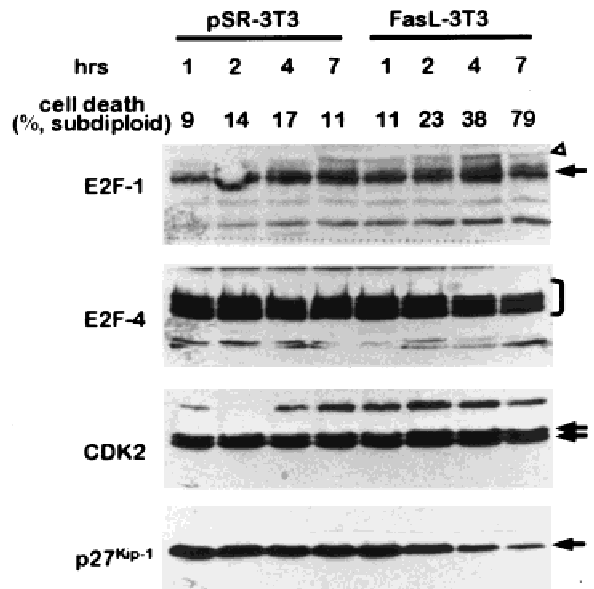

Fig. 4. Expression of transcription factor, E2Fs, CDK2 and p27^{Kip-1} in CD95 engaged thymocytes. The experiment was performed as described in Figure 1. E2F-1 and E2F-4 are indicated as arrow (E2F-1) and a bracket (E2F-4). Arrowheads, in case of E2F-1, indicate hyper-phosphorylated molecules. Expression of CDK2 and p27^{Kip-1} is shown as arrows.

1993; Vlach et al., 1996; Alevizopoulos et al., 1997; Leone et al., 1997; Bouchard et al., 1998]. In order to understand a possible role of c-myc in CD95-mediated apoptosis, the level of c-myc expression was examined. Incubation of thymocytes with CD95L-expressing 3T3 cells for 7 h caused a reduction in c-myc expression than in control thymocytes and the down-regulation was time-dependent. It is well known that c-myc acts as a transcription factor for cdc25A, which controls the activity of cdk2 [Jinno et al., 1994; Iavarone and Massague, 1997]. Therefore, we next examined the protein level of cdc25A. Figure 5 showed the decreased level of cdc25A expression by CD95 ligation in thymocytes. Decreased expression of c-myc and cdc25A was evident after 7 h incubation with CD95L-expressing fibroblasts (Fig. 5).

Blockade of the Regulation and Degradation of Cell Cycle Proteins by Caspase Inhibitor

Table I showed an increase of the percentage of S phase cells as well as that of subdiploid cells caused by CD95 ligation. This was not the case in lpr-cg mice (Table I) suggesting that the death domain (DD), which resides in the cytoplasmic tail of CD95, may play an important role in cell cycle progression as well as apoptosis. To test this role of the DD, we treated cells with zVAD-fmk at 50 μM final concentration to block the function of the caspases. When zVAD-fmk was added to the thymocytes, apoptosis was completely blocked (by PI staining, data not shown). We next examined the expression and the degradation of cyclin E. Both accumulation and the degradation of cyclin E were inhibited by a pan caspase inhibitor, zVAD-fmk (Fig. 6). Likewise, the down-regulations of $p27^{Kip-1}$ and c-myc were completely blocked (Fig. 6). Therefore, the DD might play an important role in cell cycle progression, as well as its role in CD95-mediated apoptosis in thymocytes. On the contrary, in case of thymocytes, it appeared consistent with Figure 4 that levels of CDK2 show hardly any changes with the engagement of CD95L in the absence or presence of zVAD-fmk (Fig. 6).

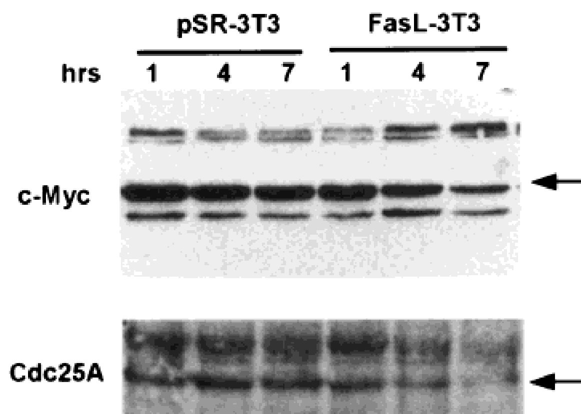

Fig. 5. Down-regulation of c-myc and cdc25A by CD95 ligation in thymocytes. As in Figure 1, c-myc and cdc25A are detected by Western blot analysis and each molecule is indicated as arrows. In both cases, the densities were reduced by CD95 ligation.

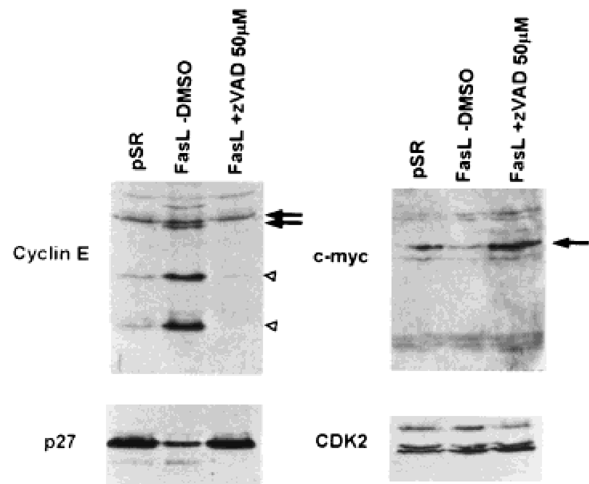

Fig. 6. Caspase dependent modulation of cell cycle proteins upon CD95 engagement. Thymocytes were co-cultured for 5 h with either control (pSR-3T3) or CD95L-expressing (FasL-3T3) cells in the presence or absence of zVAD-fmk. Blockade of caspase action by pan-caspase inhibitor, zVAD-fmk, abolished the induction and cleavage of cyclin E, down-regulation of c-myc and deregulation of $p27^{Kip-1}$ as probed by Western blot analysis. Arrows indicate intact molecules (cyclin E and c-myc), and in case of cyclin E, arrowheads indicate the cleaved products.

The Regulation of Cell Cycle Related Molecules is Observed in Jurkat Human T Cell Line

Most thymocytes are in CD4 + CD8 +, double positive stage, and they are known to be non-cycling and are sensitive to many apoptotic signals. We therefore addressed the question whether the regulation of cell cycle molecules through CD95 signals is restricted to the thymocytes. Jurkat cells are known to be the targets of CD95-mediated apoptosis. We checked the expression levels of a number of cell cycle-related molecules at the protein level. As shown in Figure 7, the regulation patterns of

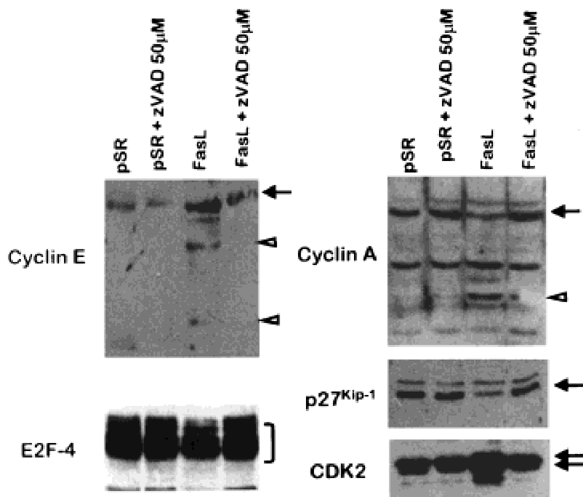

Fig. 7. Changes of cell cycle regulatory proteins in Jurkat cells by CD95 ligation in the presence or absence of pan-caspase inhibitor, zVAD-fmk. Western blot analysis was performed with the corresponding antibodies. In the cases of cyclin E and cyclin A, arrows and arrowheads indicate intact molecules and the cleaved products, respectively. Arrows in $p27^{Kip-1}$ and CDK2 show the expression of each molecule.

these molecules appeared to be similar as in thymocytes. There were no changes in the expression of cyclin D3 (data not shown) upon CD95 engagement, but we were able to detect the accumulation and the degradation of cyclin E (Fig. 7). As expected, the differential regulation of cyclin E by CD95 ligation was inhibited upon the treatment of zVAD-fmk. However, the blockade was not observed by DEVD-CHO treatment (data not shown). As described above, we could hardly detect cyclin A in case of thymocytes. Surprisingly, in the case of Jurkat cells, there was a reduction in cyclin A caused by CD95 ligation, and the reduction was inhibited by zVAD-fmk. As was evident in thymocytes, $p27^{Kip-1}$ expression was also decreased by CD95 ligation and the down-regulation of $p27^{Kip-1}$ was completely blocked by the caspase inhibitor, zVAD-fmk in Jurkat cells. Furthermore, in the case of CDK2, CD95 engagement induced the expression and the augmentation was dependent on caspase activity in Jurkat cells (Fig. 7).

DISCUSSION

Both immature cortical thymocytes and mature peripheral T cells are sensitive to apoptosis caused by ligation of several receptors including the T cell receptor (TCR) and CD95 [Dao et al., 1997; Hakem et al., 1999; Hingorani et al., 2000]. The link between cell death and the cell cycle is suggested by numerous studies in mature T cells and cell lines [Crispe, 1994; Green et al., 1994; Dao et al., 1997; Lissy et al., 1998; Zhou et al., 1998; Hingorani et al., 2000]. CD95 is one of the death signals, which induces apoptosis in double positive thymocytes as well as activated T cells.

It was previously reported that S-phase cells were protected from activation-induced cell death (AICD) caused by CD95 signaling [Dao et al., 1997; Hingorani et al., 2000]. The present study is designed to understand the relations between CD95-mediated apoptosis and the cell cycle, and to test the hypothesis that the cell cycle is linked to CD95-mediated apoptosis in T cells. In this report, we first chose thymocytes as targets of CD95 signaling, since they are sensitive to CD95-mediated apoptosis. In addition, some other investigators have used cell cycle inhibition to determine the effects of death stimuli on cells at different stages, but we have avoided this approach since we do not want to perturb the cell cycle control mechanisms that are the subject of study. Table I showed that the frequency of S phase thymocytes was increased upon CD95 signaling, while the frequency of G1 phase cells was reduced. This implies that S phase cells were not the targets of the death signal from CD95–CD95L interaction. This result reproduces the previous finding that S phase cells are resistant to CD95-mediated death in AICD [Dao et al., 1997; Hingorani et al., 2000]. The percentage of S phase cells was even increased (Table I) by CD95 ligation.

There are two possibilities that explain the increase of S-phase cells. One is simply due to the selective sparing of S phase cells. However, this is not likely to be a complete explanation, as biochemical analysis showed changes in the expression of cell-cycle proteins before apoptosis was observed. For example, Figure 3 showed that the induction of cyclin E occurred before apoptosis. A likely second possibility is that thymocytes in G1 that are exposed to CD95L are induced to cell cycle progression. These cells are in G1/S transition and could be sensitive to apoptosis. Therefore, the increase of S phase cells shown in Table I was a complex phenomenon in which the sparing of S phase cells was accompanied by cell cycle progression. In addition, the reduced G2/M population

(Table I) might be due to apoptosis of these cells after progression through G1.

The changes in cell cycle-related proteins presented in this report are the characteristics of the G1–S phase transition. This includes the degradation of p27^{Kip-1}, cyclin D1 induction, phosphorylation of the pocket, proteins, and the induction of cyclin E. This implies that CD95 ligation induces cell cycle progression and along with the activation of caspases, cells in G1–S phase transition were susceptible to apoptosis. In addition, another independent study has suggested that the G1–S transition of the cell cycle is the most susceptible point to apoptotic signals [Meikrantz and Schlegel, 1995].

However, as thymocytes are known to be mainly in a resting state, the relation between cell cycle and the CD95-mediated apoptosis is not clearly understood. In addition, there have been controversial findings. For instance, Boehme and Lenardo. [1993] reported that S phase T cells are sensitive to apoptotic signals. Dao et al. [1997] and Lissy et al. [1998], however, reported that G1 phase cells are the targets for the CD95-mediated apoptosis and TCR-induced AICD, respectively. Furthermore, we recently found that there is reduced CD95-mediated cell death in thymocytes and activated T cells from p21$^{cip-1/WAF-1}$ deficient mice [Hingorani et al., 2000]. It is well known that p21$^{cip-1/WAF-1}$, one of the CDK inhibitors, plays an important role in G1 arrest. Therefore, our recent findings suggest that without the CDK inhibitor, p21$^{cip-1/WAF-1}$, more cells may exit from G1 to S phase and become resistant to CD95-mediated death.

There have been a number of studies showing that the eukaryotic cell cycle is regulated by the sequential activation of different cyclins and CDKs [Hunter and Pines, 1994; Morgan, 1995; Arellano and Moreno, 1997; King and Cidlowski, 1998]. These two groups of molecules are assembled into heterodimeric complexes. Different cyclin–CDK complexes are required to catalyze the phosphorylation of a number of protein substrates including Rb pocket proteins and are thought to be important to drive cell-cycle events [Hunter and Pines, 1994; Arellano and Moreno, 1997]. Therefore, we next examined the expression patterns of cell cycle related molecules including cyclins and CDKs in CD95-engaged thymocytes in order to test for evidence of cell cycle progression. We used NIH-3T3 cells expressing CD95L on their surfaces, which induced apoptosis in thymocytes as well as in activated T cells more efficiently than anti-mouse CD95 Ig [Dao et al., 1997; Hingorani et al., 2000]. This can be explained partly by the requirement for trimerization of CD95 molecules to induce apoptosis.

First, we analyzed the expression levels of cyclins. Figures 1 and 2 show the levels of cyclin D1, D2, and D3, cyclin E and cyclin A to determine the status of the cell cycle in cells that are undergoing CD95-mediated apoptosis. Low levels of cyclin D molecules were detected in all cases (Fig. 1) but cyclin D1 was upregulated after 4 h in response to CD95 ligation. There was, however, no obvious difference in the degree of cyclin A expression and, as most thymocytes are not cycling, we could hardly detect cyclin A expression.

Surprisingly, cyclin E is upregulated at the protein level by CD95 ligation (Fig. 3). This cyclin is expressed in late G1, prior to cyclin A expression, and is critical for the G1–S transition. However, the bands for the cyclin E induced by CD95 ligation were doublets including these slightly smaller in size, as compared to those in control thymocytes. This was probably due to differential phosphorylation, or to ubiqutinization. For the last few years, it has been known that cell cycle-related molecules such as Rb [Tan and Wang, 1998] and CDK inhibitors including p21$^{cip-1/WAF-1}$ and p27^{Kip-1} [Levkau et al., 1998] can be the targets of caspases. Apart from these, we found that a number of other cell cycle-related proteins have caspase-3 recognition sites (i.e., a DxxD motif) in the sequence. These include the pocket proteins, p130 and p107, all members of the E2F family, and cyclin E. Interestingly, we could detect two different sized fragments of cyclin E in thymocytes engaged by CD95 (Fig. 3), which appeared to be caspase-cleaved products (Fig. 4). In fact, cyclin E has three caspase recognition sites. Among cyclins, only cyclin E has internal caspase recognition sites. This suggests that cyclin E is one of the substrates of caspases, and we propose that this cyclin may have a critical role in regulating the link between apoptosis and the cell cycle.

The pocket proteins play a role in cell cycle progression and in apoptosis, and pRb is cleaved followed by apoptotic signals [Tan and Wang, 1998]. This occurs in TNFR1 and CD95-

mediated apoptosis. In addition to pRb [Tan and Wang, 1998], p130 and p107 appeared to be the substrates for caspase(s) (Fig. 2) as their cleavage was blocked by the treatment of caspase inhibitor, zVAD-fmk (data not shown). However, we failed to detect the degraded products of E2F-1 or E2F-4, although they have a conserved caspase recognition site at the C-termini. One possible explanation is that the C-terminus cleaved product was too small to be resolved. The antibodies raised were polyclonal, from a rabbit immunized with 15 amino acids from the C-terminus of each E2F molecule, and therefore these antibodies might not be able to recognize the N-terminal cleaved products. However, in the case of E2F-1, the band densities between control and CD95-ligated thymocytes seemed to be almost identical suggesting that there was no caspase action on E2F-1. The other possibility, in the case of E2F-4, is that the caspase(s) may act only on phosphorylated E2Fs. If this is the case, it might be due to steric hindrance. For instance, E2Fs are known to associate with other molecules such as DP or pocket proteins. Therefore the partner molecule, the DP and/or pocket proteins, may block the action of caspase(s) on E2F-4 molecules by physical interaction. In fact, the density of E2F-4 was slightly decreased by CD95 ligation, but the decrease seemed to be restricted to the high molecular weight form that might be the phosphorylated E2F-4 (Fig. 4). The structures of the E2F family members support this idea, as the conserved caspase recognition site resides at the C-terminus, which is known to be the binding site for the p110Rb pocket proteins [Sardet et al., 1995]. This is more likely as it is obvious that the decreased density of higher molecular weight E2F-4 has been detected in the case of Jurkat cells by CD95 ligation (Fig. 7).

C-Myc is involved in cell proliferation, and is known as a positive regulator of the S phase transition [Vlach et al., 1996; Alevizopoulos et al., 1997; Bouchard et al., 1998] which functions as a transcription factor for the cdc25 A, B, and C genes. Among these c-myc target genes, cdc25A acts on G1–S progression [Jinno et al., 1994; Bouchard et al., 1998]. Consequently, we tested the effect of CD95 ligation on the levels of c-myc and cdc25A. The down-regulation of c-myc may result in the inhibition of G1/S transition implying that CD95-mediated death of thymocytes occurs during the G1/S transition.

We next asked if the signals resulting in differential expression and degradation of cell cycle proteins, cyclin E, CDK2, c-myc, and $p27^{Kip\text{-}1}$, are through the death domain of the CD95 molecule. zVAD-fmk, an inhibitor for a broad spectrum of caspases, was used to block the signals through death domain prior to CD95 ligation. Figure 6 shows that the modulations of cyclin E, c-myc, and $p27^{Kip\text{-}1}$ appear to be caspase-dependent.

Although thymocytes are the most sensitive type of cells to CD95-mediated death in the physiological condition, CD95 is known to play a pivotal role in protection from the development of autoimmune disease, by eliminating activated T cells in the periphery. We examined the human leukemic T cell line, Jurkat, to determine if there are changes similar to those in thymocytes from CD95-mediated apoptosis. Among cell cycle proteins, we focused on the expression of cyclins E and A, as they are the cyclins differentially expressed in G1 and S phases (Fig. 7). Findings were almost identical in Jurkat cells to those in thymocytes. As expected, cyclin A expression was generally high in Jurkat cells in contrast to that of thymocytes. Upon CD95 ligation on Jurkat cells, surprisingly, cyclin A was down-regulated (Fig. 7). CDK2 was much more upregulated, as compared with that in mouse thymocytes, by CD95 engagement and the induction was blocked by zVAD-fmk in the case of Jurkat cells (Fig. 7). This implies that CDK2 regulation through CD95 signaling in Jurkat cells is more striking than that in mouse thymocytes.

In summary, CD95-mediated apoptosis in thymocytes and a leukemic T cell line, Jurkat, seems to occur in G1–S transition. We therefore suggest that, like p53, CD95 has a critical role in G1 check point in T cells. In addition, CD95-mediated death might occur at similar time point as that of TCR antigen-induced cell death in the late G1 phase [Lissy et al., 1998]. However, as there was no cyclin E induction in the case of TCR antigen-induced cell death [Lissy et al., 1998], CD95 mediated death is not the same as that of AICD. Boehme and Lenardo. [1993] reported that S-phase cells are the targets of AICD while Dao et al. [1997] and Hingorani et al. [2000] showed that G1 phase cells are susceptible to CD95-mediated

apoptosis in T cells. It could be understood that CD95-mediated death might be different from AICD caused by other stimuli.

The present report showed the differential regulation and degradation of a number of cell cycle proteins upon CD95 ligation. We have suggested here and previously [Dao et al., 1997; Hingorani et al., 2000] that S phase cells are protected from CD95-induced death. This model makes the explicit prediction that the overexpression of molecules inducing S phase progression, including cyclin A and c-myc or cdc25A, could block CD95-mediated death in T cell. It is an interesting possibility that some tumors with dysregulated cell cycle control proteins, leading to constitutively active G1–S progression factors, may be intrinsically CD95-resistant for this reason.

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