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Release of preformed Fas ligand in soluble form is the major factor for activation-induced death of Jurkat T cells

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SUMMARY

Interaction of Fas/APO-1 (CD95) and its ligand (FasL) plays an important role in the activation-induced cell death (AICD) of T lymphocytes. In the present work, the contribution of soluble FasL to AICD of the human T-cell line Jurkat has been studied. Jurkat cells prestimulated with phytohaemagglutinin (PHA) induced the death of non-activated Jurkat cells, and also of L1210Fas, but not that of Fas-negative L1210 cells. Culture supernatants from prestimulated Jurkat cells were highly toxic to their non-activated counterparts. Time–course analysis revealed that PHA-stimulated Jurkat cells quickly release (less than 15 min) to the medium a toxic molecule following a biphasic pattern, with maximal cytotoxic activities at 1 hr and 7 hr after stimulation. The cytotoxic effect of those supernatants was prevented by the addition of a blocking anti-Fas monoclonal antibody, suggesting that PHA-stimulated Jurkat cells exert Fas-based cytotoxicity mainly through the release of soluble FasL. The constitutive intracellular expression of FasL in non-activated Jurkat cells and its release as a consequence of PHA activation were detected by immunostaining and immunoblotting using an anti-FasL antibody. These data indicate that, at least in Jurkat cells, AICD is mainly mediated by the rapid release of preformed FasL in soluble form upon stimulation.

INTRODUCTION

Fas/APO-1 (CD95) is a type I transmembrane glycoprotein belonging to the tumour necrosis factor (TNF) receptor superfamily that is expressed in thymocytes, activated and tumoral T and B cells, and in several non-immune tissues. Fas induces apoptosis when cross-linked with specific monoclonal antibodies (mAb) or with its natural ligand, FasL.[1,2] Several recent studies have demonstrated that the interaction of Fas with FasL mediates the activation-induced cell death (AICD) of T-cell hybridomas[3–5] and of mature normal T lymphocytes,[6–8] controlling the down-modulation of cellular immune responses and peripheral tolerance.[9,10] FasL, a type II membrane glycoprotein, is a member of the TNF family, and is primarily expressed in activated T cells.[11,12] Spontaneous mutations of the Fas and FasL genes in *lpr* and *gld* mice, respectively, cause lymphoproliferation and a generalized lupus-like autoimmune disease as a result of defective AICD.[13–15] FasL expression is not constitutive in T cells and pharmacological [phorbol 12-myristate 13-acetate (PMA) plus ionomycin] or antigenic stimulation is needed to induce its expression.[11,16–18] It has been recently reported that supernatants of anti-CD3-activated Jurkat cells[3] or of FasL-transfected COS cells[19] induce apoptosis in Fas-expressing cells. A functional soluble 26 000 mol.wt. protein present in supernatants of FasL-transfected COS cells or in PMA/ionomycin-activated human T cells,[20,21] has been identified as the soluble form of human FasL (sFasL). Little is known about the regulation of FasL expression in T cells or about the mechanisms involved in the production of the functional sFasL. Recently, and similarly to TNF, it has been characterized that the cleavage of human FasL and production of sFasL by T cells is mediated by a still unidentified Zn^{2+}-dependent metalloprotease.[21–23]

In the present work, we have used the human T-cell leukaemia Jurkat as a model to study the contribution of sFasL to AICD. This cell line constitutively expresses Fas, is sensitive to Fas-induced apoptosis and also undergoes AICD.[3] By using cytotoxicity bioassays, immunofluorescence staining and Western blotting analysis, we report here evidence that Jurkat T cells constitutively express intracellular FasL, which is rapidly ($\leq$15 min) secreted as functional sFasL after phytohaemagglutinin (PHA) stimulation.

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Abbreviations: AICD, activation-induced cell death; FasL, Fas/APO-1 ligand; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; sFasL, soluble Fas/APO-1 ligand.

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MATERIALS AND METHODS

Cells

Jurkat E6-1, a human T-cell leukaemia which naturally expresses Fas, was obtained from the American Type Culture

Collection (ATCC, Rockville, MD). L1210, a mouse lymphocytic leukaemia which does not express Fas,[16] or L1210 cells transfected with Fas cDNA (L1210Fas) and expressing high levels of Fas[16] were used as negative and positive controls, respectively, for Fas-based cytotoxicity induced by Jurkat cells. The cytolytic hybridoma d11S, that expresses rat Fas ligand after PMA/ionomycin stimulation,[11] was used as a positive control in anti-FasL immunoblots. L1210, L1210Fas and d11S cells were kindly provided by Dr Pierre Golstein (Marseille, France). Cell lines were cultured in RPMI-1640 medium (BioWhittaker, Barcelona, Spain) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (referred to hereafter as complete medium).

Cytotoxicity assays

Jurkat cells (5×10^5 cells/ml in complete medium) were incubated for different times at 37° with either PHA (ranging from 0·1 to 50 μg/ml), anti-human CD3 mAb OKT3 (20 μg/ml), or anti-human Fas mAb CH-11 (UBI, Lake Placid, NY) (10–100 ng/ml).[24] OKT3 was purified from supernatants of the corresponding murine hybridoma (ATCC) using Protein-G immobilized on Sepharose (Pharmacia, Barcelona, Spain). In other experiments, Jurkat cells (4×10^5 cells/ml) were prestimulated by incubation with 50 μg/ml PHA at 37° for 5 min. PHA was then removed by brief centrifugation in a Beckman Minifuge (12 000 *g*, 5 seconds), cells washed three times with RPMI-1640, resuspended in complete medium, seeded into flat-bottom 96-well plates (25 000 cells/well) and cultured for different times alone or mixed with non-stimulated Jurkat cells. The degree of apoptosis was evaluated by nuclear staining with *p*-phenylenediamine,[25] and cell viability by a modification of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method of Mossman, as described elsewhere.[26] In some experiments extracellular Ca^{2+} was chelated with 1 mM ethylene glycol-bis(β-aminoethyl)ether (EGTA), containing 1·5 mM $MgCl_2$ to ensure a Mg^{2+} excess. In cytotoxicity tests using the MTT detection method, target cell death was calculated as follows:

% of target cell death =

$$\frac{A_{550}(\text{effectors} + \text{targets}) - A_{550}(\text{effectors})}{A_{550}(\text{targets})} \times 100$$

The absorbance at 550 nm (A_{550}) of effector or target cells was determined in separate cultures containing either effector or target cells alone.

Cytotoxicity of PHA-prestimulated Jurkat cells was also evaluated on L1210 or L1210Fas cells labelled with [^{125}I]deoxyuridine. Target cells were mixed with prestimulated Jurkat cells at different effector to target ratios and incubated for 20 hr. DNA fragmentation was calculated from ^{125}I c.p.m. obtained in 0·5% Triton-X-100 cell lysates, as described elsewhere.[27] Spontaneous [^{125}I]deoxyuridine release from target cells was always lower than 20% of the total. Jurkat cells could not be used as targets in [^{51}Cr]- and [^{125}I]deoxyuridine release assays since their spontaneous release was ≥70% of the total.

Detection of sFasL

To analyse the production of sFasL by Jurkat cells in response to PHA stimulation, cells (4×10^5 cells/ml) were prestimulated with 50 μg/ml PHA for 5 min and, after PHA removal by centrifugation as described above, were resuspended in complete medium and cultured at 37° for different times (15 min to 24 hr). Cells were recovered after brief centrifugation and aliquots of supernatants (100 μl) assayed for their cytotoxic activity using non-activated Jurkat cells (5×10^4 cells/well) as targets. Supernatants from non-activated cells were used as controls. After incubation for 12 hr, cytotoxicity of supernatants was determined by the MTT assay. To verify that the cytotoxic activity was due to secreted sFasL, non-stimulated Jurkat cells were incubated in parallel experiments with supernatants containing 100 ng/ml of the specific anti-human Fas mAb SM1/23 (Bender MedSystems, Barcelona, Spain). This mAb is not cytotoxic due to poor Fas cross-linking and blocks Fas/FasL interaction.[28]

Effect of inhibitors of transcription and translation on cytotoxicity of supernatants

Cells were preincubated with 0·1 μg/ml actinomycin D or with 10 μg/ml cycloheximide in complete medium for 15 min, stimulated with 50 μg/ml PHA for 5 min and resuspended in complete medium containing the indicated concentrations of each of the inhibitors. This medium was replaced again by fresh medium after 3 hr of incubation. Cell supernatants were collected at different times and their toxicity was tested on non-activated Jurkat cells by the MTT method. The effect of actinomycin D and cycloheximide alone on Jurkat AICD itself was also tested on cells treated with 50 μg/ml PHA during 12 hr in the presence or absence of the metabolic inhibitors. Non-activated Jurkat cells incubated with the same concentrations of each inhibitor were used as appropriate controls. Growth of Jurkat cells treated with actinomycin D or cycloheximide alone was 30% and 50%, respectively, of that of control cells.

Inhibition of FasL secretion

The implication of metalloproteases in FasL secretion was analysed by using the Zn^{2+} chelator 1,10-phenanthroline (Merck, Barcelona, Spain).[22] Cells were preincubated for 10 min with 1 mM phenanthroline in complete medium, prestimulated with PHA for 5 min, washed, resuspended in complete medium containing 1 mM phenanthroline and further incubated for 30 min. To eliminate the toxic effect of phenanthroline on target cells during 12-hr bioassays, supernatants from cells prestimulated in the presence of phenanthroline were extensively dialysed by ultrafiltration in Centricon-10 units (Amicon, Barcelona, Spain) and reconstituted to their initial volume with fresh medium. Aliquots of complete medium containing the same amount of phenanthroline were treated under similar conditions and used as suitable controls. Cytotoxicity of supernatants was tested on non-activated Jurkat cells by the MTT assay, as described above.

Immunocytochemistry

The intracellular localization of FasL was analysed by immunofluorescence using an IgG anti-FasL polyclonal antibody (N-20, Santa Cruz Biotechnology, CA). This antibody recognizes the intracellular N-terminus region of human, murine and rat FasL and has been proved useful for immunocytochemistry.[29] Non-activated Jurkat cells and cells prestimulated with 50 μg/ml PHA at 37° for 5 min were fixed by resuspension in 4% of paraformaldehyde in phosphate-buffered saline (PBS), pH 7·4, for 15 min. Fixed cells were

washed with PBS, centrifuged onto glass coverslips coated with 1% poly-L-lysine, permeabilized at room temperature for 15 min with 0·1% Triton-X-100/0·5% bovine serum albumin (BSA) in PBS and subsequently blocked by incubation with a 1/10 dilution of normal goat serum in PBS. Cells were then washed and incubated at room temperature with 0·2 μg/ml of anti-FasL antibody for 1 hr followed by a 1/500 dilution of a goat anti-rabbit IgG (H+L) antibody conjugated with fluorescein isothiocyanate (FITC; Caltag Laboratories, CA) for 1 hr. Specificity of labelling was checked by adding 100 μg/ml of a control peptide (sc-384P, Santa Cruz Biotechnology) during incubations with the anti-FasL antibody. After extensive washing with PBS, cells were mounted on a glass slide over a drop of *p*-phenylenediamine/glycerol,[30] examined by epifluorescence microscopy and photographed.

Anti-FasL immunoblotting

Non-activated Jurkat cells (5×10^6 in 100 μl) or cells prestimulated with 50 μg/ml PHA for 5 min, were resuspended in fresh culture medium and incubated at 37° for different times (ranging from 30 min to 24 hr). The d11S cells activated with 10 ng/ml PMA and 500 nM ionomycin and the untreated L1210 cells were used as positive and negative controls, respectively, for FasL expression.[17] At the end of the incubations, cells were lysed at 4° in a buffer containing 1% Triton-X-100 and protease and phosphatase inhibitors, as previously described.[31] Cell lysates were analysed by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and separated proteins were transferred to nitrocellulose membranes. Membranes were sequentially incubated with 1 μg/ml of rabbit anti-FasL in PBS containing 3% BSA and with 0·2 μg/ml of goat anti-rabbit IgG coupled to alkaline-phosphatase (Sigma, St Louis, MO) and revealed with the BCIP/NBT substrate (Sigma).

RESULTS AND DISCUSSION

PHA-activated Jurkat cells induce death of non-activated Jurkat cells

As a model of AICD, Jurkat T cells were stimulated with PHA or with the anti-CD3 mAb OKT3 and this type of cell death was compared with that induced by anti-Fas mAb CH-11 treatment. Cell nuclei staining with *p*-phenylenediamine indicated that T-cell receptor (TCR) stimulation or Fas triggering induced a similar type of apoptotic death in Jurkat cells (data not shown). To determine if AICD could still proceed after the removal of stimuli, Jurkat cells were briefly (3–5 min) treated with either 50 μg/ml PHA or 20 μg/ml OKT3, then washed and cultured for 8 or 24 hr in fresh medium. Then, membrane integrity was analysed by the trypan blue exclusion test and apoptosis by staining with *p*-phenylenediamine. Results indicate that, after 8 hr incubation, only 15% were permeable to trypan blue but more than 50% of PHA-prestimulated cells were apoptotic. In the case of OKT3 stimulation, only 25% of cells exhibited chromatin condensation and almost no disruption of plasma membrane permeability was observed. After 24 hr, the number of apoptotic and trypan blue-positive cells increased and was comparable, both in the case of PHA and OKT3 stimulation. These results

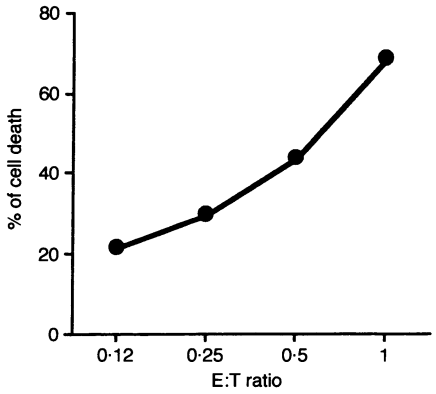

Figure 1. Prestimulation of Jurkat cells with PHA induces cytotoxicity on non-activated Jurkat cells. Cells were treated with 37° with 50 μg/ml PHA for 5 min. After removal of stimuli, fresh medium was added and cells were incubated for 12 hr in the presence of non-activated Jurkat cells as targets. The effector to target ratios are indicated in each case. Percentage of cell death was measured by the MTT assay, as described in the Materials and Methods.

suggest that after TCR/CD3 triggering, Jurkat cells are already committed to apoptosis and the process is irreversible.

Taking advantage of this observation, we designed cytotoxicity tests in which prestimulated Jurkat cells were used as effectors and non-activated cells as targets, in the absence of stimuli. PHA-prestimulated cells were mixed with target cells at different ratios and the extent of cell death was determined by the MTT assay. As shown in Figure 1, prestimulated Jurkat cells induced death of their non-activated counterparts. The percentage of cell death increased with the effector to target ratio, being already detectable at ratios as low as 0·12 : 1 and reaching 80% at the higher ratio assayed (1 : 1). As mentioned in the Materials and Methods, cytotoxicity tests based on [^{125}I]deoxyuridine release were not suitable when using Jurkat cells as targets. However, using L1210 and L1210Fas as targets, it was observed that Jurkat cells prestimulated with PHA were cytotoxic to L1210Fas (30% of specific [^{125}I]deoxyuridine release after 20 hr incubation at a 10 : 1 effector to target ratio), but not to L1210 cells, suggesting that PHA-induced cytotoxicity involved the Fas/FasL system. Induction of FasL expression has been shown to be Ca^{2+}-dependent,[18] probably through the implication of calcineurin activation,[17] while the execution of Fas cytotoxicity is not.[16] To analyse the effect of Ca^{2+} in AICD, cells were incubated with EGTA/Mg^{2+} prior to the treatment with PHA. Cells prestimulated in the presence of EGTA/Mg^{2+} were unable to kill non-activated Jurkat cells (data not shown). This further supported the implication of the Fas/FasL system as the sole mediator of Jurkat AICD.

Release of sFasL by PHA-stimulated cells

Activation of mature T cells induces the transcription of the FasL gene and the transient expression of functional FasL on their surface.[11,17,18] Recent studies have shown that interaction of Fas with FasL provides a death signal which triggers apoptosis during the process of AICD. This process could take place as a fratricide, if FasL is expressed on an activated T cell and Fas is expressed on a neighbour cell, but it could also occur

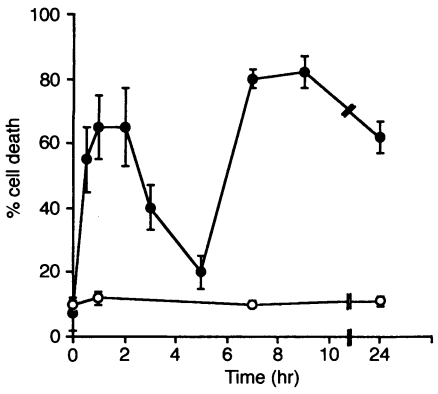

Figure 2. Time–course release of cytotoxic activity from PHA-prestimulated Jurkat cells. Cells were prestimulated with 50 μg/ml PHA for 5 min and resuspended in fresh medium as described in the Materials and Methods. At the times indicated, their corresponding supernatants were collected and added to non-activated Jurkat cells. Target cells were incubated for 12 hr with those supernatants in the presence (open circles) or absence (solid circles) of 100 ng/ml of blocking anti-Fas mAb SM1/23. Culture medium from non-activated Jurkat cells was used as control. Cell death was determined by the MTT assay and is expressed as percentage of control cells. Data are the mean of quadruplicate determinations on five different experiments; vertical bars indicate SD.

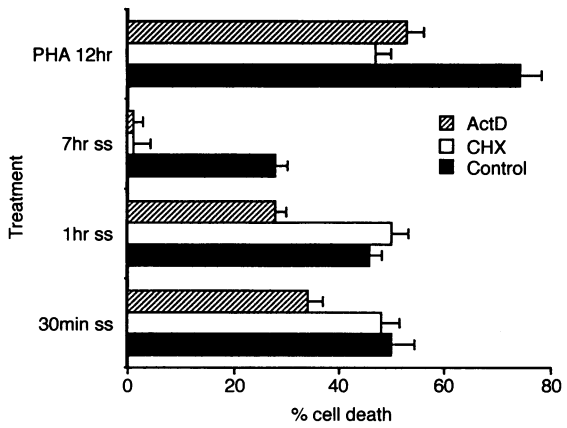

Figure 3. Effect of inhibitors of transcription and translation on cytotoxicity of supernatants from activated Jurkat cells. Cells were preincubated for 15 min with medium alone (solid bars), with 10 μg/ml cycloheximide (open bars), or with 0·1 μg/ml actinomycin D (hatched bars), stimulated with 50 μg/ml PHA for 5 min and resuspended in fresh medium or in medium with the indicated concentrations of inhibitors. After 30 min, 1 hr, or 7 hr, the corresponding supernatants (ss) were collected and their toxicity was tested on non-activated Jurkat cells by the MTT method. To analyse the toxicity due to the second peak (Fig. 2), the culture medium of prestimulated cells was replaced by fresh medium at 3 hr of incubation. The effect of actinomycin D or cycloheximide on AICD itself was also tested on cells treated with 50 μg/ml PHA for 12 hr, as indicated. Non-activated Jurkat cells incubated with fresh medium or with medium containing the corresponding inhibitor were used as controls.

in an autocrine or paracrine manner, if FasL were released in soluble form.[2] Indeed, it has been recently described that activated human T cells and certain types of leukaemias release a soluble form of FasL (sFasL) upon activation.[3,20,21,23]

To study the time–course release of the soluble toxic molecule during Jurkat AICD, cells were prestimulated with PHA for 5 min, washed and cultured in fresh complete medium for different times. Supernatants from prestimulated cells were collected and their cytotoxicity tested on non-activated Jurkat cells by the MTT assay. Cytotoxic activity was already detectable (15% of cell death) in 15 min supernatants and increased rapidly showing a first maximum (around 65% of cell death) in 1–2 hr supernatants. The cytotoxic activity decreased in the next 2 hr and then increased again exhibiting a second maximum in 7–9 hr supernatants (Fig. 2). Similar results were obtained after OKT3 stimulation, although supernatant cytotoxicity was lower, inducing around a 20% cell death (data not shown). This latter result agrees with a previous report[3] which found 10% of cell death caused by supernatants from anti-CD3-stimulated Jurkat cells.

To verify that the two peaks of cytotoxic activity were due to the presence of sFasL in supernatants, parallel experiments were performed in the presence of 100 ng/ml of SM1/23, a non-cytotoxic anti-Fas mAb that blocks Fas/FasL interaction.[28] Treatment with this antibody completely prevented the cytotoxicity of 1 hr or 7 hr supernatants, suggesting that sFasL was the cytotoxic mediator in both cases. PHA-induced cytotoxicity itself was also completely prevented by this blocking mAb (not shown). These results indicate that functional sFasL is rapidly released from Jurkat cells upon PHA activation. Release was detectable at 15 min, a time insufficient for completion of the *de novo* mRNA transcription and protein synthesis process, which suggested that FasL might be already preformed in Jurkat cells. The release of preformed FasL was complete after 30–60 min of PHA stimulation and its cytotoxic activity remains high for at least another hour. The subsequent decrease in cytotoxic activity may be due to sFasL instability and/or to the uptake of sFasL by PHA-prestimulated cells, reducing the amount of sFasL in the supernatants. Cytotoxic activity was still high in 24 hr supernatants suggesting either a continuous FasL synthesis and release or a reduced uptake of sFasL by dying Jurkat cells.

Previous works have indicated that expression of FasL mRNA is induced at a high level after 3–4 hr of TCR/CD3 stimulation.[3,17] According to this, the second maximum of cytotoxicity observed in our experiments would correspond to FasL synthesized *de novo* after mRNA transcription induced by PHA, while the first peak would correspond entirely to preformed FasL. This possibility was tested by using the protein synthesis inhibitor cycloheximide and the transcription inhibitor actinomycin D. Cycloheximide did not affect toxicity induced by 30 min or 1 hr supernatants, while completely blocking toxicity due exclusively to the second peak (Fig. 3). Actinomycin D partially inhibited the toxicity due to 30 min or 1 hr supernatants, and completely the toxicity of the 7 hr supernatants (Fig. 3). The difference between cycloheximide and actinomycin D regarding 30 min or 1 hr supernatants may be explained by the higher intrinsic toxicity of actinomycin D, that could kill some PHA-activated Jurkat cells before releasing any sFasL. According to the results obtained with the supernatants and with previous observations in Jurkat cells,[3] the metabolic inhibitors only partially blocked AICD itself (Fig. 3, PHA 12 hr).

Western blot analysis of FasL

The presence of preformed FasL in Jurkat cells was further characterized by Western blotting using a polyclonal antibody against human FasL. Cell lysates from non-activated Jurkat cells contained important amounts of the mature form of FasL, with a mol. wt. of 42 000 (Fig. 4a) but the intensity of this band decreased rapidly after PHA stimulation. The anti-FasL antibody also detected other bands (Fig. 4a, arrowheads) that probably correspond to different glycosilated forms of the FasL.[19,20] The intensity of these bands also decreased in cell lysates upon activation. On the contrary, very low levels of rat FasL were present in non-activated d11S cells, which substantially increased after activation with PMA/ionomycin (Fig. 4a). Rat FasL had a slightly higher mol. wt. than human FasL, probably due to differences in the glycosylation pattern.[11,19] These results confirmed that mature FasL is present in proliferating Jurkat cells and they show that its level rapidly decreases upon PHA stimulation. This is probably due to the cleavage of the 42 000 mol. wt. mature form of FasL to the 26 000 mol. wt. soluble form, as reported.[20,21] We could not detect sFasL in the supernatants from activated cells since the antibody does not recognize the extracellular portion of FasL. However, cytotoxicity bioassays and experiments using blocking anti-Fas mAb demonstrate the presence of sFasL in supernatants and suggest that this molecule is the main mediator of AICD in Jurkat cells. A continuous release of sFasL seems to occur upon PHA stimulation since no FasL accumulation was observed at any time tested (Fig. 4a). Nor was any accumulation of FasL found in the membrane of PMA/ionomycin-stimulated human MOLT-4 cells.[22] However, a similar treatment caused a great increase in membrane expression of FasL in murine RMA cells,[22] according to our FasL blotting results obtained with d11S cells. This could indicate that release of sFasL is a particularity of human T cells, and a property not shared by murine or rat T cells, as previously suggested.[20]

On the other hand, it has been recently reported that sFasL release from PMA/ionomycin-activated human T cells is dependent on metalloprotease activity.[21–23] Inhibition of metalloproteases by high phenanthroline concentrations (2·5 mM) resulted in FasL plasma membrane accumulation.[22] Membrane-bound FasL did not accumulate in the absence of the inhibitor probably due to the continuous FasL cleavage and sFasL release. Treatment with 1 mM phenanthroline during 30 min almost completely inhibited the observed decrease in FasL content of PHA-stimulated Jurkat cells (Fig. 4b), confirming the implication of metalloproteases in FasL cleavage and secretion. To prevent the intrinsic cytotoxicity of phenanthroline in bioassays, it was removed from supernatants by ultrafiltration and dialysis before their addition to non-activated Jurkat cells for FasL bioassays, as described in the Materials and Methods. As shown in Fig. 4(c), treatment with phenanthroline eliminated the cytotoxic activity of 30 min supernatants, probably by blocking the secretion of sFasL from PHA-stimulated Jurkat cells.

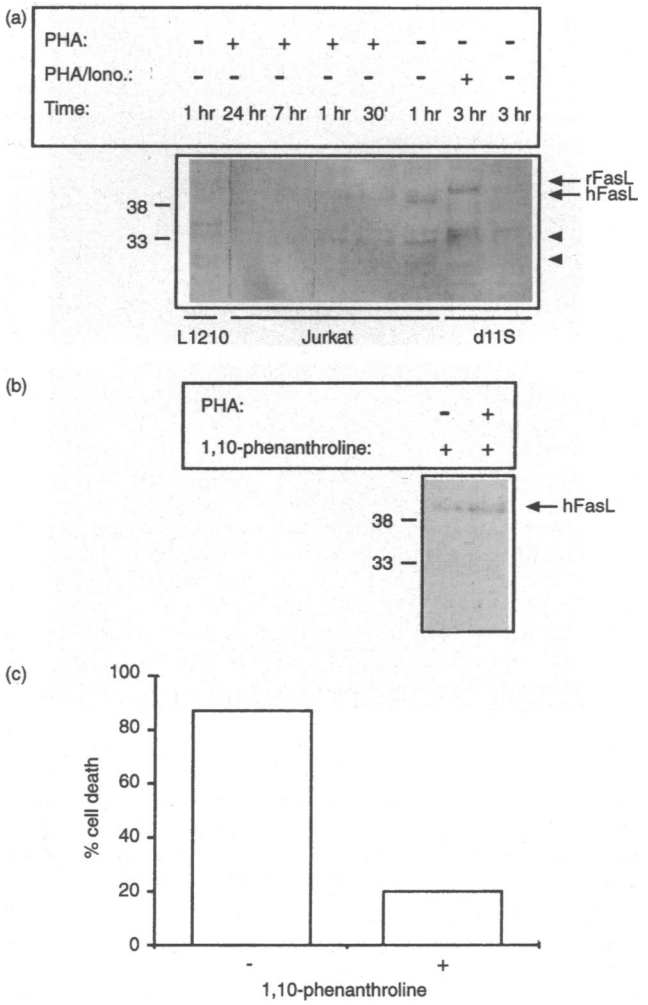

Figure 4. Expression of FasL in non-activated Jurkat T cells and inhibition of its secretion by phenanthroline upon triggering of AICD. (a) Cells were incubated at 37° with or without 50 μg/ml PHA for 5 min, resuspended in fresh complete medium, cultured for the times indicated and lysed. The d11S cells, treated with 10 ng/ml PMA and 500 nM ionomycin, and the L1210 cells were used as positive and negative controls, respectively, for FasL expression. Cell proteins were electrophoresed, transferred to nitrocellulose and immunoblotted with anti-human FasL polyclonal antibody and phosphatase–alkaline-labelled secondary antibodies followed by BCIP/NBT staining. Sizes and position of the mol. wt. markers are indicated on the left. Arrows mark the position of the mature form of FasL. Arrowheads indicate bands corresponding to different glycosylated forms of FasL. (b) Cells were incubated at 37° with or without 50 μg/ml PHA for 5 min in the presence of 1 mM phenanthroline, washed, resuspended in culture medium containing the metalloprotease inhibitor and incubated for another 30 min. Cell proteins were electrophoresed and immunoblotted with the anti-human FasL antibody. The arrow marks FasL position. (c) Cells were prestimulated with PHA for 5 min in the presence or absence of 1 mM phenanthroline and supernatants were collected and processed as described in the Materials and Methods. Cytotoxic activity of supernatants were determined on non-activated Jurkat cells by the MTT assay.

Cellular localization of FasL

Localization of FasL in Jurkat cells was determined by immunofluorescence microscopy using a specific anti-FasL antibody. Non-activated Jurkat cells exhibited numerous brilliant dots in the cytoplasm, mainly located in the cell periphery. In addition, an intense bright spot in the juxtanuclear region could be appreciated in most cells (Fig. 5a). This characteristic labelling

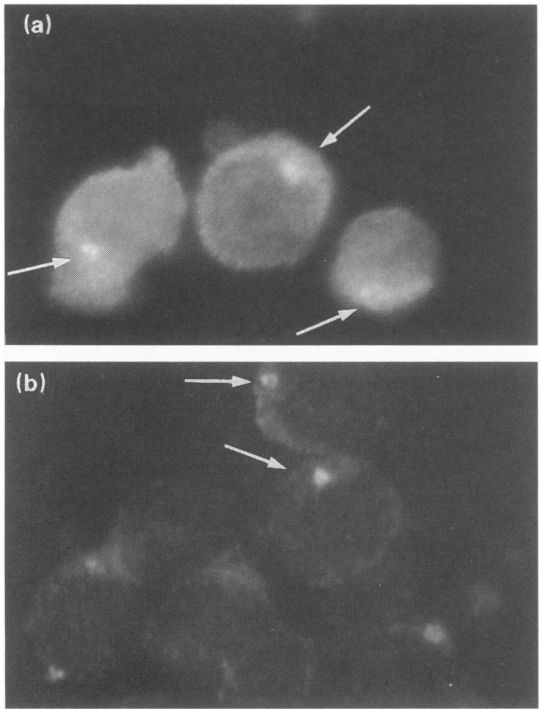

Figure 5. Immunofluorescence localization of FasL in Jurkat cells. Non-activated cells (a) or cells prestimulated with 50 μg/ml PHA for 5 min (b) were fixed at room temperature, permeabilized, incubated sequentially with rabbit anti-FasL antibody and FITC-conjugated goat anti-rabbit IgG and photographed under epifluorescence illumination. Arrows indicate the labelling in the TGN region. Original magnification, ×700.

has been previously observed in myeloma cells stained with anti-transferrin receptor antibody and identified as the *trans* Golgi network (TGN).[30] Inclusion of specific control peptide (sc-834P) during incubation with anti-FasL abolished cell labelling (not shown). When cells were prestimulated for 5 min with PHA, the cytoplasmic labelling greatly decreased (Fig. 5b). Fluorescence was mainly distributed as a rim in the plasma membrane and the intensity of the spot in the TGN region diminished in some cells (Fig. 5b). These results confirm the constitutive presence of FasL in non-activated cells detected by Western blot (Fig. 4) and suggest its rapid translocation to the cell membrane upon PHA stimulation. These observations are also consistent with other experiments indicating that FasL is not localized at the membrane surface in non-activated Jurkat cells, since no self-toxicity was found even if cell-to-cell contact was forced by centrifugation and pelleting (not shown). These results, taken together, indicate that culture supernatants of Jurkat cells become cytotoxic promptly after PHA or anti-CD3 stimulation, suggesting that TCR engagement activates the release of functional sFasL. In this connection, the interaction of SH3 domains of $p59^{fyn}$ with the proline-rich cytoplasmic segment of FasL has recently been reported.[32] This interaction could target newly synthesized FasL to the cell surface via *fyn* and/or *lck* SH3 domains in polarized vesicle–cell membrane fusion. Alternatively, SH3 domains could result in a high local accumulation of FasL in a region close to the activated TCR complex.[32]

The importance of sFasL secretion versus plasma membrane-expressed FasL in the physiological regulation of the immune system is not clear.[10] The presence of low amounts of sFasL has been previously demonstrated in supernatants from Jurkat cells stimulated with anti-CD3 mAb,[3] and from T-cell blasts stimulated with PMA/ionomycin.[20,21] We show here that TCR engagement, by anti-CD3 mAb and, in a greater extent, by PHA, produces the rapid secretion of preformed intracellular FasL. It may be then possible that sFasL and other homologous molecules such as TNF and the soluble form of CD40 ligand, which are released after T-cell activation,[33] play a crucial role in the communication of activated T cells with other cells of the immune system and in the regulation of the immune response. The presence of preformed FasL in Jurkat T cells and its secretion upon activation, offer a new insight in the problem and are worthwhile to study in normal human T cells. In this connection, the presence of intracellular FasL in normal murine T cells from a 6-day mixed lymphocyte culture has recently been reported.[34] Further studies will be necessary to characterize the exact cellular location of preformed FasL and the biochemical mechanisms controlling the generation and release of sFasL.

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