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Characterization of Apoptosis-resistant Jurkat T Cell Clones

Abstract

The T-acute lymphoblastic leukemia cell line Jurkat is sensitive to various apoptotic inputs, including stimulation of the T cell antigen receptor and CD95, receptor systems essential for the regulation of the immune response. Characterization of apoptosis-resistant variant clones from this cell line indicates that the T cell receptor can induce apoptosis by both CD95-dependent and -independent mechanisms and reveals the existence of a protein that regulates CD95 sensitivity upstream from CPP32 (caspase-3) activation.

Introduction

The T cell antigen receptor (TcR) recognizes antigenic peptides in the context of molecules of the major histocompatibility complex. As a result, resting lymphocytes are activated to divide and differentiate into helper- or cytolytic effector cells. However, renewed TcR triggering on activated T cells can induce apoptosis, which most likely serves to attenuate the immune response (Russell et al., 1991). Also, during T cell development in the thymus, TcR triggering can either rescue thymocytes from programmed cell death and induce further maturation (positive selection), or induce apoptosis and consequently delete potentially autoreactive cells (negative selection). These observations raise the question how the same receptor system can induce such vastly different responses: cellular activation or death?

Stimulation of the TcR/CD3 complex activates cytosolic protein tyrosine kinases, allowing recruitment and activation of Src-homology 2 (SH2)-domain containing molecules (Weiss and Littman 1994). Tyrosine kinase substrates include phospholipase C (PLC)-γ1 and the Shc-Grb2/Sos complex (Ravichandran et al., 1993), which initiate relatively well known signalling cascades. PLC-γ1 generates inositol trisphosphate and diacylglycerol. Inositol trisphosphate mobilizes Ca^{2+} ions from intracellular stores, leading to activity of the phosphatase calcineurin, which plays a role in the activation of the NFAT transcription factor complex (Rao 1994, Crabtree et al., 1994). Diacylglycerol is a cofactor for PKC, the enzyme thought to be required for activation of the NFκB transcription factor (Siebenlist et al., 1994). Formation of the Shc-Grb2/Sos complex induces the Ras pathway, resulting in activation of Erk 2

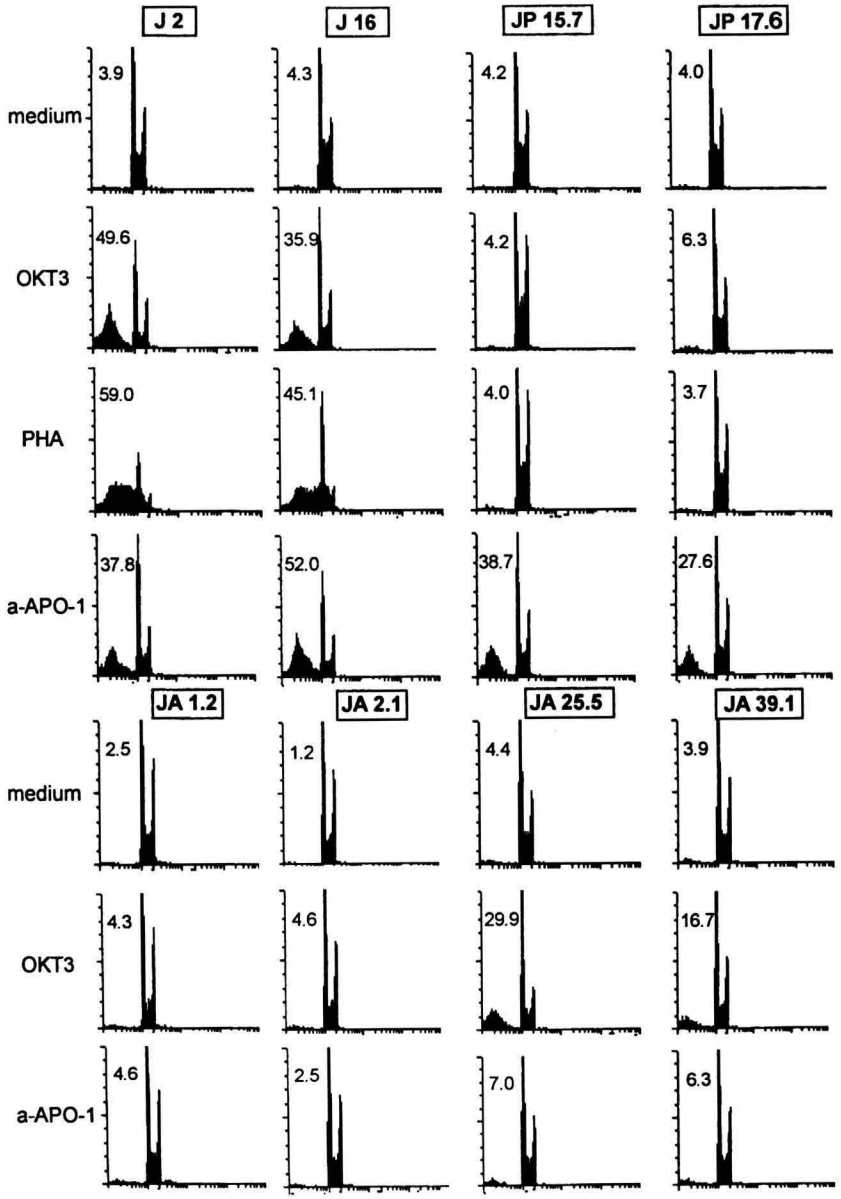

Fig. 1. Sensitivity to TcR- and CD95-induced apoptosis of wild type and variant Jurkat clones. Clones were cultured for 24 h in the presence or absence of the indicated stimuli. To stimulate the TcR/CD3 complex, OKT3 mAb was coated onto the tissue culture wells at 10 μg/ml or PHA was used in soluble form at 2 μg/ml. To stimulate CD95, soluble anti-APO-1 mAb was used at 1 μg/ml. Cells were harvested and lysed in a hypotonic buffer, which leaves the nuclei intact. DNA was stained with propidium iodide and fluorescence intensity of intact or apoptotic nuclei was measured using a FACScan. The numbers indicate the percentage of apoptotic nuclei in each sample, recognizable as a subdiploid DNA peak. J 2 and J 16 are wild type clones; JP 15.7 and JP 17.6 were selected for resistance to PHA-induced apoptosis and JA 1.2, JA 2.1, JA 25.5 and JA 39.1 were selected for resistance to anti-APO-1-induced apoptosis.

Table 1. Sensitivity of Jurkat and variant clones to TcR- and CD95-induced apoptosis

	medium	OKT3	anti-APO-1
Jurkat	4.8[a]	28.0	40.9
J 2	3.9	49.6	37.8
J 16	5.3	39.3	59.1
JP 15.7	5.4	6.6	55.0
JP 17.6	5.9	6.2	53.5
JA 1.2	2.5	4.3	4.6
JA 2.1	1.2	4.6	2.5
JA 9.3	1.4	2.4	5.7
JA 10.2	3.2	5.2	13.9
JA 16.6	1.7	7.2	3.8
JA 21.3	1.1	2.7	2.1
JA 22.3	1.7	5.7	1.9
JA 25.5	4.4	29.9	7.0
JA 35.2	6.1	20.9	10.7
JA 39.1	3.9	16.7	6.3

[a] Percentage of apoptosis 24 h after addition of stimulus, as determined according to Nicoletti et al. (22). Data are representative of four independent experiments.

mitogen activated protein kinases (MAPK) and the Fos transcription factor (Izquierdo et al., 1993). In addition, in concert with the costimulatory molecule CD28, the TcR induces Jun phosphorylation by the stress-activated protein kinases or Jun kinases (Su et al., 1994). The various transcription factor complexes can bind to promoter sites in a great variety of genes, encoding products which drive cell proliferation and differentiation (Crabtree et al., 1994).

Like TcR-induced proliferation and differentiation, TcR-induced apoptosis is also dependent on mRNA and protein synthesis (Ucker et al., 1989). Recent studies (Ju et al., 1995, Brunner et al., 1995, Dhein et al., 1995) have indicated that the TcR does not directly activate the apoptotic machinery but can induce apoptosis via the CD95 receptor system. Stimulation of the TcR/CD3 complex appeared to increase CD95 ligand (L) mRNA levels and protein expression, while TcR-induced apoptosis could be inhibited by soluble CD95 molecules. From these observations, one can conclude that TcR triggering induces signal transduction events leading to upregulation of CD95L mRNA levels. Resulting increased CD95L protein production is most likely responsible for the induction of apoptosis via the CD95 molecule.

CD95 was originally identified by anti-Fas (Yonehera et al., 1989) and anti-APO-1 (Trauth et al., 1989) mAbs selected for the capacity to induce cell death. CD95 belongs to the TNF receptor family, which includes a number of molecules implicated in the positive and/or negative regulation of cell survival (Bazan 1993). These receptors have no intrinsic enzymatic activity and employ novel signal transduction mechanisms. Apoptosis-inducing members of the TNF receptor family appear to couple directly, in a protein synthesis-independent manner to the caspase family of cysteine proteases that are thought to constitute the apoptotic machinery (Yuan 1997). These

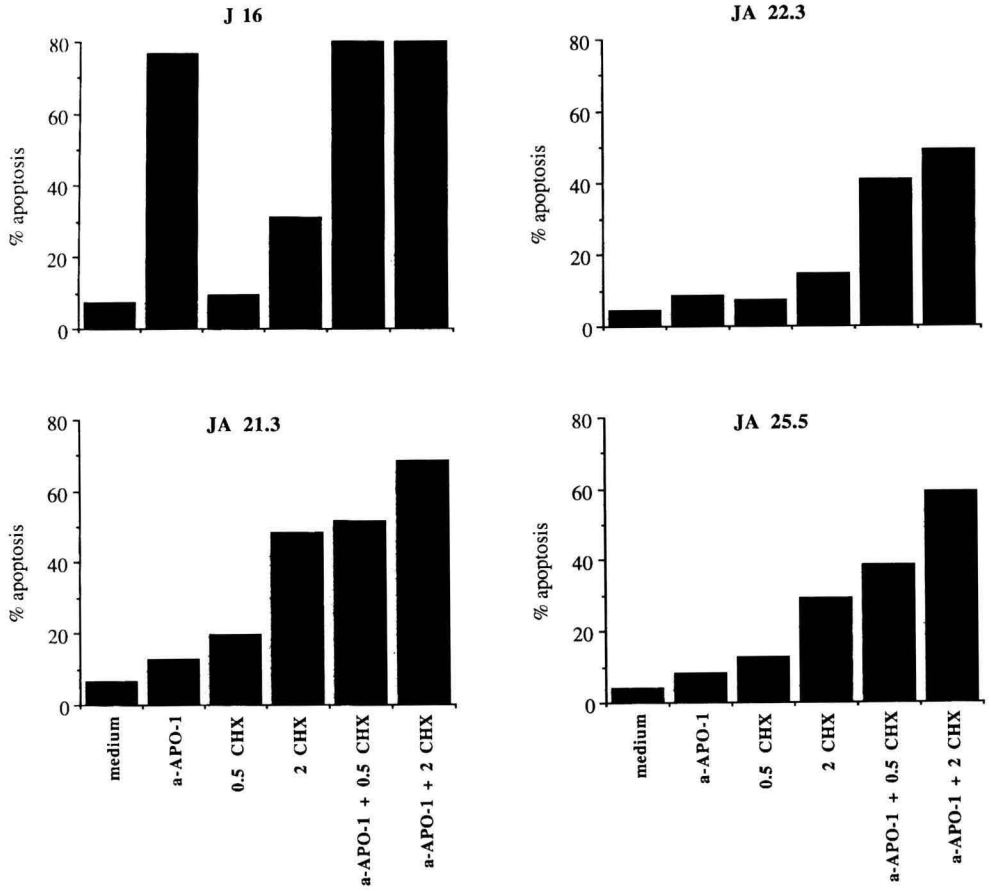

Fig. 2. The CD95 signalling pathway in JA clones is inhibited at, or upstream from CPP32 activation. Resistance can be alleviated by protein synthesis inhibition. (A) Proteolytic processing of the CPP32 protease as determined by immunoblotting of total lysates of J 16 and JA cells. Cells were preincubated with 0.5 μg/ml CHX for 4 h, CH-11 anti-CD95 mAb was added at 1 μg/ml and cells were further incubated for 1-6 h. Indicated by a dash is the precursor CPP32 molecule at 32 kDa and indicated by an arrow is its degradation product at about 20 kDa. (B) Percentage apoptosis after 20 h incubation of wild type J 16 and three JA clones with 1 μg/ml anti-CD95 mAb (anti-APO-1), CHX at 0.5 or 2 μg/ml, and anti-CD95 mAb in combination with eithr concentration of CHX. Cells were preincubated with CHX for 4 h prior to addition of anti-CD95 mAb or control stimulus.

receptors share a so-called 'death domain' in their cytoplasmic tail. In case of CD95, this domain allows interaction with a homologous region in the FADD adaptor protein upon receptor stimulation (Chinnayian et al., 1996a). With its amino-terminal 'death effector domain' FADD recruits the FLICE protease (caspase-8) to the receptor system, which allows its proteolytic activation ((Muzio et al., 1996, Boldin et al., 1996). CD95 can also activate CPP32 (caspase-3) and ICE-LAP-3 (caspase-7), which most likely operate downstream from FLICE (Chinnayian et al., 1996b).

To gain insight into the requirements for TcR- and CD95-mediated apoptosis, we have characterized a number of clones, derived from the human T- cell line Jurkat, which were selected for resistance to either TcR- or CD95-induced apoptosis. We conclude that the TcR/CD3 complex can induce apoptosis in a CD95-dependent manner, but also reveal that a CD95-independent mechanism exists. We find evidence for a protein of high turnover, that regulates sensitivity to CD95-mediated apoptosis upstream from caspase-3 activation. Given the connection between TcR- and CD95-mediated apoptosis, this protein may also play a role in life/death decisions upon TcR stimulation.

Materials and Methods

Antibodies and immunofluorescence. Purified anti-APO-1 mAb (IgG3, κ) (Trauth et al, 1989) was provided by dr. P. Krammer (DKFZ, Heidelberg, FRG). The CH-11 anti-CD95 mAb was purchased from Immunotech (Marseille, France). Mouse anti-human CD3 mAb OKT3 (IgG2a, κ) (Reinherz and Schlossman 1980) was a gift from Ciba Geigy, Basel, Switzerland. Anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology (Lake Placid, NY) and used in biotinylated form for immunoblotting. Streptavidin-conjugated horse radish peroxidase was used as second step reagent. Polyclonal antiserum to CPP32 was prepared in our institute by dr. G. Gil-Gomez. A rabbit was immunized with a glutathione-S-transferase fusion protein of human CPP32, amino acids 29-176. For immunofluorescence analysis, cells were incubated with OKT3 or anti-APO-1 mAb at 10 μg/ml, followed by FITC-conjugated goat anti-mouse $F(ab')_2$ fragments. Fluorescence intensity of 10.000 cells per sample was measured on a FACScan (Becton Dickinson, Mountain View, CA). All antibodies were used as purified Ig.

Cell culture. The human T- acute lymphoblastic leukemia cell line Jurkat and clones derived from it were cultured in Iscove's modified Dulbecco's medium with 10% FCS, 2 mM glutamine and antibiotics. Wild type clones were derived from Jurkat by limiting dilution and selected for TcR/CD3 expression. JP variant clones were derived by culturing the parental Jurkat line in the presence of 0.4 μg/ml phytohemagglutinin (PHA; HA 16, Murex Diagnostics Ltd., Dartford, U.K.) for about one month. Surviving cells were cloned and subcloned by limiting dilution, selected for expression of all TcR/CD3 components at both mRNA and protein level and for resistance to OKT3-induced cell death. JA variant clones were generated by culturing Jurkat cells in the presence of 1 μg/ml anti-APO-1 mAb for about five weeks and subsequent cloning and subcloning of surviving cells. All cells in the bulk culture resistant to anti-APO-1 mAb had maintained CD95 expression as determined by FACS analysis. Clones were selected on the basis of TcR/CD3 expression and high resistance to CD95-induced apoptosis. TcR/CD3 and CD95 expression were routinely checked by immunofluorescence.

Apoptosis assay. For induction of apoptosis, OKT3 mAb was coated onto flat-bottom microtiter plates at 10 μg/ml in PBS overnight at 4°C. Culture vessels were washed before use to remove free mAb. Anti-CD95 mAb and PHA were added in soluble form, at 1 μg/ml and 2 μg/ml respectively. Cells were seeded at 1×10^6/ml,

Table 2. Sensitivity of Jurkat-cells and variant clones to PHA-induced apoptosis

	medium	OKT3	anti-APO-1
Jurkat	4.1[a]	46.7	62.9
J 2	3.1	59.1	37.2
J 16	5.4	47.0	68.0
JP 15.7	5.3	6.3	55.0
JP 17.6	6.2	8.9	58.8
JA 1.2	1.3	22.3	5.6
JA 2.1	1.3	14.2	8.1
JA 16.6	6.8	33.3	13.8
JA 21.3	2.1	39.0	6.4
JA 22.3	2.4	31.1	6.5
JA 25.5	3.3	37.0	8.0
JA 39.1	3.8	42.9	7.0

[a] Percentage of apoptosis 24 h after addition of stimulus, as determined according to Nicoletti et al. (22). Data are representative of five independent experiments.

100 µl/well in flat- or round bottom 96 well microtiter plates in culture medium, stimulus was added and cells were incubated at 37°C, 5% CO_2 for 24 h. To detect apoptotic cells, cells were washed with PBS after incubation and lysed in hypotonic buffer consisting of 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/ml propidium iodide (Nicoletti et al., 1991). In this lysis buffer, nuclei remain intact and propodium iodide can diffuse in to stain the DNA. Fluorescence intensity of nuclei was determined on a FACScan (Becton Dickinson) and data were analysed using Lysys software. Segmented, apoptotic nuclei are recognizable in this assay as a population with diminished and rather heterogeneous fluorescence intensity as compared to intact diploid nuclei (Nicoletti et al., 1991). The percentage of nuclei present in this sub-diploid peak are considered to reflect the percentage of apoptotic cells.

Ca^{2+} mobilization assay. For measurement of the intracellular Ca^{2+} ion concentration, Jurkat clones were incubated at 10×10^6/ml with 3 µM Indo-1-AM (Molecular Probes, Eugene, OR) for 30 min. at 37°C in medium with 0.1% BSA, followed by dilution to 2×10^6/ml and a further incubation for 30 min. Cells were washed and resuspended at 2×10^6/ml in the same medium and stored on ice until analysis. After equilibration at 37°C, cells were stimulated with 1 µg/ml OKT3 mAb and mobilization of Ca^{2+} ions was determined with a FACStar flow cytometer (Becton Dickinson) using Lysys software for analysis as described (Ossendorp et al., 1992).

Detection of CD95L mRNA. Cells at 4×10^6/ml were stimulated in culture flasks coated with OKT3 mAb for 2, 4 or 6 h periods at 37°C, 5% CO_2, washed in PBS and snap frozen in liquid nitrogen. 40×10^6 cells were used per time point. Unstimulated control cells were incubated for 2 h at 37°C RNA was isolated by NP-40 lysis (Auffray and Rougeon 1980). Reverse transcription for synthesis of first strand cDNA was performed with SuperScript II (Gibco BRL, Technologies) and random hexamer

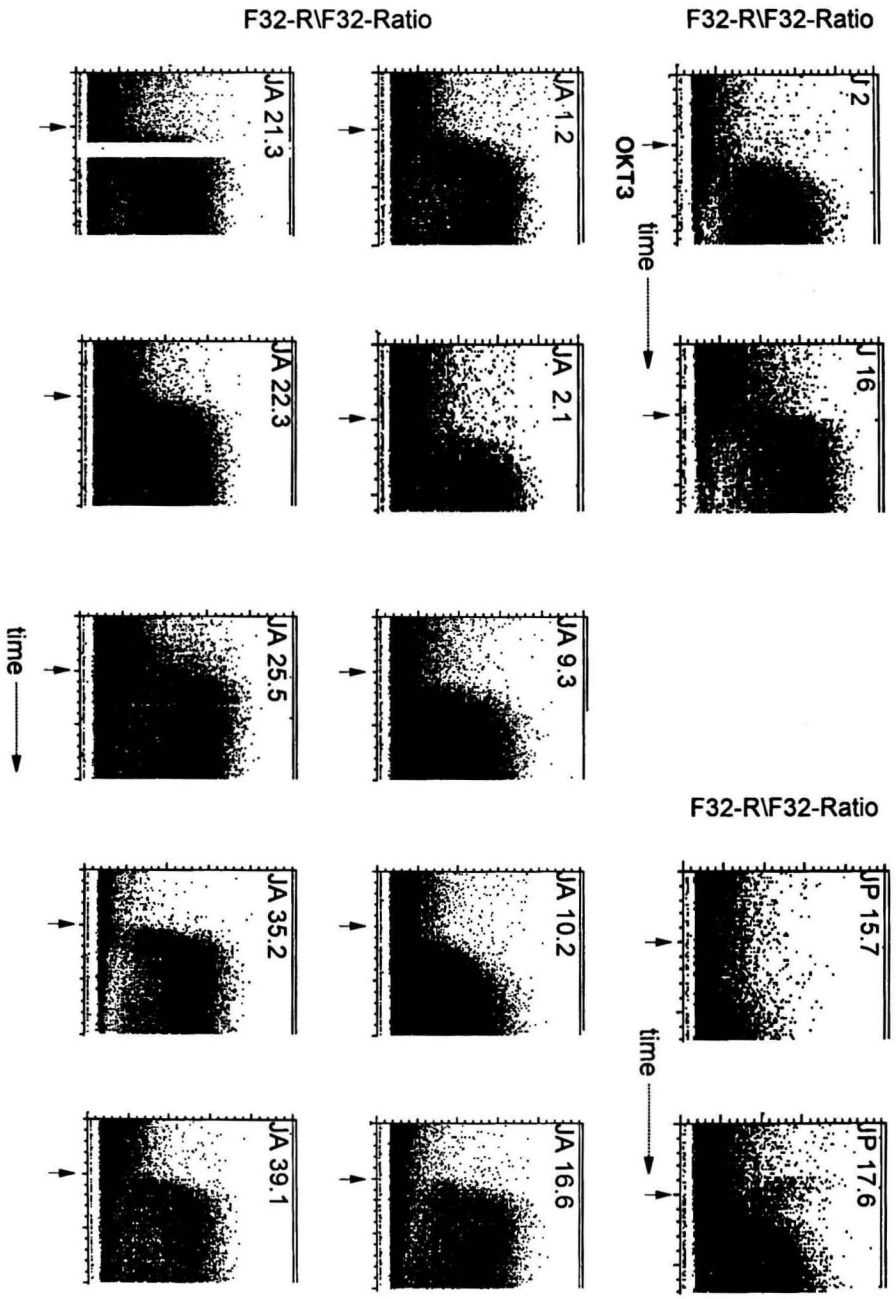

Fig. 3. Triggering of the TcR/CD3 complex mobilizes intracellular Ca^{2+} ions in all wild type and variant clones, except JP 15.7. Cells were loaded with Indo-1-AM and equilibrated in medium at 37°C for 3 min in a chamber attached to the FACStar. Measurement started in the absence of stimulus and was followed for 100 seconds; at this timepoint (arrow), OKT3 mAb was injected to a final concentration of 0.5 μg/ml and measurement was continued for another 50-100 seconds.

primers (Perkin Elmer). For PCR amplification of CD95L cDNA the forward primer 5'-TGGCAGAACTCCGAGAGTCTA and the reverse primer 5'-CACTGGTAA-GATTGAACACTGC were used. As a control, expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was determined using the forward primer 5'-GACCCCTTCATTGACCTC and the reverse primer 5'-CCAAAGTTGTCATG-GATG. PCR was carried out at 58°C for 25 cycles in case of CD95L and 20 cycles for GAPDH. Products were electrophoresed in agarose gels, transferred to Hybond membranes and detected with random primed ^{32}P-labeled probes. The CD95L PCR product was detected with an EcoRI-StyI fragment of the full-length cDNA clone of human CD95L. This cDNA was isolated in the laboratory of Dr. J. Tschopp (University of Lausanne, Epalinges, Switzerland). The GAPDH PCR product was detected with a rat GAPDH cDNA (Schuuring et al., 1992). Signals on the autoradiographs were quantitated with a Fuji BAS 2000 TR phosphorimager.

Immunoblotting. For analysis of CPP32 processing, cells were suspended at 10×10^6/ml in a 24 well culture plate, stimulated with medium or CH-11 mAb for various time periods at 37°C, 5% CO_2, and lysed with 50 µl 1% Nonidet P-40 in 0.01 M triethanolamine-HCl pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, 0.02 mg/ml trypsin inhibitor and 0.02 mg/ml leupeptin. Lysates were centrifuged at 14.000 g for 15 min and the supernatants were mixed with concentrated reducing SDS sample buffer. Equivalents of 10^6 cells per lane were separated on a 10% SDS polyacrylamide minigel. Proteins were transferred to nitrocellulose membrane (Schleicher and Schüll, Dassel, FRG). Blots were blocked with 5% non fat dry milk in PBS, 0.1% Tween 20, incubated with purified anti-CPP32 Ig at 10 µg/ml in PBS, 0.1% Tween 20, 1% non fat dry milk, followed by a 1: 7500 dilution of horse radish peroxidase-conjugated swine anti-rabbit Ig (DAKO A/S, Glostrup, Denmark) and developed by enhanced chemiluminescence (Amersham, U.K.). For anti-phosphotyrosine immunoblotting, cells at 10^7/ml in medium were stimulated with medium or 10 µg/ml OKT3 mAb at 37°C, lysed at 4°C with 1% Nonidet P-40 in 50 mM Tris.HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 1 µg/ml each of leupeptin, aprotonin, pepstatin and chymostatin. Lysates were centrifuged at 14.000 g for 15 min. Supernatants were mixed with SDS sample buffer and separated on a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane. Blots were blocked with 5% BSA in PBS, 0.1% Tween 20, incubated in PBS with 0.1% Tween 20 with biotinylated 4G10 mAb, followed by streptavidin-conjugated horseradish peroxidase and developed by enhanced chemiluminescence.

Results

Generation of Apoptosis-resistant Jurkat Clones.

The Jurkat T cell line is susceptible to apoptosis-induction via both the TcR/CD3 complex and CD95 (Table 1). Since about 10-20% of cells within the Jurkat line lack TcR/CD3 expression, we have derived wild type clones by limiting dilution. These clones were selected for TcR/CD3 and CD95 cell surface expression by

immuno-fluorescence. The wild type clone J 16 was selected for high sensitivity to TcR- and CD95-induced apoptosis.

JP variant clones were derived by limiting dilution from Jurkat cells cultured for about 1 month in the presence of 0.4 μg/ml PHA, a lectin which binds to the TcR (Chilson et al., 1994) and induces both IL-2 production and death in Jurkat cells (Gillis and Watson 1980). Only five out of fifteen JP clones thus generated contained functional TcRβ mRNA and expressed the TcR/CD3 complex at levels comparable to the wild type clones. JP 15.7 and JP 17.6 were selected from these five PHA-resistant clones for concomitant resistance to apoptosis-induction by anti-CD3 monoclonal antibody. These two subclones express TcR/CD3 and CD95 at levels comparable to wild type clones.

JA variant clones were derived by limiting dilution from the Jurkat line, cultured for about five weeks in the presence of 1 μg/ml anti-APO-1 mAb. According to limiting dilution analysis, about 1 in 10^4 cells survive this treatment. Ten clones were selected for use in further experiments on the basis of TcR/CD3 expression and resistance to growth inhibition with anti-APO-1 mAb. To ensure clonality, JA clones were sub-cloned by limiting dilution. Like JP clones, they were maintained in medium without selecting stimulus after cloning from the apoptosis resistant bulk culture. Although TcR/CD3 plasma membrane expression varies between JA clones, all are positive and give rise to intracellular Ca^{2+} mobilization upon TcR/CD3 stimulation, while CD95 expression is within the wild type range (results not shown).

Sensitivity of Wild Type and Variant Jurkat Clones to TcR- and CD95-Induced Apoptosis

Apoptosis sensitivity is illustrated for wild type, JP and JA clones in Fig. 1 and listed in full in Table 1. Like the Jurkat line, wild type clones J 2 and J 16 are sensitive to apoptosis-induction with immobilized OKT3 mAb, which triggers the TcR/CD3 complex. Also, PHA induces apoptosis in the wild type clones, whereas the JP 15.7 and JP 17.6 clones, derived from the PHA-resistant Jurkat bulk culture, are fully resistant to both PHA- and OKT3-induced apoptosis. Despite their resistance to TcR-induced apoptosis, JP 15.7 and JP 17.6 are sensitive to CD95-induced apoptosis at wild type levels (Fig. 1, Table 1).

All ten JA clones repeatedly showed greatly diminished (JA 9.3 and JA 10.2) or no sensitivity to apoptosis-induction by anti-APO-1 mAb (Fig. 1, Table 1), indicating that the pathway leading to apoptosis from the CD95 molecule is inhibited in these clones. The CD95 signalling pathway is blocked upstream from, or at the level of CPP32 (caspase-3) activation, since this occurs in wild type, but not in resistant JA clones in response to CD95 stimulation (Fig. 2A). The cytoplasmic tail of the CD95 molecule in all clones is wild type, according to nucleotide sequence analysis (results not shown). Interestingly, apoptosis-sensitivity can be restored in JA clones by inhibition of protein synthesis with cycloheximide (Fig. 2B). This same treatment also allows CPP32 processing in response to CD95 stimulation (Fig. 2A). These findings indicate that in JA clones the CD95 signalling pathway and the apoptotic machinery are intact. Most likely, the CD95 signalling pathway is inhibited by a protein of high turnover that acts in between the receptor system and CPP32.

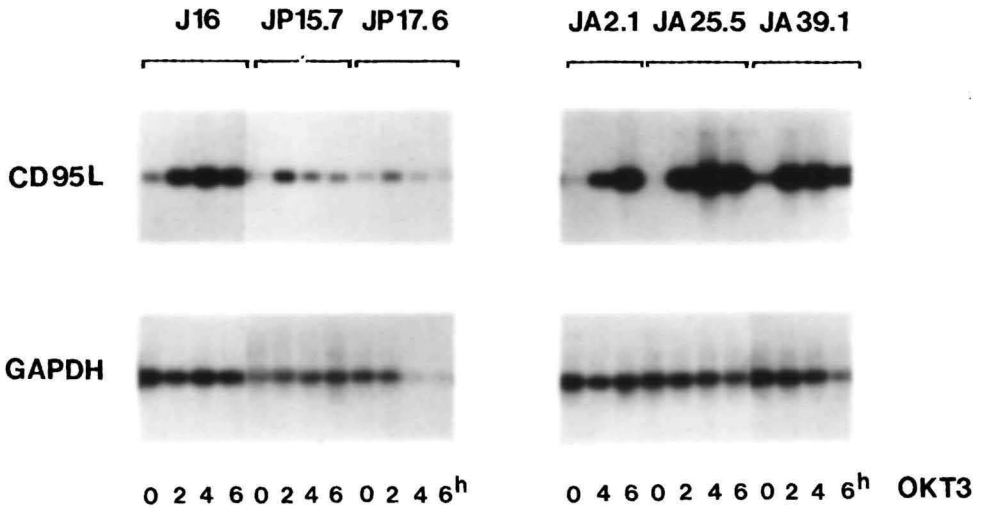

Fig. 4. Detection of CD95L mRNA after TcR stimulation in wild type J 16, TcR-resistant JP clones and JA clones. Cells were incubated in medium alone, or stimulated with OKT3 mAb for 2, 4 or 6 h at 37°C, 5% CO_2. RNA was isolated and reversed transcribed. cDNA was used as template for PCR with primers specific for CD95L and GAPDH. PCR products were run on gel, transferred to Hybond and hybridized with ^{32}P-labeled cDNA probes specific for CD95L and GAPDH.

The majority of JA clones displayed concomitant resistance to apoptosis-induction by TcR/CD3 stimulation with OKT3 mAb (Fig. 1, Table 1). In all JA clones, the TcR/CD3 complex can generate intracellular signals, since triggering with OKT3 mAb gave rise to Ca^{2+} ion mobilization (Fig. 3). The cross-resistance indicates that TcR-mediated signalling to apoptosis depends on a functional CD95 signalling pathway. Out of twenty TcR/CD3$^+$ wild type clones tested, none was resistant to TcR-induced apoptosis, indicating that the correlation between CD95- and TcR resistance in the JA clones is significant.

Connection Between TcR- and CD95-Induced Signalling Pathways Leading to Apoptosis

Recently, it was found that, at least in certain human and murine T cell lines, the TcR signalling pathway does not directly activate the apoptotic machinery, but operates via CD95. TcR stimulation led to elevation of CD95L mRNA levels and TcR-induced apoptosis could be inhibited by soluble, recombinant CD95. In one of these studies, the J 16 Jurkat wild type clone described here was used (Dhein et al., 1995). We have investigated whether the two JP clones, which are resistant to TcR-induced apoptosis, but have a functional CD95 pathway, are defective in inducing CD95L mRNA expression. Wild type clones J 2 and J 16, as well as TcR signalling defective clones JP 15.7 and JP 17.6 were stimulated with immobilized OKT3 mAb for 2, 4 or 6 h. Analysis by PCR showed clear induction of CD95L mRNA after TcR stimulation in the wild type clones, whereas in both JP clones CD95L mRNA levels did not increase significantly (Fig. 4).

The TcR/CD3 complex on the JP clones is functional, since stimulation with OKT3 mAb induces protein tyrosine kinase activity, revealed by anti-phosphotyrosine blotting of overall intracellular substrates. In wild type cells, as well as in both JP 15.7 and JP 17.6, the 42 kDa Erk 2 MAP kinase was phosphorylated upon TcR triggering (Fig. 5), as confirmed by anti-phosphotyrosine blotting of anti-Erk 1, 2 immunoprecipitates (results not shown). However, JP 15.7 is defective in TcR-induced tyrosine phosphorylation of a 150 kDa substrate (Fig. 5), and does not give rise to PLCγ-mediated mobilization of intracellular Ca^{2+} ions (Fig. 3). JP 17.6 gives a wild type response in both assays. We conclude that TcR-induced Erk 2 MAP kinase activation is not sufficient to induce elevation of CD95L mRNA levels and that PLCγ activation is most likely required.

In response to TcR stimulation, JA clones give rise to Ca^{2+} ion mobilization (Fig. 3) and CD95L mRNA upregulation (Fig. 4) indicating that the TcR signalling pathway is functional and can give rise to the production of CD95L. Resistance to TcR-induced apoptosis in the JA clones is therefore best explained by inhibition of the apoptotic CD95 signalling pathway.

Residual TcR-Mediated Apoptosis in CD95-Resistant Clones

The majority of JA clones were concomitantly resistant to CD95- and TcR-induced apoptosis. However, some CD95-resistant JA clones, particularly JA 25.5 and JA 39.1, repeatedly displayed significant sensitivity to apoptosis induction by OKT3 mAb (Fig. 1, Table 1). This finding suggests that the TcR can also induce apoptosis by a CD95-independent mechanism. When JA clones were stimulated with PHA, rather than with anti-TcR/CD3 mAb, the majority of clones was sensitive to apoptosis-induction (Table 2), even though clones like JA 1.2 and JA 16.6 do not respond even to high concentrations of strongly agonistic CH-11 anti-CD95 mAb, or recombinant CD95 ligand (data not shown). These data indicate that triggering of the TcR, possibly in concert with co-stimulatory molecules activated by PHA can activate an apoptotic pathway that does not involve CD95.

Discussion

In this paper, we describe a number of variant clones isolated from the Jurkat T cell line, which were selected for resistance to apoptosis-induction by the TcR or CD95. The JP clones, selected for TcR-resistance, undergo apoptosis in response to CD95 triggering and other stimuli known to induce apoptosis in wild type Jurkat cells, such as cycloheximide and certain anti-cancer drugs (not shown). This indicates that these cells have no defect in the apoptotic mechanism and suggests that they are specifically disturbed in signalling induced by the TcR. TcR stimulation in JP 15.7 and JP 17.6 fails upregulate CD95L mRNA, which is most likely the cause of their resistance to TcR-induced apoptosis. These clones are useful to delineate which TcR-induced signalling events are required to increment CD95L mRNA levels. In both clones, TcR stimulation induces tyrosine phosphorylation of multiple intracellular

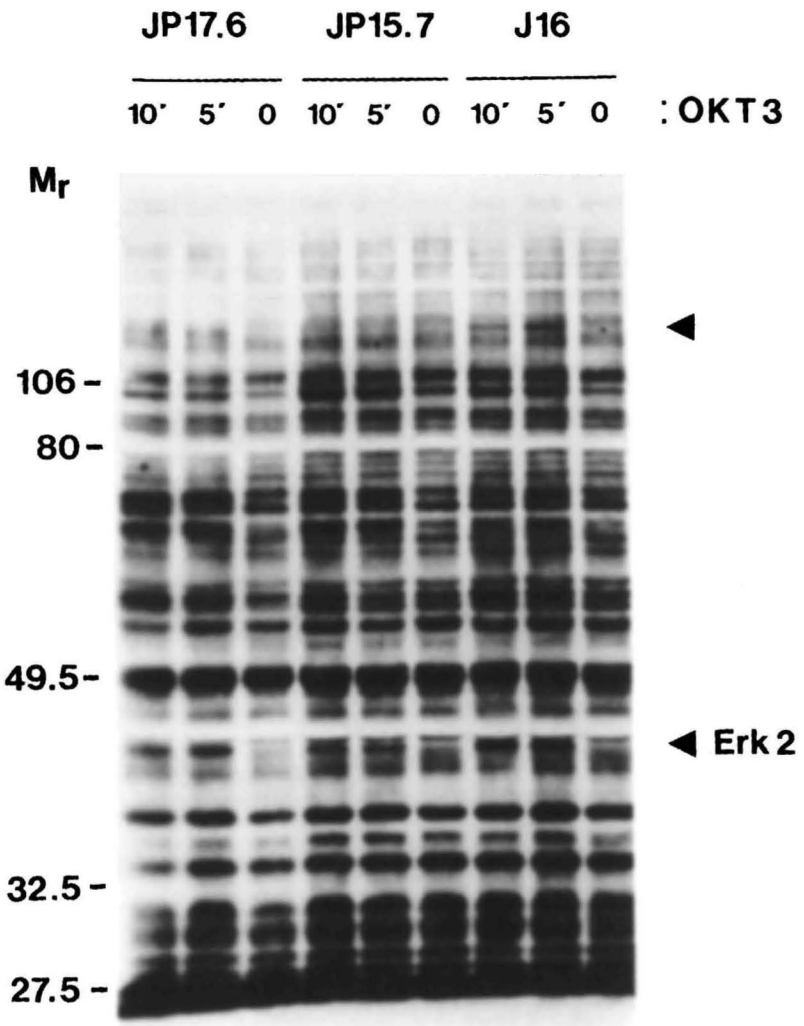

Fig. 5. TcR-induced tyrosine phosphorylation of intracellular substrates in wild type J 16 and TcR-resistant JP 15.7 and JP 17.6 clones. Cells were stimulated with OKT3 mAb and lysed with 1% NP-40. After centrifugation, lysates were mixed with reducing SDS sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and subjected to anti-phosphotyrosine immunoblotting with 4G10 mAb. The position of Erk 2 MAP kinase, which was confirmed by parallel immunoblotting with anti-Erk 1, 2 serum (not shown), is indicated. The arrow indicates the position of a substrate for TcR-induced tyrosine kinase activity of about 150 kDa, which is lacking in JP 15.7. M_r are indicated in kDa.

substrates, indicating that receptor-proximal Src-related tyrosine kinases and ZAP 70 are most likely not defective. However, JP 15.7 virtually lacked TcR-induced phosphorylation of a 150 kDa substrate, which is PLC-γ1 according to preliminary

immunoblot analysis (results not shown). Moreover, JP 15.7 is defective in TcR-induced Ca^{2+} mobilization and degradation of the IκB inhibitor of NFκB (not shown), supporting a defect in PLC-γ1 activation. Inhibition studies using cyclosporin A (CsA) have already indicated that PLCγ-induced signalling events are required for CD95L mRNA upregulation. CsA inhibits the PLCγ pathway downstream from Ca^{2+} ion mobilization and impedes activation of calcineurin and NFAT (4, 5). CsA was shown to block TcR-induced CD95L mRNA upregulation (Dhein et al., 1995, Anel et al., 1994). The second TcR-resistant clone, JP 17.6, is not defective in TcR-induced Ca^{2+} mobilization. However, preliminary evidence indicates that JP 17.6 is defective in TcR-induced degradation of IκBα, the inhibitor of NFκB. This would suggest a role for PKC-induced events, possibly NFκB activation, in CD95L mRNA upregulation. In both JP 15.7 and JP 17.6, Erk 2 MAP kinase was phosphorylated on tyrosine upon TcR stimulation and therewith presumably activated. Apparently, Erk 2 activation is not sufficient to induce CD95L mRNA elevation.

The JA clones were selected for resistance to CD95-mediated apoptosis, as induced by anti-APO-1 mAb. Clonogenic assays have indicated that about 1 in 10^4 cells survives prolongued culture with this antibody. Since the line was not mutagenized, we have selected Jurkat variant clones, which were present in the starting population. The JA clones do not display loss of function alterations, since CD95-mediated apoptosis could be restored by concomitant incubation with inhibitors of protein or mRNA synthesis. In the JA clones, the CD95 signalling pathway is inhibited in between the receptor and CPP32. The presumed inhibitory factor most likely does not act on CPP32, since this caspase can be activated by exogenous ceramide, which bypasses apoptosis-resistance in the JA clones (results not shown). Known components of the CD95 pathway upstream from CPP32 are FADD and FLICE (caspase-8). FADD variants lacking the death effector domain, which is required for coupling to FLICE, and FLICE variants lacking a functional protease domain can act as dominant negative signalling molecules which inhibit receptor mediated apoptosis (Boldin et al., 1996, Chinnayian et al., 1995, Hsu et al., 1996). It will be of interest to determine whether overexpression of endogenous versions of such molecules is responsible for inhibition of the CD95 signalling pathway in the JA clones.

The majority of CD95-resistant JA clones are resistant to TcR-induced apoptosis, in line with the mechanism proposed earlier by various groups (Ju et al., 1995, Brunner at al 1995, Dhein et al., 1995). CD95 can play a role in TcR-mediated, so-called 'activation-induced cell death' in peripheral T lymphocytes, which serves to attenuate the immune response. This follows from *in vitro* and *in vivo* analysis of CD95-deficient murine T cells (Russel et al., 1991, Singer and Abbas 1994). However, it has been evident for quite some time that the TcR can also induce apoptosis in a CD95-independent fashion, at least in thymocytes, since negative selection of autoreactive thymocytes is not impeded in CD95-deficient *lpr/lpr* mice (Singer and Abbas 1994, Sidman et al., 1992). In a TcR transgenic model, it was recently found that activation-induced cell death in peripheral T cells induced by the specific antigen influenza hemagglutinin, was independent of CD95 (Sytwu et al., 1996). Whereas a role for TNF was found in another system (Zheng et al., 1995), blocking of TNF receptors did not interfere with TcR-mediated apoptosis of peripheral T cells in the

influenza hemagglutinin system (Sytwu et al., 1996). Also in Jurkat cells TNF does not play a role in TcR-induced death, since Jurkat cells are TNF-resistant unless they are treated with inhibitors of *de novo* protein synthesis (results not shown). Possibly, other apoptosis-inducing members of the TNF receptor family can play a role in activation induced cell death in peripheral T cells and deletion of autoreactive thymocytes, such as TRAMP/Wsl-1/Apo-3/DR3 (Chinnayian 1996c, Kitson et al., 1996, Marsters et al., 1996, Bodmer ey al 1997) or the Trail receptor (Pan et al., 1997). Alternatively, no second receptor system is involved and the TcR directly activates the apoptotic machinery.

The CD95-resistant clones, with the exception of JA 9.3 and JA 10.2, are cross-resistant to apoptosis-induction by the anti-cancer drug etoposide (Boesen et al., submitted). Etoposide is a topoisomerase II inhibitor, which induces double strand DNA breaks. Friesen et al., have recently provided evidence that the DNA damaging drug doxorubicin can induce CD95L upregulation and CD95-mediated apoptosis in a human leukemic T cell line (Friesen et al., 1996). This mechanism would explain the cross-resistance as observed in the JA clones. However, in wild type Jurkat cells, nor in primary thymocytes, do we find evidence for role of CD95 in etoposide-induced apoptosis (Boesen et al., submitted). Most likely, as for TcR-induced apoptosis, there are CD95-dependent and -independent mechanisms to induce apoptosis in response to DNA damaging drugs.

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