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Apoptosis in a Fas-resistant, T-cell receptor-sensitive human leukaemic T-cell clone

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SUMMARY

The Fas (CD95) antigen plays a key role in regulating T-cell activation and survival. We have generated a Fas-resistant subclone of the human T-cell leukaemia line, H9, which is still able to undergo apoptosis in response to T-cell receptor ligation. Molecular analyses revealed that resistance to Fas-mediated apoptosis was due to a heterozygous mutation in the death domain of the *Fas* gene which generates a stop codon, and thus encodes a truncated Fas molecule. Fas ligation was able to induce apoptosis in the presence of cycloheximide, indicating that the mutant Fas molecule retained some signalling capability, which is death-domain independent. These cells will provide a useful tool for dissecting the complexities of Fas signalling pathways.

INTRODUCTION

The control of T-cell responses to antigen plays a critical role in regulation of the immune response. Depending on their differentiation state and the availability of costimulatory molecules, T-cell recognition of antigen mediated by the T-cell receptor (TCR) may trigger activation, anergy, or apoptosis (or no response at all). The molecular events controlling the choice of response in an individual cell are still incompletely understood, but the Fas antigen is thought to play an important role in regulating the cell death pathway.[1]

Fas (CD95) is a 45 000 MW cell surface receptor belonging to the tumour necrosis factor-α (TNF-α)/NGF receptor family[2,3] that is expressed by a number of cell types, including activated T cells and a variety of lymphoid cell lines.[4–6] Studies with T-cell lines have shown that ligation of the Fas molecule by Fas ligand or anti-Fas monoclonal antibodies[4,7–9] results in the rapid induction of apoptosis that appears to involve distinct signalling pathways from those triggered in response to cytokine deprivation or by TCR ligation.[10] Recent evidence has suggested that TCR stimulation mediates the expression of FasL which then interacts with Fas at the cell surface to trigger the apoptotic process.[11–13]

The molecular mechanisms involved in signal transduction through Fas have been the focus of intensive study.[14,15] In particular, a number of proteins have been isolated that associate with the cytoplasmic portion of Fas and appear to mediate apoptosis.[15–20] Interestingly, FADD/mort-1[16,17] associates both with Fas and a recently identified interleukin-1 converting enzyme (ICE) family protease, FLICE/MACH,[21,22] providing the first evidence of a direct link between apoptotic signals at the cell surface and apoptotic effector proteases. However, the role of other Fas-associated proteins remains incompletely understood.

Here we report the characterization of a subclone of the human leukaemic line H9 (H9-3D8FR), which is resistant to Fas-mediated apoptosis, but remains sensitive to apoptosis induced by TCR stimulation suggesting that, contrary to recent reports, TCR-mediated signals may be able directly to trigger apoptosis in some cells. The Fas pathway defect in H9-3D8FR is in part due to a heterozygous mutation which produces a death-domain-defective Fas protein. However, apoptosis can be induced through Fas ligation in the presence of cycloheximide, suggesting that death signalling pathways are activated in the presence of a mutant Fas molecule.

MATERIALS AND METHODS

Reagents

Staphylococcal enterotoxin SEC1 was purchased from Toxin Technology Inc. (Sarasota, FL). The anti-CD3 monoclonal antibody (mAb), OKT3 (Ortho Diagnostics, Raritan, NJ) was conjugated to sheep anti-mouse IgG2a-coated magnetic beads, (Dynabeads m-450, Dynal, Great Neck, NY). The IgM anti-human Fas mAb, clone CH-11 was purchased from Upstate Biotechnology Inc (Lake Placid, NY). Isotype-matched, non-reactive control IgM myeloma protein used for both activation assays and immunofluorescence was purchased from Sigma (St Louis, MO) The secondary antibody used in immunofluorescence assays was a phycoerythrin (PE)-conjugated goat anti-mouse kappa (Becton Dickinson, San José, CA). The protein synthesis inhibitor, cycloheximide was purchased from Boehringer Mannheim (Indianapolis, IN).

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Abbreviations: APC, antigen-presenting cell; CHX, cycloheximide; SEC1, staphylococcal enterotoxin C1.

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T-cell cultures

H9 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin. The subclone H9-3D8 was derived from the parental H9 by limiting dilution. 3D8 can be triggered to both die by apoptosis and secrete interleukin-2 (IL-2; unpublished data) in response to cross-linked anti-CD3, superantigen SEC1, or anti-Fas antibody. The anti-Fas-resistant subline 3D8-FR was obtained by culturing H9-3D8 cells in the presence of anti-Fas antibody (500 ng/ml) for 5 days, followed by expansion of the surviving cells in complete medium.

Apoptosis assays

Propidium iodide (P/I) staining of DNA was used as a convenient and semi-quantitative measure of apoptosis. For these studies, 3D8 and 3D8-FR were cultured (in triplicate) at 10^5 cells/well in 96-well round bottom Costar plates in the presence of complete medium alone (RPMI + 10% FBS), 250 ng/ml SEC1 or anti-CD3 coated beads (1:1, bead:cell ratio). Cultures were incubated overnight at 37° in a humidified atmosphere containing 5% CO_2. Cells were then pooled from triplicate wells, washed in phosphate-buffered saline (PBS) and fixed in an ice cold mixture of PBS:70% methanol (1:2). Cells were washed and resuspended in 0·5 ml of PBS, treated with 5 mg/ml DNAse-free RNAse (Boehringer Mannheim) for 30 min and stained with propidium iodide (P/I) (5 mg/ml) (Boehringer Mannheim). Cell samples (5000 events/sample) were acquired on a fluorescence-activated cell sorter (FACScan) flow cytometer using CellFit and analysed by LYSYS II software packages provided by the manufacturer (Becton Dickinson Immunocytometry Systems). Data are depicted as histograms with markers defining regions with cells undergoing apoptosis (M1) and cells undergoing normal cell cycle (M2). The percentage of acquired events falling within marker region M1 corresponds to the apoptotic index used for data presentation in some figures.

Northern blotting

Messenger RNA was isolated from 5×10^7 unactivated 3D8 and 3D8-FR cells using the PolyATract System 1000 (Promega, Madison, WI). Northern blotting followed established procedures[23] with minor modifications. Briefly, mRNA (2 μg) was dissolved in 30 μl of 1 M glyoxal (deionized), 50% dimethylsulphoxide (DMSO) and 12 mM Tris, 6 mM sodium acetate, 0·3 mM ethylene diamine tetraacetic acid (EDTA; pH 7·0). After incubating at 50° for 15 min, the samples were cooled and analysed by electrophoresis on a 1·5% agarose gel in 10 mM phosphate buffer. After separation, the samples were transferred to Hybond N+ membrane (Amersham, UK) by capillary blotting in 25 mM phosphate buffer. The blot was cross-linked using a Stratagene ultraviolet cross-linker on the automatic setting (1200 μJ), and then incubated in prehybridization solution [5 × saline-sodium phosphate EDTA (SSPE), 10 × Denhardts' solution, 100 μg/ml salmon sperm DNA, 50% formamide, 2% sodium dodecyl sulphate (SDS)] for 3–6 hr at 42°. Hybridization was overnight at 42° with a ^{32}P-labelled heat-denatured DNA probe prepared by random-primed labelling (Amersham, UK). The blot was washed several times with 2 × saline-sodium citrate (SSC), 0·05% SDS at room temperature, followed by a 15 min wash with 0·1% × SSC, 0·1% SDS at 50°. The blots were then exposed to film (XAR-5, Kodak). All DNA probes used were derived from complete human cDNA clones.

Molecular analyses of Fas

Messenger RNA (2 μg) from 3D8 and 3D8-FR (as prepared for Northern blotting) was used to prepare cDNA by reverse transcription-polymerase chain reaction (RT-PCR) according to the manufacturers established protocol (Perkin-Elmer, Foster City, CA). This cDNA was then amplified (60° annealing, 30 cycles) using oligonucleotide primers specific for the Fas death domain, as follows:

5′-TTGAGTAAATATATCACCACTATTGCTGG (sense primer) and 5′-ATAGTCTGAATTTTCTCTGCAAGAGTA (antisense primer).

The resulting PCR products were cloned into the pCRII vector (InVitrogen) and sequenced using Sequenase.

RESULTS

TCR- and Fas-induced apoptosis in H9 clones 3D8 and 3D8-FR

As part of a cloning effort to produce subclones of the T-cell leukaemic line H9 with differing activation requirements for IL-2 production, we identified a single clone (3D8) which could be activated by the superantigen staphylococcal enterotoxin C1 (SEC1) in the absence of an antigen-presenting cell population. The ability to present SEC1 is most likely due to the expression of HLA-DR molecules by these cells, but the lack of requirement for APC-derived costimulatory signals is less clear (unpublished observations). Subsequent analysis revealed that apoptosis was also induced in this clone by SEC1, as well as by cross-linking TCR with anti-CD3-coated beads or the Fas antigen with the IgM monoclonal anti-Fas antibody CH11 (Fig. 1). To evaluate further the mechanisms

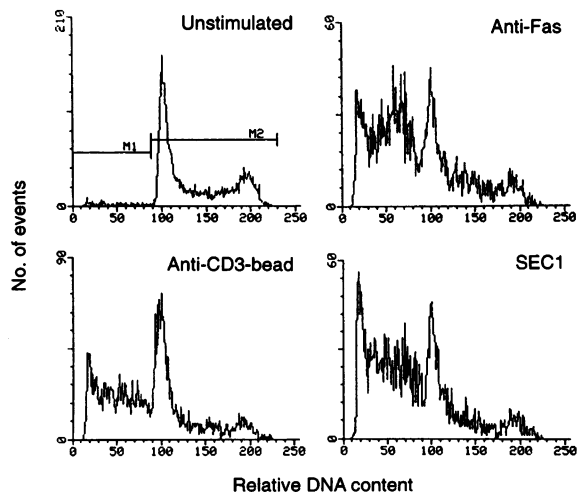

Figure 1. Flow cytometric detection of activation-induced apoptosis in H9-3D8 cells. Cells were cultured for 24 hr with or without anti-Fas (100 ng/ml CH-11 antibody), anti-CD3-coated beads (1:1 bead:cell ratio) or SEC1 (100 ng/ml) as indicated. Cells were stained with P/I and analysed as described in the Materials and Methods. Data are depicted as histograms with markers defining regions with cells undergoing apoptosis (M1) or cells in normal cell cycle (M2).

involved in apoptosis induction in these cells, we generated a Fas-resistant subclone of 3D8 (3D8-FR) by exposing these cells to CH11 for 5 days and expanding the few (<0·1%) surviving cells. The 3D8 and 3D8-FR cells expressed similar levels of Fas antigen on their surface by FACS analysis (Fig. 2), but 3D8-FR were completely resistant to apoptosis induction by anti-Fas antibody (Fig. 3). In marked contrast with recent reports that TCR-mediated apoptosis is completely dependent on an intact Fas signalling pathway[11–13], the apoptotic response to the TCR signals SEC1 and anti-CD3 remained intact in 3D8-FR cells (Fig. 3).

Since members of the Bcl2 family of proteins play key roles in regulating apoptosis in lymphoid cells[24] we investigated whether an alteration in expression of these molecules might account for the resistance of 3D8-FR cells to Fas-mediated apoptosis. Northern blotting for Bcl2, Bcl_x, Bax and Bak failed to reveal any differences between 3D8 and 3D8-FR cells (data not shown). Even though both cells readily undergo apoptosis in response to TCR ligation, they expressed high constitutive levels of mRNA for the apoptosis inhibitory molecule Bcl_x. Whether or not this reflects signalling differences in these cells, compared to other T cells, or expression of other apoptosis regulatory molecules is not known.

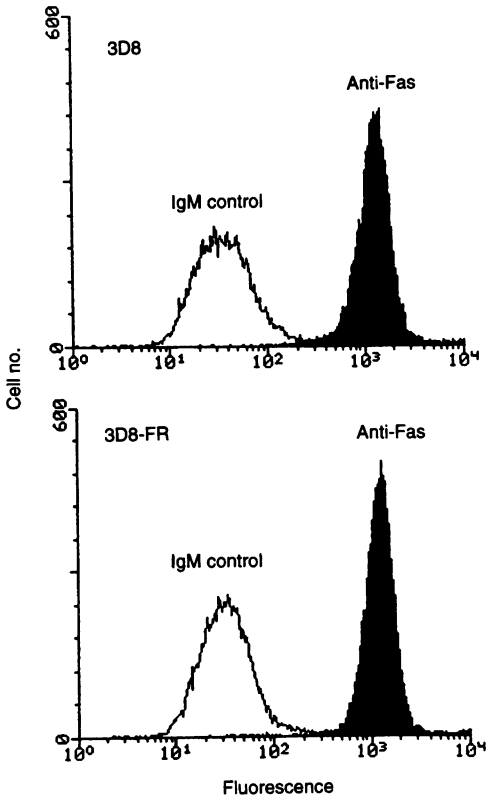

Figure 2. Flow cytometric analysis of Fas expression on 3D8 and 3D8-FR cells. 3D8 and 3D8-FR cells were stained with anti-Fas antibody (solid area) or a murine IgM myeloma control (open area), followed by PE-conjugated anti-mouse κ, and analysed with a FACScan.

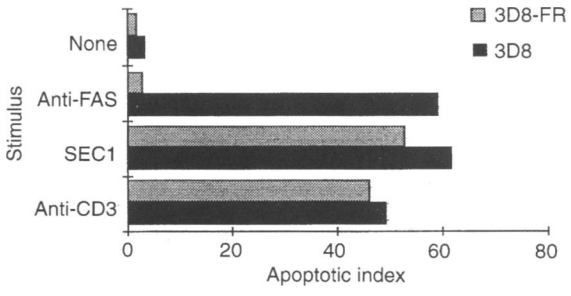

Figure 3. Induction of apoptosis in 3D8 (solid bars) and 3D8-FR (stippled bars) by different activation pathways. Cells were cultured and analysed as described in Fig. 1.

3D8-FR cells express a mutant Fas allele

We next examined the structure of the Fas molecules expressed by 3D8 and 3D8-FR cells, since Fas gene mutations have been shown to lead to resistance to apoptosis. Using pairs of PCR primers covering the full length of the human Fas-coding sequence there appeared to be no gross deletions or truncations of the Fas mRNA in 3D8-FR cells (not shown). To look for more subtle differences, we used PCR primers to clone and sequence the Fas death domain[3] from 3D8-FR cells. Three of four clones sequenced showed an identical single base substitution (CAG to TAG) which introduced a stop codon in place of the Gln_{257} codon in the middle of the death domain (Fig. 4). Thus, the 3D8-FR cells harbour a heterozygous Fas mutation. This result predicted that one of the Fas alleles in 3D8-FR cells should produce a truncated form of Fas. This was confirmed by Western blotting of 3D8 and 3D8-FR cell extracts with anti-Fas antibody (not shown).

Effects of cycloheximide and FK-506 on apoptosis in 3D8 and 3D8-FR cells

In order to begin to dissect the TCR and Fas signalling of apoptosis in 3D8 and 3D8-FR cells, we examined the effects of cycloheximide (CHX) on this system. As shown in Fig. 4, TCR-stimulated apoptosis (either anti-CD3 or SEC1) was not inhibitable by CHX in either 3D8 or 3D8-FR cells. Fas-mediated and background apoptosis in 3D8 cells were never inhibited by CHX, but usually slightly enhanced. However, in the presence of CHX, Fas antibody was able to induce

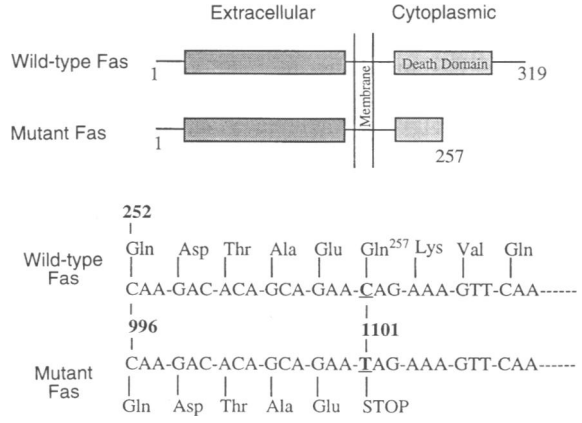

Figure 4. Depiction of the wild-type and mutant Fas alleles expressed by 3D8-FR. The stop codon at position 1101 leads to a premature termination of translation producing the illustrated Fas protein with a truncated cytoplasmic death domain.

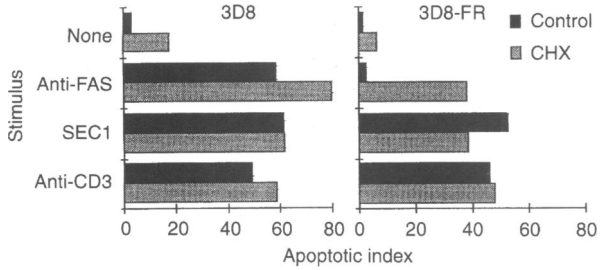

Figure 5. Effect of cycloheximide (CHX) on apoptosis in 3D8 and 3D8-FR cells. 3D8 and 3D8-FR were treated with the different stimuli as described in Fig. 1, in the presence (stippled bars) or absence (solid bars) of 10 μM CHX. TCR-mediated activation of apoptosis was insensitive to CHX in both cell populations. The Fas-resistant 3D8-FR cells could be induced to undergo apoptosis in response to anti-Fas antibody if stimulated in the presence of CHX (but not either agent alone).

apoptosis in 3D8-FR cells, indicating that Fas was able to signal in these cells and that newly synthesized proteins may be repressing the apoptotic response.

DISCUSSION

In this paper, we have described a human T-cell lymphoma line (3D8-FR) which expresses high levels of cell surface Fas, yet (unlike sister clones) is resistant to Fas-mediated apoptosis. In view of recent reports that TCR-stimulated apoptosis is mediated through Fas–FasL interactions[11–13], we were surprised to find that TCR-mediated apoptosis, induced with either anti-CD3 or SEC1, was intact in these cells. This suggests that, at least in these cells, apoptosis can be directly triggered by TCR-signalling pathways. These results are supported by the recent demonstration of anti-CD3-induced apoptosis in lymph node T cells from Fas-deficient MRL-*lpr*/*lpr* mice,[25] and the observation that *in vivo* anergized T cells are also Fas-resistant.[26] Compared with other H9 subclones that we have generated, 3D8 is very sensitive to TCR stimulation and can be triggered with soluble anti-CD3 and SEC1 in the absence of an APC population.

The Fas mutation in 3D8-FR cells is identical to that described for a patient (patient 5) with autoimmune lymphoproliferative syndrome by Fisher *et al.*[27] In these patients, heterozygous Fas mutations produce a dominant negative phenotype for Fas signalling. However, cells from heterozygous parents of these children do not exhibit the same defects, suggesting that other signalling alterations may also be required for expression of the dominant negative Fas defect. In our experiments, treatment of 3D8-FR cells with anti-Fas in the presence of CHX led to significant apoptosis, whereas either treatment alone did not. This observation indicates that Fas signalling is at least partially intact in these cells and that it may be regulated by an inducible protein(s). This situation is similar to TNF-induced apoptosis in some fibroblast lines, which requires the presence of CHX, suggesting that there may be some commonality to the regulation of TNF and Fas signalling, despite their activation of distinct early signalling events.[28] An increasing number of proteins have been shown to associate with Fas, and in particular the components of the DISC complex[19] suggest the possibility that apoptosis signalling proteins may associate with regions of Fas other than the death domain itself. The 3D8-FR cells containing the mutation described here, which deletes most of the recognized death domain, may allow the contribution of other Fas-associated proteins to the apoptotic signalling mechanism to be determined. One of these proteins, FAP-1,[20] has been shown to regulate Fas signalling negatively and could be considered a candidate 'repressor protein' that is blocked by CHX. However, FAP-1 associates with the C-terminal 15 amino acids which are deleted by the 3D8-FR mutation. Since the mutation is heterozygous, and cell surface Fas is thought to exist as a trimer, it is possible that trimerization is not required for FAP-1 association and inhibition of signalling. This will be the subject of further experiments.

Although 3D8-FR are leukaemic T cells, and thus not 'normal', their Fas-signalling defect(s) may provide insight to the Fas-resistant phenotype of peripheral T cells; resting memory ($CD45RO^+$) T cells express Fas on their surface yet are resistant to anti-Fas-mediated apoptosis. Sensitivity to Fas ligation is induced following TCR-mediated T-cell activation and subsequent IL-2-induced proliferation, suggesting that apoptosis signalling may be dependent on signals generated through the TCR or perhaps linked to the cell cycle.[26,29] Thus, the 3D8-FR cells will provide a useful tool for investigating the regulation of Fas signalling in T cells.

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