Differential role of tyrosine phosphorylation in the induction of apoptosis in T cell clones via CD95 or the TCR/CD3-complex

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Abstract

Activated T cells undergo apoptosis when the Fas-antigen (APO-1, CD95) is ligated by Fas Ligand (FasL) or agonistic anti-Fas antibodies. Repeated stimulation of T lymphocytes via the TCR/CD3-complex induces activation-induced cell death (AICD) associated with FasL surface expression. FasL binding to Fas molecules triggers the Fas-dependent death signaling cascade. Since it is still controversial whether Fas-induced cell death is associated with tyrosine kinase activity, we investigated the tyrosine kinase activation requirements in anti-Fas antibody-induced cell death and AICD in human T cell clones. We report that cell death triggered by anti-Fas antibody is not accompanied by an increase in tyrosine phosphorylation and cannot be blocked by inhibitors of protein tyrosine kinases (PTK). Under the same conditions, AICD of T cell clones is clearly associated with tyrosine kinase activation. In fact, semiquantitative RT-PCR analysis of FasL mRNA expression triggered in T cell clones via the TCR/CD3-complex revealed that tyrosine phosphorylation is required for functional FasL mRNA and surface expression.

Keywords: human, T lymphocytes, apoptosis, signal transduction, tyrosine phosphorylation, CD95, T cell receptor

Abbreviations: AICD, activation-induced cell death; FasL, Fas ligand; PI, Propidium iodide; PTK, protein tyrosine kinase

Introduction

At least two major cell death pathways exist in activated mature T cells. The first pathway is induced via the interaction of cell surface Fas antigen (CD95, APO-1) with the corresponding Fas ligand (FasL) or ligation of the Fas molecule with agonistic anti-Fas antibodies (see Nagata and Golstein, 1995 for review). The second cell death pathway,

also referred to as activation-induced cell death (AICD), can be initiated in preactivated mature T lymphocyte by ligation of the TCR-CD3-complex or other surface structures such as CD2 (Janssen *et al*, 1991; Wesselborg *et al*, 1993a,b; Kabelitz *et al*, 1993). It has been shown recently that the two pathways are causally related and in concert play a critical role in immune selection, target cell destruction and immune response termination (Russel, 1995).

The intracellular mechanisms of Fas-induced or activation-induced cell death are not precisely understood. Cross-linking antibodies to Fas or FasL in its trimeric form induce apoptosis in most Fas-positive cells indicating that close proximity of the cytoplasmic tails of several Fas-molecules is required to activate downstream molecules (Dhein *et al*, 1992; Suda and Nagata, 1994). The Fas-triggered pathway is independent of extracellular Ca^{2+} (Rouvier *et al*, 1993), does not require macromolecular synthesis (Yonehara *et al*, 1989; Itoh *et al*, 1991; Klas *et al*, 1993; Stalder *et al*, 1994; Golstein *et al*, 1994) and can proceed in the absence of a nucleus (Schulze-Osthoff *et al*, 1994a). It has been suggested that Fas-signals are conveyed via the activation of acidic sphingomyelinases resulting in the production of ceramide, a complex lipid which induces rapid apoptosis in different cell types when added to the culture medium (Cifone *et al*, 1994; Jarvis *et al*, 1994). Ceramide mediates Fas-induced apoptosis by activating the ras signaling pathway (Gulbins *et al*, 1995). Using the cytoplasmic tail of Fas in the yeast two-hybrid system, other putative components of the Fas-mediated death pathway have been identified. Two of these, termed FADD and RIP contain death domains which interact with the death domain of Fas (Chinnaiyan *et al*, 1995; Stanger *et al*, 1995). Other potential regulators of Fas-mediated apoptosis are the FAP-1 phosphatase which when overexpressed in Jurkat cells inhibits anti-Fas antibody-initiated cell death (Sato *et al*, 1995), or the FAST serine/threonine kinase that is activated upon Fas- but not upon TCR/CD3-ligation in Jurkat cells (Tian *et al*, 1995).

By co-immunoprecipitation strategies with anti-CD95 mAb, four cell death associated proteins (CAP, for cytotoxicity-dependent APO-1-associated proteins) have been identified which in concert with the intracellular part of CD95 form a death inducing signaling complex (DISC) (Kischkel *et al*, 1995). Two of these proteins (CAP1, CAP2) were recognized by anti-MORT-1 (anti-FADD) antibodies (Kischkel *et al*, 1995). CAP4 encodes a novel 55 kDa protein termed FLICE (also MACH) which shares homology to FADD and ICE-like cysteine proteases (Muzio *et al*, 1996; Boldin *et al*, 1996). It was also suggested that CAP3 represents the FLICE prodomain which remains bound to Fas upon proteolytic activation (Muzio *et al*, 1996).

Eischen and colleagues reported that in Jurkat T cells as well as in CD95⁺ U937 and NK cells, tyrosine kinase activation provides an early and requisite signal for Fas-induced apoptosis (Eischen *et al*, 1994). However, so far no tyrosine kinase has been shown to directly interact with the cytoplasmic portion of the Fas molecule or to be selectively activated following Fas ligation. In addition, the finding that Fas-induced apoptosis can be blocked very efficiently with protein kinase inhibitors such as Herbimycin A, Genistein and Staurosporine (Eischen *et al*, 1994) is controversial (Schulze-Osthoff *et al*, 1994b; Schraven and Peter, 1995; Janssen *et al*, 1996). In line with the aforementioned report (Eischen *et al*, 1994), in a previous study with Jurkat cells, we also observed a rapid and transient tyrosine phosphorylation when the Fas molecule was ligated with cross-linking antibodies (IgM isotype) on parental Jurkat cells (Janssen *et al*, 1996). The pattern of tyrosine phosphorylated proteins resembled the pattern seen upon TCR/CD3-ligation and was similar to the one described by Eischen *et al*. However, our

analysis of various Jurkat-subclones clearly revealed that Jurkat variant cells were efficiently killed by anti-Fas mAb in the absence of detectable tyrosine phosphorylation (Janssen *et al*, 1996).

Similar results were obtained by other investigators when various Jurkat subclones were analyzed that lacked p56^{lck} or CD45 activity (Schraven and Peter, 1995; Oyaizu *et al*, 1995; Latinis and Koretzky, 1996). These studies clearly demonstrated that neither p56^{lck} nor CD45 phosphatase activity are required to mediate Fas-induced cell death. In addition, in the same study, Latinis and Koretzky provided preliminary evidence that Fas-triggering induces serine/threonine kinase activity (Jun kinase) rather than tyrosine phosphorylation. Furthermore, when analyzing the formation and phosphorylation of the death inducing complex formed around the cytoplasmic tail of CD95, Kischkel and colleagues failed to detect tyrosine phosphorylation but found serine phosphorylation of associated CAPs (Kischkel *et al*, 1995).

Figure 1 Tyrosine phosphorylation in whole cell lysates following short-term TCR/CD3- or CD95-stimulation. Clone cells (clones D894/25 (**A**) and D798/18 (**B**)) were kept in medium or preincubated with Herbimycin A for three hours prior to stimulation. 1.5×10^6 cells per sample were then incubated for 1 to 10 min with the anti-CD3, anti-CD95 or anti-TCR antibodies. Rabbit anti-mouse (RaM) antibodies used to cross-link IgG isotype antibodies against the TCR or CD3 served as a control. Following incubation in a prewarmed antibody solution (37°C), the cells were washed once in cold PBS and immediately lysed in NP40 lysis buffer. Proteins separated by SDS-PAGE were transferred to Nitrocellulose membranes and phosphotyrosine-containing proteins were detected with mAb 4G10, horseradish-

To further characterize the role of tyrosine kinase activation in cell death of IL-2-dependent, non-transformed human T lymphocytes, we compared the tyrosine phosphorylation requirements in TCR/CD3-mediated AICD and anti-Fas antibody-induced apoptosis in different T cell clones. Our data clearly demonstrate that Fas-mediated cell death is independent of detectable short-term tyrosine phosphorylation. In addition, in all T cell clones tested, CD95-induced cell death could not be blocked by inhibitors of PTKs such as Herbimycin A. By contrast, TCR/CD3-mediated AICD was almost completely abrogated when AICD-sensitive clone cells were pretreated with the inhibitor of src-related kinases. As analyzed by a semiquantitative RT-PCR approach, we also demonstrate that Herbimycin A blocks AICD at the level of FasL mRNA expression. The data presented here fit into the concept that tyrosine kinase activity plays a pivotal role in TCR/CD3-triggered AICD being necessary for the induction of FasL expression. The induction of CD95-mediated death, however, is independent of PTK activity and likely to be regulated 'more directly', e.g. due to the intrinsic protease activity of Fas-associated FLICE/MACH (Muzio *et al*, 1996; Boldin *et al*, 1996).

Results

CD95-ligation does not induce tyrosine phosphorylation in human T cell clones

Since there is a still on-going discussion whether or not CD95-induced cell death is associated with rapid changes in the tyrosine phosphorylation in some cell types and not in others (Eischen *et al*, 1994; Schulze-Osthoff *et al*, 1994b; Schraven and Peter, 1995; Janssen *et al*, 1996), we analyzed the phosphoprotein patterns within whole cell lysates of non-transformed human T cell clones stimulated with mAb against the TCR/CD3-complex or CD95, respectively. All tested clones expressed comparable levels of TCR/CD3 and CD95 antigen (not shown). As demonstrated in Figure 1 for two representative clones (D894/25 (A), D798/18 (B)), CD3- or TCR-ligation induced the rapid phosphorylation of a characteristic and similar set of proteins as detected by a sensitive Western blotting technique using enhanced chemiluminescence. In agreement with our previous report on tyrosine phosphorylation-independent CD95-induced apoptosis in leukemic human T cell lines (Janssen *et al*, 1996), we did not detect a significant increase in tyrosine phosphorylation upon stimulation (here shown up to 10 min) with anti-CD95 antibodies or rabbit anti-mouse cross-linking antibodies used as a control for TCR/CD3-stimulation (Figure 1). We also failed to detect changes in tyrosine phosphorylation upon longer exposure (up to 2 h) of clone cells to anti-CD95 mAb (data not shown).

Inhibition of AICD but not Fas-induced cell death by Herbimycin A

Next, we analyzed whether TCR/CD3- and/or CD95-induced death of T cell clones depended on protein

tyrosine kinase activity and thus could be influenced by PTK-inhibitors. As also shown in Figure 1, TCR/CD3-induced tyrosine phosphorylation was efficiently reduced by pretreatment with the tyrosine kinase inhibitor Herbimycin A. We therefore determined the fraction of clone cells that underwent apoptosis during overnight incubation in medium alone or with anti-CD3-, anti-CD95- or anti-TCR mAb in the presence or absence of PTK inhibitors. We used two different methods to analyze cell death, i.e. PI-staining of dead cells and determination of absolute numbers of viable cells by standard cell dilution assay (SCDA). Representative experiments for clones D894/25 (A), and D798/18 (B), are shown in Figure 2 (PI-staining) and for all tested clones in Figure 3 (SCDA), respectively. As judged by PI-staining, in individual experiments, the fraction of dead cells following overnight incubation in the presence of 2 μ g/ml anti-Fas antibody 7C11 was between 43 and 91% (mean $74 \pm 17\%$). All five clones underwent AICD when stimulated with anti-CD3 or anti-TCR mAb (24 to 91% apoptotic cells (mean $65 \pm 23\%$)). In the experiments shown in Figure 2, incubation of clones D894/25 (A) or D798/18 (B) with soluble anti-CD3 or anti-TCR mAb resulted in a significant increase in the number of apoptotic cells. The fraction of dying cells was calculated based on fluorometric analyses following PI-staining with small PI-negative cells with reduced forward and side scatter properties considered to be in the process of undergoing apoptosis. Thus, when compared to 10% apoptotic cells in the absence of antibodies, 31% (A) or 24% (B) of the cells underwent apoptosis upon CD3-stimulation and 67% or 61% upon

Figure 2 PI-staining of dead clone cells. 10^6 cells (clone D894/25 (A) and D798/18 (B)) were incubated overnight in 1 ml of culture medium in the presence or absence of soluble anti-CD3, anti-CD95 or anti-TCR antibodies with or without Herbimycin A. Dead cells were stained with Propidium iodide. The cell size (forward scatter=FSC, x-axis) is plotted against the relative cell number (z-axis) and the red fluorescence intensity (FL-2, y-axis).

Figure 3 Inhibition of AICD but not Fas-induced apoptosis by Herbimycin A. Cell viability of clone cells was analyzed using the SCDA protocol. The cells were incubated in IL-2-containing medium alone or with anti-CD3, anti TCR or anti-CD95 mAb in the absence (white bars) or presence (black bars) of Herbimycin A. Five different T cell clones were tested (A–E). Values are given as per cent viable cells normalized to the medium control in the absence of Herbimycin A (set to 100%).

TCR-stimulation. All clones were susceptible to 7C11-mediated cell death. The fraction of dead or apoptotic cells upon incubation in the presence of anti-CD95 mAb was 43, 80 or 87%, respectively (Figure 2). Although slight changes in the cell size were noted, treatment of D894/25 or D798/18 clone cells with 1.5 μ M Herbimycin A did not significantly affect the viability in medium alone (e.g., 15% and 12% apoptotic cells, respectively, in the experiment shown in Figure 2). However, in these clones, Herbimycin A very efficiently blocked AICD in the presence of anti-CD3 or anti-TCR mAb – but enhanced anti-Fas antibody induced cell death.

Similar results were obtained when cell viability instead of cell death was analyzed in a standard cell dilution assay (SCDA, Figure 3). The data shown in Figure 3 were normalized to the medium control in the absence of Herbimycin A (set to 100%). Cell viability in TCR/CD3-stimulated cultures was significantly restored in all five T cell clones in the presence of Herbimycin A (Figure 3 A–E). By contrast, anti-CD95-induced cell death was unaffected or even enhanced in the presence of PTK inhibitor. Herbimycin A alone did not protect any of the clones from CD95-induced cell death but rather significantly increased the number of dead cells in four of the five tested clones exposed to anti-CD95 mAb.

Herbimycin A inhibits TCR/CD3- but not CD95-driven DNA-fragmentation

We also analyzed the DNA-degradation in methanol-fixed cells following overnight incubation with or without Herbimycin A in the presence or absence of anti-TCR, anti-CD3 or anti-CD95 mAb (Figure 4). Cell death as analyzed in Figure 2 was accompanied by the appearance of hypodiploid DNA-fragments indicative of endonuclease activity. Shown for clones D894/25 (A) and D798/18 (B) in Figure 4, anti-CD3 or anti-TCR-induced DNA-degradation was markedly reduced in the presence of Herbimycin A. By contrast, CD95-induced DNA fragmentation was not affected or even enhanced. The results of other experiments using the different T cell clones strictly correlated with the data presented for PI-staining and SCDA.

Effects of other kinase inhibitors on AICD or Fas-induced apoptosis

The effects of other inhibitors of signaling proteins such as Staurosporine (Tamaoki *et al*, 1986), Genistein (Akiyama *et al*, 1987), Tyrphostin (Gazit *et al*, 1989), and Wortmannin (Powis *et al*, 1994) on AICD or CD95-induced apoptosis were analyzed primarily with regard to the inhibition of cell death in

Figure 4 Inhibition of TCR/CD3-induced but not CD95-induced DNA fragmentation by Herbimycin A. Clone cells (clones D894/25 (A) and D798/18 (B)) were stimulated overnight in the presence or absence of Herbimycin A with medium alone or soluble anti-CD3, anti-CD95 or anti-TCR antibodies, respectively. Cells were either kept in medium alone or in the presence of Herbimycin A for three hours prior to stimulation. The DNA content was analyzed following fixation with methanol and DNA-staining with Propidium iodide. Red fluorescence intensity (FL-2, x-axis) is plotted against the relative number of events acquired.

overnight cultures of clone cells stimulated with anti-TCR/CD3 reagents or anti CD95 antibodies, respectively. It should be noted that higher doses of all tested inhibitors (especially of Staurosporine) resulted in the induction of cell death in a significant fraction of clone cells by themselves. The 'non-toxic' concentrations that we used for inhibition experiments usually were 1 : 2 or 1 : 5 dilutions of doses that induced low level but significant apoptosis in the absence of any other stimulus. In such experiments, all four inhibitors sometimes showed a minor inhibitory effect on AICD (maximum inhibition 10–20%). However, in our hands, Herbimycin A was the only inhibitor that reproducibly and significantly blocked activation-induced cell death as demonstrated in the experiment with clone D894/25 shown in Figure 5. As can be seen, Herbimycin A not only blocked AICD triggered by anti-CD3 or anti-TCR mAb, but also AICD triggered by more physiological TCR-dependent stimuli such as superantigen SEA (Kabelitz and Wesselborg 1992). Of note, in most experiments, Genistein also accelerated but never inhibited CD95-induced death.

Herbimycin A blocks FasL mRNA and surface expression

It has been shown that in different T cell populations, TCR-mediated cell death is associated with the tyrosine kinase-

Figure 5 Effects of kinase inhibitors on AICD. Clone cells (clones D894/25) were stimulated overnight in the presence or absence of PTK-inhibitors at concentrations indicated in the Materials and Methods section and with medium alone or soluble anti-CD3 or anti-TCR antibodies, or Staphylococcal enterotoxin A. Cell viability was analyzed by staining with Propidium iodide.

dependent activation of FasL expression (Oyaizu *et al*, 1995; Migita *et al*, 1994; Orchansky and Teh, 1994; Vignaux *et al*, 1995; Anel *et al*, 1994). To test whether the inhibitory effect of Herbimycin A can be explained by differences in the

Figure 6 TCR/CD3-induced FasL mRNA (A) and FasL surface expression (B) in AICD is blocked by PTK inhibitor pretreatment. (A) RT-PCR-analysis of FasL mRNA expression in D894/25 cells with or without pretreatment with Herbimycin A and with or without stimulation with anti-CD3 mAb. The clone cells were pretreated for 3 h in Herbimycin A and thereafter incubated for 3 h with or without anti-CD3 (OKT3). The cells were then harvested and total RNA was subjected to RT-PCR. GAPDH served as a control for equal amounts of RNA prior to the PCR. (B) FACS-analysis of FasL expression on D894/25 cells with or without stimulation with anti-CD3 and anti-TCR mAb, respectively. The cells were pretreated for 3 h with Herbimycin A, then incubated for 3 h with or without anti-TCR/CD3 mAb, and analyzed using a biotinylated anti-FasL mAb and Streptavidin-PE.

inducibility of FasL mRNA or protein expression in T cell clones, we analyzed FasL mRNA induction in clones following stimulation with anti-TCR/CD3 mAb. As shown in Figure 6A, RT-PCR analyses of FasL mRNA from unstimulated or CD3-stimulated D894/25 cells pretreated with Herbimycin A revealed that in fact the PTK-inhibitor significantly prevented the CD3-stimulated induction of FasL mRNA expression. We also analyzed FasL surface expression using a biotinylated anti-FasL mAb with Streptavidin-PE (Figure 6B). Again, we compared the expression of FasL surface molecules on clone cells pretreated with the PTK-inhibitor and incubated in medium alone or in the presence of anti-CD3 mAb. As expected from the RT-PCR analysis of D894/25 cells, we detected a lack of the induction of cell surface FasL expression on clone cells that had been pretreated with Herbimycin A.

Discussion

We have analyzed the role of tyrosine phosphorylation in Fas- and TCR/CD3 induced cell death in several non-transformed, IL-2-dependent human T cell clones. Using the agonistic anti-human CD95 mAb 7C11 (IgM), we clearly demonstrate that at a clonal level, anti-Fas antibody-triggered cell death is not dependent on early tyrosine phosphorylation. By contrast,

AICD triggered via ligation of the TCR/CD3-complex is associated with and dependent on rapid activation of tyrosine, probably src-related, kinases. Therefore, AICD can be efficiently reduced by Herbimycin A, a potent inhibitor of src-type protein tyrosine kinases (June *et al*, 1990; Uehara *et al*, 1989) which blocks tyrosine phosphorylation-dependent FasL mRNA (and surface) expression. The data presented here extend our previous results with transformed leukemic Jurkat cells (Janssen *et al*, 1996) to the analysis of non-transformed normal T cells at the clonal level.

However, our results contradict the study of Eischen *et al*, reporting that in Jurkat T cells, tyrosine kinase activation provides an early and requisite signal for Fas-induced cell death (Eischen *et al*, 1994) and also a more recent study by Mollereau *et al*, describing tyrosine-phosphorylation-dependent CD2-induced apoptosis and also (less prominent) tyrosine phosphorylation upon Fas ligation in activated human T cells (Mollereau *et al*, 1996). It should be stressed that in our previous study (Janssen *et al*, 1996), we also reported that CD95-ligation induced tyrosine phosphorylation in parental Jurkat cells. In our experiments, we used similar concentrations of anti-CD95 antibodies (although we used different clones) and similar stimulation and lysis conditions as reported by Eischen and colleagues. These 'normal' Jurkat cells expressed Fas molecules and high levels of TCR and especially CD3 molecules. However, when testing a set of CD3-negative Jurkat subclones that undergo CD95-induced cell death, we had to conclude that CD95-induced cell death occurred independent of PTK activation and that the observed phosphorylation in parental Jurkat cells was likely to be a result of co-cross-linking of PTK-associated molecules such as the TCR/CD3-complex (Janssen *et al*, 1996).

Our results are in full agreement with those reported by Schraven and Peter (1995), Oyaizu and colleagues (1995), and Latinis and Koretzky (1996) for p56^{lck}- or CD45-negative Jurkat variants, respectively. In their studies, PTK-inhibitors also failed to block CD95-induced apoptosis. Oyaizu *et al*, reported that in their hands, TCR/CD3-induced cell death is abrogated in p56^{lck}-deficient JCaM cells when compared to parental JE6.1 cells. Taken together, from these studies one has to conclude that at least src-type kinases are not involved in CD95-mediated apoptosis as had been suggested by Eischen *et al*. By contrast, although the significance has to be proven, Latinis and Koretzky, (1996) suggested a Fas-mediated JNK (Jun Kinase, a serine-threonine kinase) activation with a clearly different time course kinetics as compared to the fast tyrosine phosphorylation reported by Eischen and colleagues.

On the other hand, it should also be mentioned that a recent report by Atkinson *et al* (1996) suggested a physical interaction between Fas and p59^{fyn} and a role of fyn in the regulation of Fas-mediated death and killing in murine T cells. From their observations in fyn knockout mice, it was concluded that fyn plays an important role in Fas signal transduction. However, one could also envision that in fyn knockout mice, activation-induced expression of the FasL is reduced which could also explain the elevated lifespan of activated T cells. Further, it has been suggested that fyn

plays an important role in the stabilization of FasL expression due to an interaction of the intracellular part of FasL with SH3 domains of the kinase (Hane, 1995). Our data showing that CD95-ligation does not directly increase tyrosine phosphorylation and that a specific inhibitor of src kinases does not block anti-CD95-induced apoptosis in untransformed human T cell clones, do not exclude that fyn and/or lck do have some impact on e.g. the lifespan via a potentially tyrosine phosphorylation-independent adaptor function through SH2 or SH3 domains.

Tyrosine phosphorylation plays a crucial role in TCR/CD3- (and CD2-) triggered AICD. It is well established that TCR/CD3-engagement induces tyrosine kinase-dependent signaling cascades which may or may not be accompanied by an increase in tyrosine phosphorylation of downstream second messenger molecules (Wange and Samelson, 1996; Rudd, 1994). However, it is still unclear which intracellular signaling molecules form switch points for the different outcomes (proliferation, cytokine production, AICD) following receptor activation. It is also not known how these molecules are activated or silenced in favour of the apoptosis pathway in stimulated cells. Thus, it has been reported for thymocytes that protein tyrosine kinase inhibitors such as Herbimycin A or Genistein prevent anti-CD3- but not glucocorticoid-induced apoptosis (Migita, 1994). Further, in proliferating murine T cells, AICD has been shown to be associated with altered phosphorylation of the various CD3 subunits (Orchansky, 1994) and in murine T cell hybridomas, signaling events most probably involving tyrosine kinase-dependent phosphorylation of tyrosine based motifs within the CD3 ζ -chains lead to rapid expression of FasL (Vignaux 1995). Importantly, in mouse cytotoxic T cell clones, the TCR-induced FasL expression could be blocked by PTK inhibitors Genistein and Herbimycin A (Anel, 1994).

Whether TCR/CD3-triggered AICD in previously stimulated T cells is mediated exclusively by Fas/FasL interactions or can at least in part also proceed when the Fas/FasL interaction is not operational is still not known. Several reports addressing the role of Fas/FasL in AICD indicate that blocking the Fas/FasL interaction during AICD (e.g. by Fas-Ig fusion proteins or anti-Fas antibody fragments) results in a substantial reduction of cell death (Alderson *et al*, 1995; Dhein *et al*, 1995; Brunner *et al*, 1995; Ju *et al*, 1995). These data in conjunction with many data on TCR/CD3-induced tyrosine phosphorylation suggest that the initial activation of protein tyrosine kinases as a consequence of surface receptor ligation (Wange and Samelson, 1996) may in fact be required as a crucial and indispensable step in TCR/CD3-triggered, Fas-dependent apoptosis. Our data presented here demonstrate that AICD as well as TCR/CD3-induced DNA-fragmentation can be efficiently blocked in AICD-susceptible human T cell clones by inhibition of src-type PTKs with Herbimycin A. Our analysis of FasL mRNA and surface expression clearly shows that this PTK-inhibitor blocks the level of FasL expression at an early stage before mRNA transcription and that on a clonal level this inhibition is sufficient to almost completely block AICD. The fact that Herbimycin A, which is a specific inhibitor of

src-related kinases was the only inhibitor in our hands to reproducibly reduce AICD in all experiments, points to an essential role of the src-type kinases p56^{lck} or p59^{lyn} in TCR/CD3-AICD. This is compatible with data from p56^{lck}-deficient leukemic cells (Oyaizu *et al*, 1995; Latinis and Koretzky, 1996). Of note, all inhibitors that we have tested induced apoptosis in IL-2-dependent T cells when used in higher concentrations. The partial (Genistein) or complete ineffectiveness (Staurosporine, Tyrphostin, Wortmannin) of other inhibitors to reduce AICD in human T cell clones further indicates that src-type PTK activity forms an initial step in AICD. Activation of src-related kinases is one of the earliest events in T cell activation. It results in the initiation of a variety of different signaling cascades including the recruitment and activation of ZAP-70 or PI 3-Kinase, and recruitment of multiple enzymes or adapter proteins to the activation complex (Wange and Samelson, 1996; Rudd *et al*, 1994). The blocking effect of the src-type kinase inhibitor Herbimycin A on AICD is thus compatible with the essential role of src-related kinases in TCR/CD3 signaling.

The Fas molecule itself most likely utilizes a tyrosine kinase-independent signaling machinery when triggered by agonistic anti-Fas mAb and presumably by the natural ligand on FasL expressing cells. Various groups have reported that CD95 ligation like engagement of the receptor for TNF- α (Kim *et al*, 1991; Schütze *et al*, 1992; Dressler *et al*, 1992) activates acidic sphingomyelinases which hydrolyze sphingomyelin and result in the production of ceramide (see Gill *et al*, 1994 for review). It is still an open question which of the potential downstream enzymes of ceramide such as mitogen-activated protein kinase (Raines *et al*, 1993) or the protein phosphatase 2A (Dobrowsky *et al*, 1993) become activated following CD95-ligation in T cells. It is also not known whether the expression or activity of the Fas-associated Phosphatase FAP-1 (Sato *et al*, 1995) is regulated by ceramide. However, it has been reported by Gulbins and colleagues that Fas-induced apoptosis is mediated via a ceramide-initiated activation of the Ras signaling pathway (Gulbins *et al*, 1995).

Although several components of the death inducing signaling complex (DISC) formed around the Fas molecule have been characterized as key messengers for Fas-mediated cell death (Kischkel *et al*, 1995; Boldin *et al*, 1996; Muzio *et al*, 1996), in agreement with the results reported here, there is no evidence for a direct association with PTKs. Thus, we and others failed to detect Fas-dependent tyrosine phosphorylation in Fas-susceptible T cell lines and clones (Janssen *et al*, 1996; Schraven and Peter, 1995). Further, the inhibitory effect of protein tyrosine kinase inhibitors such as Herbimycin A on Fas-mediated apoptosis is controversial. Although Eischen *et al* (1994) have shown an inhibitory effect in several cell lines including Jurkat leukemia cells, Schulze-Osthoff *et al* (1994a) did not observe a significant inhibition in their analysis of L929 cells. Our data clearly demonstrate that PTK inhibitors do not reduce cell death or DNA-damage upon Fas-stimulation of cloned human T cells. By contrast, in most clones anti-CD95-induced cell death was significantly and reproducibly enhanced when cells had

been pretreated with Herbimycin A (and in some cases with Genistein) prior to CD95 ligation. This could indicate that tyrosine phosphorylation events are also important in preventing Fas-mediated apoptosis to some extent. In this scenario, it has been reported that the oncogenic PTK Abl can block Fas-induced death (McGahon *et al* 1995).

It should be emphasized, however, that other groups have reported that serine-threonine rather than tyrosine phosphorylation may play a role in Fas-signaling. Thus, Tian *et al* (1995) have demonstrated that a serine/threonine kinase (FAST, for Fas-activated serine/threonine kinase) is rapidly dephosphorylated upon CD95-ligation to phosphorylate TIA-1, a nuclear RNA-binding protein that has been implicated as an effector of apoptosis (Tian *et al*, 1991). TIA-1 and the related RNA-binding protein have been implicated in Fas-mediated apoptosis (Tian *et al*, 1995; Taupin *et al*, 1995). However, the serine/threonine phosphatase which catalyzes dephosphorylation of FAST to activate the kinases is still unknown although it is possible that ceramide-activated phosphatases link Fas-signaling to FAST activation. In addition, a link between ceramide and serine/threonine but not protein tyrosine kinases has been recently suggested by Latinis and Koretzky (1996), demonstrating that the serine/threonine kinase JNK plays a role in CD95-mediated cell death. Interestingly, JNK-activity is increased with similar kinetics in Jurkat cells following Fas-ligation or C2-ceramide-stimulation (also in the absence of p56^{lck} or CD45, respectively).

In conclusion, our results clearly demonstrate that in untransformed clonal T cell populations, Fas-mediated apoptosis does not require rapid tyrosine kinase activation and certainly not the activation of src-related kinases. By contrast, TCR-CD3-induced cell death is dependent on tyrosine kinase-regulated signaling pathways and therefore can be efficiently abolished by blocking PTK-activity with the inhibitor Herbimycin A. The data presented here also substantiate the suggested important role of src-type PTKs in mediating initial signaling events in Fas/FasL-regulated AICD.

Materials and Methods

T cell clones

IL-2-dependent T cell clones were established from normal donors as described (Wesselborg *et al*, 1991) and grown in RPMI 1640 with 10% (v/v) FCS (Biochrom, Berlin, Germany), antibiotics (penicillin at 100 U/ml and streptomycin at 100 µg/ml), L-Glutamine (2 mM) and HEPES buffer solution (10 mM) at 37°C in a humidified atmosphere with 6% CO₂. Clones were restimulated periodically with irradiated PBMC and EBV-transformed lymphoblastoid cell lines in the presence of PHA (0.5 µg/ml, Wellcome, Burgwedel, Germany). Three days after restimulation, dead cells were removed by centrifugation on Ficoll-Hypaque density gradients. The clone cells were washed and expanded in the presence of recombinant human rIL-2 (15 U/ml, EuroCetus, Frankfurt, Germany) for several days before use in the assays. All clones expressed comparable levels of surface CD95, TCR (αβ) and CD3 molecules. Four of the clones expressed CD4, clone D768/1 was CD4⁺CD8⁺.

Antibodies and reagents

For surface staining of CD95, we used anti-human Fas mAb 7C11 (IgM) and CH11 (IgM, Kamiya Biomedical Company, Thousand Oaks, CA) with FITC-conjugated goat anti-mouse IgG and IgM second step reagents (Tago Inc., Burlingame, CA). MAb 7C11 was also used for the *in vitro* induction of cell death and for short-term stimulation. For the induction of AICD and short-term stimulation prior to anti-phosphotyrosine immunoblotting, we used anti-CD3 mAb OKT3 (kindly provided by Cilag GmbH, Sulzbach, Germany), and anti-TCRαβ mAb BMA031, (kindly provided by Dr. R. Kurrle, Behringwerke AG, Marburg, Germany), respectively, cross-linked with rabbit anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). In some experiments, clone cells were stimulated with Staphylococcus aureus enterotoxin A (SEA, Serva, Heidelberg, Germany) at 1 ng/ml (Kabelitz and Wesselborg, 1992). Other antibodies used for the characterization of surface molecules included Leu2a (anti-CD8), Leu3a (anti-CD4), Leu4 (anti-CD3), Leu5b (anti-CD2), anti-TCRαβ as FITC- or PE-conjugates (Becton Dickinson, Mountain View, CA) or OKT4 and OKT8, purified from hybridoma supernatants (American Type Culture Collection, Rockville, MD). For the detection of FasL surface expression, we used a Biotin-conjugated mAb (clone NOK-1, Pharmingen, San Diego, CA) together with Streptavidin-PE from Becton Dickinson. Briefly, clone cells (pretreated or not treated with PTK inhibitors) were incubated at 5 × 10⁵ cells/ml in the presence or absence of mAb against the TCR/CD3-complex (2 µg/ml). After 3 h, the cells were washed and stained with the anti-FasL mAb. Cells kept in medium alone served as a control. All fluorometric analyses were performed on a FACScan analyzer (Becton Dickinson) using Lysis II software.

Analysis of cell death, cell viability and DNA fragmentation

The relative number of dead cells was determined after overnight (18 to 24 hours) incubation in the presence or absence of the indicated agents by staining with the DNA dye Propidium iodide (PI, Sigma, 2 µg/ml in PBS). The absolute number of viable cells in a given population was analyzed in a standard cell dilution assay (SCDA) established in our laboratory (Pechhold *et al*, 1994). To this end, T cells were labeled with PE-conjugated anti-CD3 mAb and resuspended in staining buffer containing PI at 0.2 µg/ml and a known number of fixed FITC-labeled standard cells. The absolute number of viable T cells was calculated as the ratio of the relative proportions of PE⁺PI⁺ clone cells and FITC⁺PI⁺ standard cells. DNA fragmentation was assayed after overnight incubation of clone cells in 24 well culture plates (10⁶ cells/ml) in the presence of anti-CD95 or anti-TCR/CD3 mAb at 2 µg/ml. 400 µl of ice-cold methanol were added to 200 µl (2 × 10⁵) of resuspended cells and incubated on ice for at least 1 h. The cells were then pelleted and 100 µl of DNase-free RNase (200 U/ml) and 100 µl of PI-buffer containing 100 µg/ml PI, 0.1% (v/v) Triton X-100 and 100 µM EDTA were added (Krishan, 1975). After 30 min incubation in the dark, the DNA content was analyzed on a FACScan analyzer.

Anti-phosphotyrosine immunoblotting

Cells were washed twice in RPMI1640 with 2% FCS kept at 37°C. 1.5 × 10⁶ cells were stimulated per sample. To this end, the cells were pelleted and incubated at 37°C in 50 µl of prewarmed antibody solution with mAb at 2 µg/ml and cross-linking antibodies at 1 µg/ml. The reaction was stopped with 1 ml of cold PBS and quick centrifugation in an Eppendorf centrifuge. The pelleted cells were lysed in 30 µl of Nonidet P40 (Fluka Chemie AG, Buchs, Switzerland)

lysis buffer (1% (v/v) of detergent in 20 mM Tris-HCl (pH 8.1), 150 mM NaCl, with protease and phosphatase inhibitors Aprotinin, Leupeptin, PMSF, Sodium orthovanadate, Sodium pyrophosphate, and Sodium fluoride). Lysates remained on ice for 15–30 minutes before centrifugation at 4°C and 14000 rpm for 7 min. Proteins in the supernatants were boiled in sample buffer with β -Mercaptoethanol, separated on 10% SDS polyacrylamide gels, and transferred to Nitrocellulose membranes (Hybond C-Extra, Amersham, Braunschweig, Germany). Blots were blocked with 5% BSA (Sigma) and tyrosine phosphorylated proteins were detected with the anti-PTyr mAb 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), Horseradish peroxidase-conjugated sheep anti-mouse antibodies (Amersham) and chemiluminescence detection reagents (ECL, Amersham; Renaissance, Dupont-NEN, Bad Homburg, Germany).

Treatment of T cell clones with kinase inhibitors

To inhibit src-related tyrosine kinases and T cell activation, Herbimycin A (Calbiochem-Novabiochem, San Diego, CA) was used at a concentration of 1.5 μ M (0.86 μ g/ml). The working concentration for each inhibitor was chosen not to be toxic by itself as determined in titration experiments. We also tested a variety of other less specific kinase-inhibitors such as Genistein (75 μ M (20 μ g/ml), Calbiochem-Novabiochem, Akiyama *et al*, 1987), Staurosporine (6.7 μ M (3.125 ng/ml), Sigma, Tamaoki *et al*, 1986), or Tyrphostin (25 μ M (5 μ g/ml), Calbiochem-Novabiochem, Gazit *et al*, 1989) and the PI 3-Kinase-inhibitor Wortmannin (100 nM (42.7 ng/ml), Calbiochem-Novabiochem, Powis *et al*, 1994). The clone cells were pre-incubated for 1–3 h in inhibitor-containing medium and washed before short-term stimulation assays. For overnight incubation in the presence or absence of anti-TCR/CD3 or anti-CD95 mAb, the inhibitors remained present in the culture wells.

PCR-analysis of Fas Ligand expression

FasL mRNA was analyzed using a PCR approach based on total RNA. 5×10^6 clone cells were incubated in the presence or absence of anti-TCR or anti-CD3 mAb (2 μ g/ml). The cells were pelleted and washed in PBS. Total RNA was isolated using the Quick Prep[®] Total RNA Extraction Kit (Pharmacia Biotech, Freiburg, Germany). 0.5 μ g RNA per sample were subjected to RT-PCR using a RNA PCR kit from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ). The following oligonucleotide primers were used: for FasL: 5'-TGG-CAG-AAC-TCC-GAG-AGT-CTA-3' and 5'-CAC-TGG-TAA-GAT-TGA-ACA-CTG-C-3', and for GAPDH 5'-GCA-GGG-GGG-AGC-CAA-AAG-GG-3' and 5'-TGC-CAG-CCC-CAG-CGT-CAA-AG-3', respectively (Eurogentec, Cologne, Germany). PCR products were analyzed following separation and transfer to nylon membranes (Hybond N⁺, Amersham) using the non-radioactive ECL[™] direct nucleic acid labeling and detection system (Amersham). The DNA probe for GAPDH (399 bp) was generated by PCR from genomic DNA of PBMC using the GeneAmp PCR Reagent Kit with Amplitaq polymerase (Roche Molecular Systems). A Bluescript-based vector containing FasL cDNA was kindly provided by Dr. Nagata (Osaka, Japan). The FasL probe consisted of a purified Xba I fragment.

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