APO-1(CD95)-mediated apoptosis in Jurkat cells does not involve src kinases or CD45

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Abstract Tyrosine phosphorylation has been reported to be an early event required for APO-1/Fas(CD95) signalling in lymphocytes [Eischen, C.M., Dick, C.J. and Leibson, P.J. (1994) J. Immunol. 153, 1947–1954]. We have compared two mutant Jurkat cells, one largely deficient in expression of CD45 (J45.01) and a second one deficient in expression of p56[lck] (JCaM1.6) with wild type Jurkat cells for their ability to undergo APO-1-induced apoptosis. No significant difference was observed among the three cell lines. In the mutant Jurkat cells APO-1 triggering did not result in increased tyrosine phosphorylation of cytosolic proteins. Furthermore, herbimycin A did not inhibit but rather augmented apoptosis at concentrations which effectively degraded the src related kinases lck and fyn. The data suggest that APO-1-mediated signalling is independent from src kinases and CD45.

Key words: APO-1/Fas; lck; fyn; Herbimycin A

1. Introduction

Apoptosis plays a role in embryogenesis, metamorphosis, tissue atrophy, neural network formation, tumor regression and is essential for normal development of the immune system [1–4]. Apoptosis can be mediated via the APO-1/Fas(CD95) receptor, a member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily [5,6]. All members of this superfamily are characterized by cysteine-rich motives of about 40 amino acids located in the extracellular portion of the receptors.

The cytoplasmic region of human APO-1 consists of 145 amino acids and does not contain any known consensus sequences for an enzymatic function or binding sites for so far known signalling molecules. However, it has a homology to the intracellular death domain of the TNF type I receptor which has also been shown to be capable to transduce cytotoxic signals into the cells [7,8].

Recent studies employing inhibitors of protein tyrosine kinases (PTK) suggested that certain forms of apoptosis might require activation of PTKs [9–11]. Thus, Eischen et al. reported that activation of PTKs represents an early signal required for APO-1-mediated signalling in human lymphocytes [11]. The major PTKs known to date to be required for receptor-mediated signalling events in human lymphocytes are two members of the src family of protein tyrosine kinases, p56[lck] and p59[fyn]. Both PTKs are involved in signalling via the TCR/CD3 complex and a number of accessory receptors [12–15]. There is now compelling evidence that the enzymatic activity of lck and fyn is regulated by the protein tyrosine phosphatase (PTPase) CD45 [15,16]. Expression of CD45 has been proven to be a prerequisite for e.g. TCR/CD3-mediated activation of the PTK pathway in human lymphocytes [17]. It was therefore important to determine whether src-related PTKs and CD45 might also be involved in APO-1-mediated signalling. Using two mutant Jurkat cell lines which have previously been demonstrated to be impaired in TCR/CD3 signalling due to altered expression of p56[lck] and CD45, respectively, we show that the signals initiated by APO-1 do not depend on expression of either src-related PTKs or CD45. This assumption is further supplemented by data which demonstrate that the classical PTK inhibitor herbimycin A does not inhibit but rather augments APO-1-mediated apoptosis in wild type Jurkat cells.

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Abbreviations: APO-1L, APO-1 ligand; PBS, phosphate-buffered saline; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; MAPK, MAP kinase; Herb. A, herbimycin A.

2. Materials and methods

2.1. Cells

The leukemic T-cell line Jurkat and its mutants were maintained in RPMI 1640 (Gibco Biocult, Eggenheim, Germany) 10 mM HEPES, pH 7.3, 10% FCS (Conco, Wiesbaden, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) in 5% CO_2. The CD45 deficient Jurkat mutant J45.01 [18] and the Jurkat mutant JCaM1.6 which lacks expression of p56[lck] [13] were kindly provided by Drs. G. Koretzky (University of Iowa, IO) and A. Weiss (UCSF, CA), respectively.

2.2. Antibodies and reagents

The mouse monoclonal antibody (mAb) anti-APO-1 (IgG3, κ) recognizes an epitope on the extracellular part of APO-1 [19]. OKT3 (IgG2a) is specific for CD3. Monoclonal antibody AICD45.2 (IgG1) reacts with CD45 [20]. The anti-ERK-I-III (MAPK) rabbit Ab and the biotinylated anti-phosphotyrosine mAb 4G10 were obtained from UBI (Lake Placid, NY). The anti-p56[lck] rabbit antiserum was kindly provided by Dr. A. Veillette (McGill University, Montreal, Canada) and the rabbit antiserum Ab cst-1 (directed at a common determinant at the C-terminus of p59[fyn], p62[c-yes] and p60[c-src]) was a generous gift from Dr. S. Courtneidge (EMBL, Heidelberg, Germany). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

2.3. Western blotting, pervanadate treatment and anti-phosphotyrosine analysis

10^7 cells/ml were incubated in the presence or absence of 10 μg/ml antibody at 37°C. Stimulation was stopped by addition of 200 μl of hot reducing standard SDS sample buffer. The samples were cooled on ice. 20 μl were separated by 12.5% SDS-PAGE. For pervanadate treatment 2×10^6 cells were incubated with 0.1 or 1 mM sodium pervanadate and 1 mM H_2O_2 for 15 min at 37°C. Cells were washed twice in ice-cold TBS and lysed in a 1% Triton X-100 containing buffer. Following SDS-PAGE samples were transferred onto Hybond nitrocellulose membrane (Amersham, Braunschweig, Germany), which was blocked with 5% (w/v) dry milk in PBS for at least 1 h and then washed with PBS containing 0.02% (v/v) Tween-20 (PBS/Tween). The blot was either

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incubated with 10 μg/ml biotinylated 4G10, 1:1000 (v/v) anti-lck, 1 μg/ml cst-1 or 1 μg/ml anti-MAPK antibody for 16 h at 4°C. After washing with PBS/Tween lck/fyn/MAPK blots were incubated with a horseradish peroxidase (HRPO)-coupled anti-mouse IgG (1:30,000; v/v) (Dianova, Hamburg, Germany). 4G10-biotin blots were incubated with streptavidin-biotinylated HRPO complex (1:5,000; v/v) (Amersham). All blots were developed with the chemiluminescence method ECL following the manufacturer's protocol (Amersham).

2.4. Immunofluorescence

For indirect immunofluorescence, 10^6 cells were washed and resuspended in 100 μl of affinity-purified antibody diluted in PBS, 5% fetal calf serum, 0.1% NaN_3 (10 μg/ml). Cells were incubated for 30 min on ice, washed and resuspended in 100 μl of 1:200 diluted Phycoerythrine (PE)-conjugated goat anti-mouse IgG (Dianova), incubated for 30 min on ice, washed again and analyzed on a FACScan (Becton Dickinson).

2.5. Cell stimulation and cytotoxicity assay

10^6 cells untreated or pretreated with Herb. A were incubated with 10 μg/ml anti-APO-1 (5×10^5 cells/ml) for 4 h at 37°C unless otherwise stated in the figure legends. Quantification of DNA fragmentation to measure of apoptosis was carried out essentially as described elsewhere [21].

3. Results and discussion

It has been recently shown that TCR-induced apoptosis involves the APO-1 receptor/ligand system [22,23]. This process can be dissected into two sequential steps. (1) Triggering of the TCR/CD3-complex induces synthesis of the APO-1 ligand (APO-1L). (2) Secretion of the APO-1L and binding of APO-1L to the APO-1 receptor then induces apoptosis. In the leukemic T cell line Jurkat apoptosis can, therefore, be induced by stimulating either the TCR/CD3 complex or, alternatively, the APO-1 receptor directly (e.g. employing APO-1L or anti-APO-1 antibodies). Triggering the TCR results in a balanced activation of PTKs such as $p56^{lck}$ and PTPases such as CD45 [16]. It has also been reported that direct triggering of the APO-1 receptor in human Jurkat T cells involves activation of PTKs [11]. Since activation of the PTK pathway is a prerequisite for TCR/CD3-mediated T-lymphocyte activation, we were interested to analyze whether the mechanisms underlying signalling via TCR/CD3 and APO-1 were similar. To this end we analyzed three Jurkat cell lines, wild type Jurkat cells, J45.01 and JCaM1.6, for their ability to undergo apoptosis following triggering of the APO-1 receptor. J45.01 is largely deficient in expression of CD45 [18] whereas JCaM1.6 is deficient in expression of $p56^{lck}$ [13]. Both cell lines have previously been demonstrated to be strongly impaired in their ability to signal via the TCR/CD3 molecular complex, due to lack of expression of the two enzymes. The phenotype of both cell lines is depicted in Fig. 1 and Table 1, respectively.

We first investigated the pattern of tyrosine phosphorylation following TCR/CD3 and APO-1 triggering in wild type Jurkat cells and the two mutant cell lines. As shown in Fig. 2A, stimulation of wild type Jurkat cells employing an anti-CD3 mAb resulted in rapid phosphorylation of a particular set of proteins on tyrosine residues. In agreement with previously published data no increase in tyrosine phosphorylation was detectable in the two mutant Jurkat cell lines following CD3-mediated T-cell activation (Fig. 2A). Moreover, pervanadate treatment which has previously been shown to activate cytoplasmic PTKs through inhibition of PTPases [24] did hardly induce phosphorylation of proteins in JCaM1.6 cells whereas it lead to massive accumulation of tyrosine phosphorylated proteins in wild type Jurkat and J45.01 cells (Fig. 2C). Aside from being deficient in CD3-mediated signalling, JCaM1.6 cells seem to have a general reduction in cytoplasmic PTK activity. Perhaps more importantly, anti-APO-1 did not induce significant tyrosine phosphorylation of cytosolic proteins in all tested cells (Fig. 2B) suggesting that APO-1 does not activate a classical PTK pathway.

We next investigated J45.01 and JCaM1.6 for their ability to undergo apoptosis following triggering of the APO-1 receptor. Fig. 3 demonstrates that both cell lines were equally sensitive towards anti-APO-1-induced apoptosis when compared to wild type Jurkat cells. All three cell lines were efficiently killed by 10 ng/ml anti-APO-1 (Fig. 3). These data suggest that APO-1-mediated signalling does neither depend on expression of $p56^{lck}$ nor on expression of enzymatically active CD45.

Since we could not exclude the possibility that activation of src-related kinases different from lck were responsible for induction of apoptosis in J45.01 and JCaM1.6 cells, we investigated whether Herb. A might inhibit this pathway in wild type Jurkat cells. Herb. A represents a non-competitive inhibitor of src-related PTKs and acts by directing them towards degradation [25]. It has recently been reported to inhibit anti-APO-1-induced apoptosis at high concentrations [11]. Wild type Jurkat cells were incubated with DMSO alone or, alternatively, with increasing concentrations of Herb. A for 18 h. Subsequently, cells were stimulated with anti-APO-1 antibody for increasing periods of time. To confirm that src kinases were degraded under these conditions $p56^{lck}$ and $p59^{fyn}$ protein expression was determined by Western blotting. As shown in Fig. 4E a Herb. A concentration of 4 μM was sufficient to completely degrade lck. $p59^{fyn}$ was more resistant to the degrading effect of Herb. A. However, an in vitro kinase assay of fyn immunoprecipitates indicated complete suppression of enzymatic activity already at 1 μM Herb. A (data not shown). Importantly, concentrations of Herb. A up to 4 μM did not inhibit but rather augmented APO-1-mediated apoptosis (fig. 4A–C). Moreover, a concentration of 10 μM Herb. A induced spontaneous apoptosis of

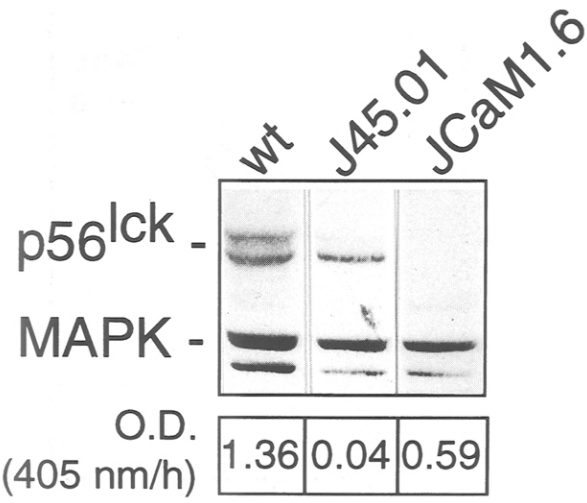

Fig. 1. Characterization of Jurkat mutants J45.01 and JCaM1.6. 2.5×10^5 cells were subjected to a Western blot analysis using an anti-lck antibody (upper panel). As an equal loading control the same blot was developed with an anti-MAPK antibody (center panel). CD45 phosphatase activity was determined as described elsewhere [20]. The background was 0.01.

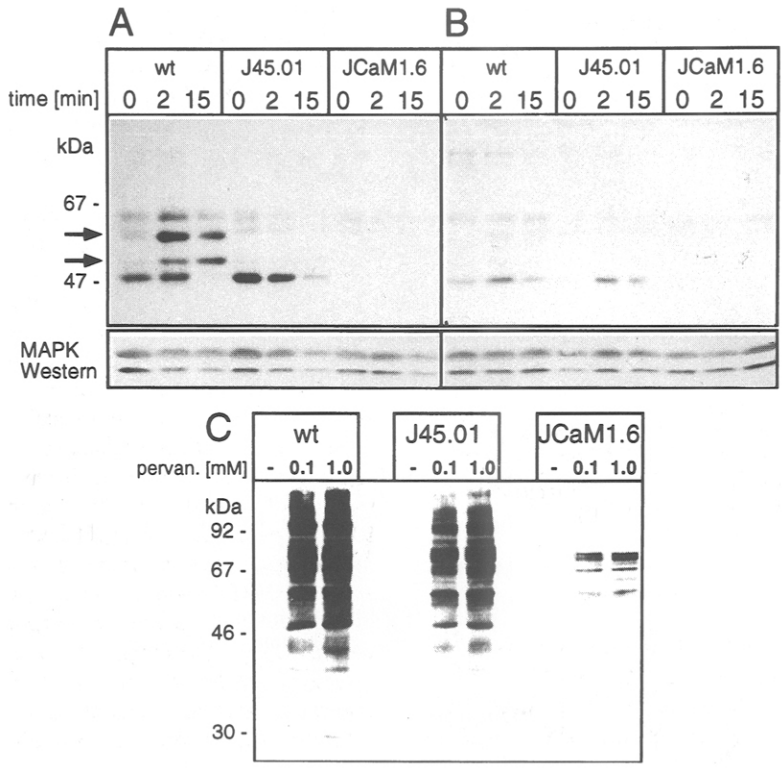

Fig. 2. Analysis of tyrosine-phosphorylated cytosolic proteins. A,B: 10^6 Jurkat cells were incubated with 10 μg/ml OKT3 (A) or with 10 μg/ml anti-APO-1 (B) and then subjected to an anti-phosphotyrosine Western blot. The same blot was probed with an anti-MAPK antibody as a control for equal lane loading. C: Cells were incubated in the absence (−) or presence (+) of pervanadate for 15 min and the lysates from 2×10^5 cells were subjected to an anti-phosphotyrosine Western blot analysis.

almost 20% of the cells even in the absence of anti-APO-1 mAb (Fig. 4D). This strongly suggests that this Herb. A concentration was toxic for the cells. These data are consistent with previous reports showing that a number of PTK inhibitors such as Genistein, Herb. A and others can induce apoptosis in lymphoid cells including Jurkat T cells [26–28]. Since it is now becoming clear that cells can only undergo anti-APO-1-induced cell death when their vitality is not affected, we interpret the failure of high concentrations of Herb. A to enhance apoptosis as being due to reduced viability of the cells. In this regard it is important to mention that a previous report could only demonstrate inhibition of anti-APO-1-induced apoptosis at Herb. A concentrations which inhibited cell growth [11]. Recently, a protein tyrosine phosphatase FAP-1 was identified that associates with APO-1 and confers resistance to APO-1-induced apoptosis [29]. However, since that enzyme is not expressed in Jurkat cells [29]. FAP-1 can not be crucial for the APO-1 signalling pathway.

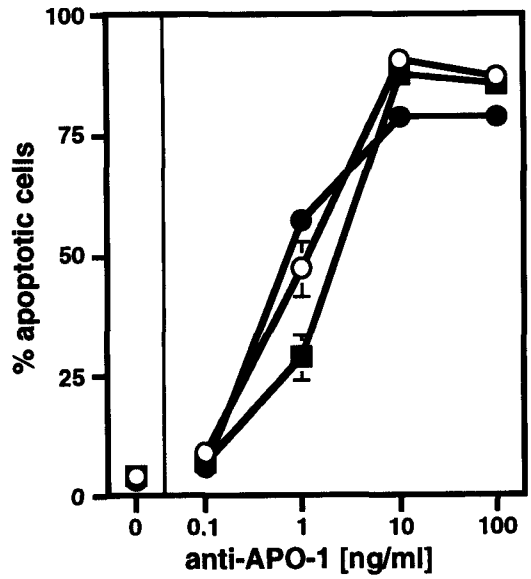

Fig. 3. The lack of CD45 or $p56^{lck}$ has no effect on anti-APO-1-induced sensitivity. 10^6 cells wt Jurkat (○), J45.01 (■) or JCaM1.6 (●) cells were incubated with different anti-APO-1 concentrations for 18 h. Apoptotic cells were determined as described in section 2.

Our data suggest that tyrosine phosphorylation might not play a critical role during APO-1-mediated apoptosis. First,

Table 1
Analysis of surface molecules on Jurkat cells

Cell line	Control	APO-1	CD45	CD3
Wt Jurkat	2.2 (4)	99.7 (54)	99.3 (50)	94.2 (94)
J45.01	1.1 (3)	98.3 (35)	6.2 (6)	99.2 (152)
JCaM1.6	1.3 (3)	99.8 (49)	97.9 (31)	87.1 (79)

In parenthesis: Mean fluorescence intensity. The following antibodies were used: anti-APO-1, OKT3 (anti-CD3) and AICD45.2 (anti-CD45). A Phycoerythrine-conjugated goat anti-mouse IgG antibody was used as second antibody. Controls were only stained with secondary antibody.

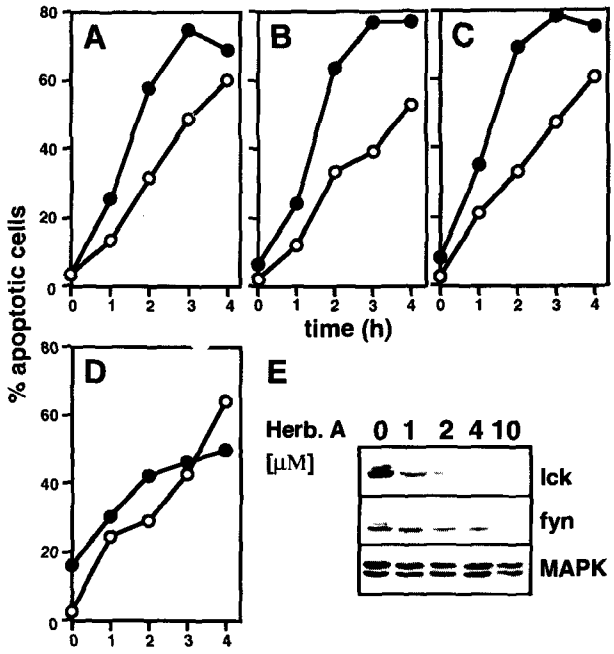

Fig. 4. Effects of Herb. A on APO-1-dependent apoptosis sensitivity. A–D: Wt Jurkat cells were preincubated with 0.05% (A), 0.1% (B), 0.2% (C) or 0.5% DMSO in the absence (○) or presence of (●) 1 μM (A), 2 μM (B), 4 μM (C) or 10 μM (D) Herb. A for 18 h and then incubated with 10 μg/ml anti-APO-1. E: 5×10^6 Jurkat cells were preincubated with Herb. A at different concentrations for 18 h. A Western blot using an antiserum directed at $p56^{lck}$ (upper panel), at $p59^{fyn}$ (center panel) or a mAb against the MAPK (lower panel).

CD45 and $p56^{lck}$ deficient Jurkat mutants (J45.01 and JCaM1.6) were as sensitive towards APO-1-mediated signalling as their wild type counterparts. Second, no increase in tyrosine phosphorylation was detectable in the severely tyrosine phosphorylation defective T cell mutant JCaM1.6. Third, the classical PTK inhibitor Herb. A did not inhibit but rather increased apoptosis at concentrations which completely inhibited src kinases. The previously published induction of PTK activity following APO-1 triggering [11] could be due to a secondary induction of src related kinases in the tested cells and the observed inhibition of apoptosis by Herb. A could be due to the properties of tyrosine kinase inhibitors to induce apoptosis themselves. Therefore, we conclude that APO-1 mediated signalling is fundamentally different from the classical TCR/CD3 mediated pathway of human T-cell activation as it does not seem to depend on protein tyrosine phosphorylation.

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