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Fungal metabolite FR901228 inhibits c-Myc and Fas ligand expression

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Activation of T lymphocytes often leads to cellular activation, production of cytokines, entry into cell cycle, and expression of Fas (CD95) and Fas ligand (FasL). Although it is well established that the interaction of Fas and FasL results in apoptosis, mechanisms for regulated expression of Fas and FasL are unclear. Our previous work with antisense oligodeoxynucleotides suggested that the protooncogene c-*myc* is obligatory for activation-induced apoptosis. To study the relationship between c-*myc* and the Fas/FasL expression, we employed the antisense method and a newly identified fungal metabolite, FR901228, which has been shown to specifically inhibit expression of c-*myc* in fibroblasts. We found that FR901228 could effectively block activation-induced apoptosis in T cell hybridomas and this was correlated with its specific inhibition of c-*myc* expression. Both FR901228 and antisense oligodeoxynucleotide to c-*myc* had similar effect in inhibiting FasL expression. These treatments did not affect activation-induced production of IL-2, nor the expression of Fas. In addition, FR901228 inhibited the expression of FasL in 3T3 fibroblasts, but not these transfected with c-*myc*, supporting a specific role of c-*myc* in this process. Thus, c-Myc plays a fundamental role in the regulation of the expression of FasL, but not Fas and IL-2. Our data further defined the requirement of c-Myc in activation-induced apoptosis in T cells.

Keywords: fungal metabolite; c-*myc*; FasL; apoptosis; T cell hybridoma

Introduction

Apoptosis functions to eliminate excessive cells during development and its deregulation leads to pathological processes (Kerr *et al*., 1972; Hale *et al*., 1996). Activation-induced apoptosis elicited by T cell receptor (TCR) crosslinking is fundamental to the mechanism for immune tolerance and cellular homeostasis (Green *et al*., 1994; Ridgway *et al*., 1994). Activation of immature lymphocytes at particular stages during development in the thymus is known to induce apoptosis, a phenomenon that could be related to negative selection (Smith *et al*., 1989; Shi *et al*., 1989; Murphy *et al*., 1991). Although stimulation of TCR on peripheral T cells usually leads to clonal expansion, primary activated mature T cells are sensitive to activation-induced apoptosis (Lenardo, 1991; Russell *et al*., 1991). T cell hybridomas have been used as models for both forms of T-cell apoptosis (Ashwell *et al*., 1987; Shi *et al*., 1990).

Activation-induced apoptosis in T cells has been attributed to the induction of Fas (CD95) and Fas ligand (FasL/CD95L) following TCR activation. Interaction of Fas and FasL triggers a suicidal process, manifested by activation of a programmed protease cascade (Brunner *et al*., 1995; Nagata and Golstein, 1995; Ju *et al*., 1995; Alderson *et al*., 1995; Dhein *et al*., 1995). Mutations in Fas or FasL gene are associated with abnormal accumulation of lymphoid cells and autoimmunity. Fas and FasL have, thus, been suggested to be fundamental to both central and peripheral tolerance (Castro *et al*., 1996; Adachi *et al*., 1996). The expression of Fas and FasL is known to be tightly regulated (Park *et al*., 1996; Yang *et al*., 1995; Brunner *et al*., 1996). In the A1.1 T cell hybridoma, Fas is minimally expressed, but it is markedly increased within 2 h after TCR stimulation. Likewise, the expression of FasL is undetectable in resting cells. It is induced within 4 h after activation (Brunner *et al*., 1995). Blocking the interaction of Fas and FasL with Fas fusion proteins prevents activation-induced apoptosis (Brunner *et al*., 1995; Ju *et al*., 1995).

The mechanisms controlling the expression of Fas and FasL are poorly understood. We previously demonstrated that c-*myc* is a component of the pathway leading to activation-induced apoptosis (Shi *et al*., 1992). The product of the c-*myc* gene is believed to be a nuclear transcription factor known to promote cell cycle progression (Spencer and Groudine, 1991). Its role in the regulation of apoptotic genes, however, remains to be elucidated. The transcription of c-*myc* can be reversibly inhibited by a newly characterized fungal metabolite, FR901228, isolated from *Chromobacterium violaceum* (Ueda *et al*., 1994a). Treatment with FR901228 specifically reduced c-*myc* expression (Ueda *et al*., 1994b). In the present study, FR901228, as well as the antisense oligodeoxynucleotides to c-*myc*, was used to investigate the role of c-*myc* in activation-induced apoptosis in T cell hybridomas. We found that following the inhibition of c-*myc* the expression of FasL was suppressed. These treatments, however, did not affect Fas expression, nor the Fas signaling pathway. We conclude that the effect of Myc on activation-induced apoptosis is exerted, at least in part, through facilitating FasL induction.

Results

FR901228 inhibits anti-CD3-induced apoptosis

The fungal metabolite, FR901228, has been shown in fibroblasts (Ueda *et al*., 1994b) to specifically inhibit

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the expression of c-*myc*, which is known to promote cell cycle progression (Spencer and Groudine, 1991). We found that FR901228 could also inhibit the proliferation of A1.1 T cell hybridoma, as well as the IL-2 induced proliferation of CTLL-2 cells. The inhibition appears to be dose dependent, with complete growth arrest observed at 5–10 ng/ml, as determined by [^{3}H]-thymidine incorporation (data not shown).

We have previously shown that inhibition of c-*myc* prevents activation-induced apoptosis (Shi *et al*., 1992). In this study we tested FR901228 for similar effects. Typically, A1.1 cells undergo apoptosis upon activation with anti-CD3 within 8 h. FR901228 showed a dose-dependent protection from apoptosis as indicated by MTT conversion (Figure 1a). Apoptosis was completely inhibited by FR901228 at 10 ng/ml. The protected cells displayed normal morphology and were devoid of DNA fragmentation (Figure 1b).

FR901228 does not block IL-2 production

Prior to apoptosis, activated A1.1 cells produce cytokines, such as IL-2 (Shi *et al*., 1990). When culture supernatants from anti-CD3-activated cells were examined for IL-2 by ELISA, it was found that IL-2 production was not inhibited by FR901228 treatment (Figure 2). Similar results were obtained with diluted tissue culture supernatant in a bioassay (data not shown). Thus, FR901228 could specifically inhibit apoptosis, without affecting the pathway leading to IL-2 production.

*FR901228 inhibits c-*myc *expression in lymphoid cells*

As shown in Figure 3, FR901228 inhibited c-*myc* expression in both A1.1 and CTLL-2 cells, as detected by Northern blotting (Figure 3b) hybridization. On the other hand, FR901228 did not inhibit the expression of β-actin, Fas, cyclin B1, cdc2 and cytochrome C (Figure 4b and data not shown) on Northern blot and Grb-2 (Figure 3b), IL-2 (Figure 2) and cyclin B1 (data not shown) on Western blot, indicating the specificity of the inhibition.

FR901228 inhibits FasL but not Fas expression

The induced expression of Fas and FasL, and their subsequent interaction, were shown to be responsible for apoptosis following T-cell activation (Brunner *et al*., 1995; Ju *et al*., 1995). The inhibitory effect of FR901228, therefore, could be due to its interference with Fas/FasL expression or Fas-mediated death signals. We first examined the effect of FR901228 on anti-Fas induced apoptosis (Figure 4a). Anti-CD3 treatment induced apoptosis in A1.1 cells as demonstrated by the hypodiploid peaks on DNA content analysis. FR901228 completely inhibited anti-CD3-induced apoptosis. Nevertheless, FR901228 rescued cells were still sensitive to anti-Fas (JO2) antibody-mediated apoptosis, indicating that FR901228 did not affect the induction of Fas, nor the Fas signaling pathway. This suggests that the effect of FR901228 should be on FasL expression, rather than interfering with death signals from Fas crosslinking. This conclusion was supported by a series of Northern analyses (Figure 4b). We activated A1.1 cells with anti-CD3 for 4 h, with or without FR901228. FR901228 abolished the expression of FasL, but not Fas. Antisense oligodeoxynucleotides to c-*myc* partially

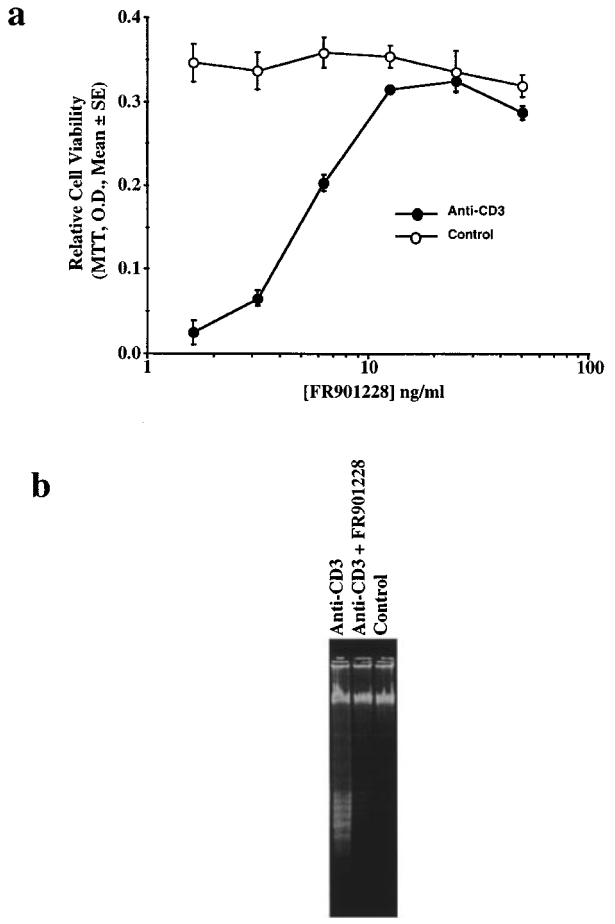

Figure 1 FR901228 inhibits activation-induced apoptosis. (**a**) A1.1 cells were activated with anti-CD3, with or without different concentrations of FR901228. Cell viability at 12 h was determined by MTT assay. (**b**) Genomic DNA fragmentation of cells treated in the same way as in (**a**). Total Genomic DNA was analysed by agarose gel electrophoresis

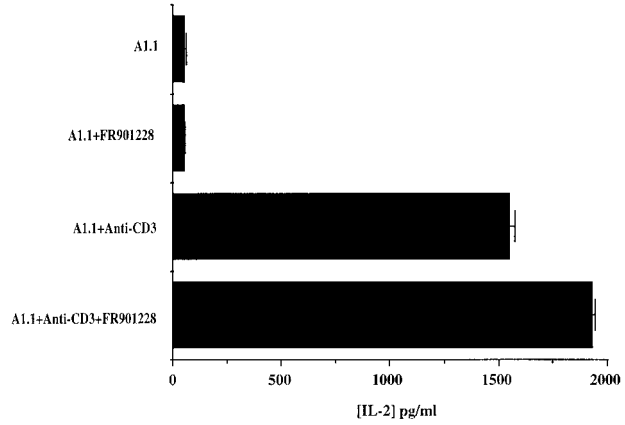

Figure 2 FR901228 does not inhibit activation-induced IL-2 production. A1.1 cells were activated with anti-CD3 in the presence or absence of FR901228 (10 ng/ml) for 12 h. IL-2 in the supernatants were detected by ELISA

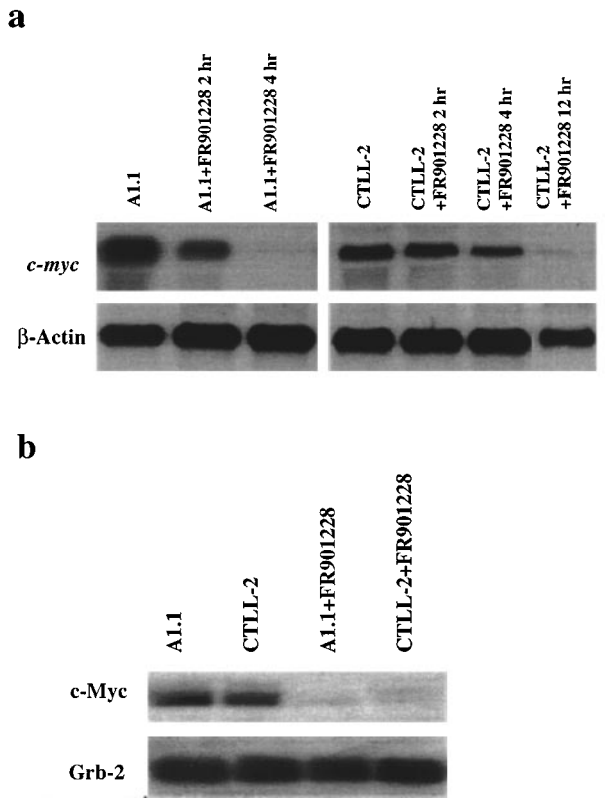

Figure 3 FR901228 inhibits c-*myc* expression. (**a**) A1.1 and IL-2 stimulated CTLL-2 cells were treated with FR901228 at 10 ng/ml for different periods and total RNA was isolated. C-*myc* expression was determined by Northern blotting. Equal RNA loading was controled in the bottom panel. (**b**) A1.1 and CTLL-2 cells were treated with FR901228 at 10 ng/ml for 10 h. Cell lysates were prepared at 4 h for Western blotting with anti-c-Myc or anti-Grb-2

inhibited FasL expression (Figure 4b). Data from these two distinct approaches strongly indicate that c-Myc is essential for FasL induction. In addition, cyclosporin A completely inhibited FasL expression, as reported (Brunner *et al*., 1996).

The inhibition of FR901228 on induced FasL expression was further demonstrated by a functional assay with L1210Fas cells. As shown previously, parental L1210 cells did not undergo apoptosis upon contact with FasL expressing cells (Brunner *et al*., 1996; Kagi *et al*., 1994; and data not shown), whereas L1210Fas cells did. Co-culture with anti-CD3 activated A1.1 cells induced DNA fragmentation in L1210Fas cells (Figure 4c). This could be compromized by FR901228 in a dose-dependent manner. Similar results were observed with another T cell hybridoma, 2B4 (data not shown).

FR901228 inhibits constitutive FasL expression

CTLL-2 cells constitutively express high levels of FasL (Suda *et al*., 1995). As shown in Figure 4d, FR901228 effectively inhibited the expression of FasL in CTLL-2 cells. Therefore, FR901228 inhibits both constitutive and activation-induced expression of FasL. Hueber *et al*. (1997) have demonstrated that c-*myc* overexpression-mediated apoptosis requires the interaction between Fas and FasL. As reported before (Ueda *et al*., 1994b), we have found that FR901228 inhibited *myc* in 3T3 cells, but not in cells constitutively expressing *myc* (data not shown). Using the same method as Hueber *et al*. (1997), we found that FasL is expressed in mouse 3T3. This expression of FasL was blocked by FR901228. On the other hand, we showed that FR901228 failed to inhibit FasL expression in c-*myc* transfected 3T3 cells. This experiment argues that FR901228 mediated inhibition of FasL expression is through c-*myc*.

Discussion

We have previously reported that activation-induced apoptosis in T cell hybridomas requires the participation of Myc (Shi *et al*., 1992). Similarly, Evan *et al*. (1992) demonstrated that deregulated c-*myc* expression promotes apoptosis following growth factor deprivation. Furthermore, Hueber *et al*. (1997), reported that this c-*myc* mediated apoptosis requires the interaction of Fas and FasL. In the present study, we found that FR901228, a potent inhibitor of c-*myc* expression (Ueda *et al*., 1994b), also inhibited both induced and constitutive expression of FasL, as well as activation-induced apoptosis. This effect was also observed in activated murine mature T cells (data not shown). Importantly, FR901228 did not affect Fas expression or Fas-mediated cell death signals. Thus, the inhibition of apoptosis by FR901228 was due to its discriminative blockade of FasL expression. Furthermore, the inhibition of FasL by FR901228 appears to be due to the suppression of c-*myc* expression. This is supported by the observation that antisense oligodeoxynucleotides to c-*myc*, which reduced c-Myc expression, exhibited similar inhibitory effect both FasL expression and the apoptosis. The inability of FR901228 to block FasL expression in 3T3 cells overexpressing c-*myc* suggests that myc is essential in FR901228 mediated inhibition of FasL expression. Recently, we found that activation-induced FasL expression seemed to be restricted to particular stages of the cell cycle, the effect of c-*myc* on FasL expression could be exerted through regulating cell cycle.

Another important observation in our studies was that FR901228 could inhibit the constitutive expression of FasL. Recent studies have suggested that the expression of FasL was associated with immune escape of tumors, with maintenance of immune privilege sites, and with the rejection of transplanted organs (Nagata and Golstein, 1995). Our results support the notion that FR901228 could be explored as a candidate for clinical therapeutical purposes.

It has been reported that activation-induced expression of FasL in T cell hybridomas could be inhibited by cyclosporin A and FK506 (Brunner *et al*., 1996), retinoic acid (Yang *et al*., 1995), as well as oxygen free radical generation inhibitors (Williams and Henkart, 1996). We have found that these reagents did not show any effect on c-*myc* expression in our T cell hybridomas (data not shown). Thus, though our data clearly demonstrated the role of c-*myc* in the regulation of FasL expression, it is conceivable that c-*myc* mediated FasL expression requires the involvement of other signals.

It is likely that FR901228 functions specifically on c-*myc* expression. We have shown that it did not affect the expression of IL-2 (Figure 2, Grb-2 (Figure 3b), Fas (Figure 4b), as well as β-actin (data not shown). Nor did it have any effect on Fas signaling pathway that leads to apoptosis (Figure 4a). Furthermore, FR901228 suppresses the proliferation of A1.1 cells in the same dose range as that in fibroblast cells (Ueda *et al*., 1994b). C-Myc is a transcriptional regulator of a number of important genes and it regulates lymphocyte

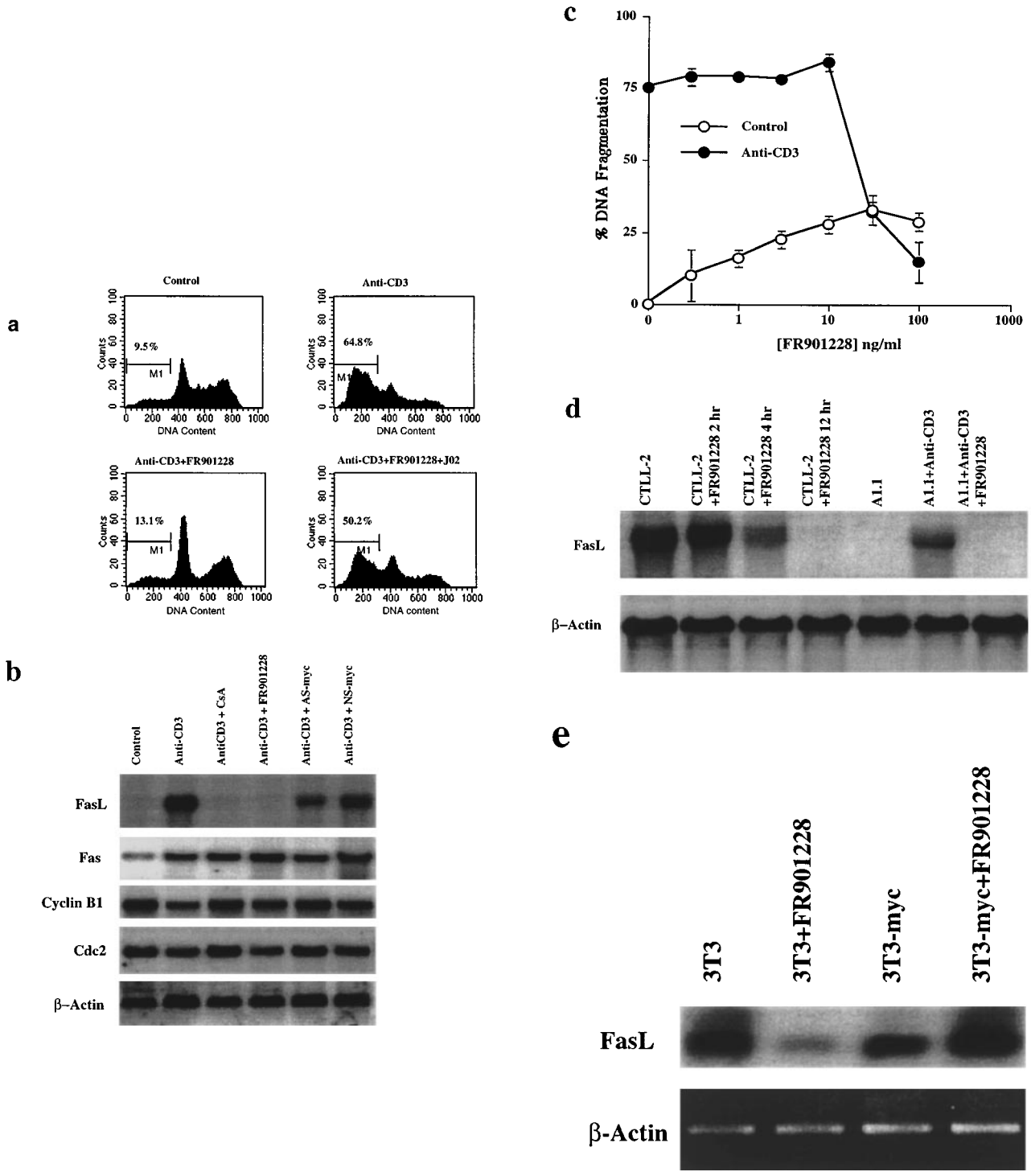

Figure 4 FR901228 inhibits FasL, but not Fas, expression. (**a**) FR901228 did not inhibit Fas-mediated apoptosis. A1.1 cells were activated with anti-CD3 with FR901228 (10 ng/ml) and anti-Fas (JO2, 500 ng/ml) for 12 h. Apoptosis was determined by DNA content analysis. The marked region represent apoptotic cells containing hypodiploid DNA. (**b**) A1.1 cells were activated with anti-CD3 in the presence of cyclosporin A (CsA, 100 ng/ml), FR901228 (10 ng/ml), antisense to c-*myc* (AS-myc, 5 μM), or sense control (S-myc, 5 μM). After 4 h, total RNA was isolated for Northern blot hybridization. The same blot was analysed for FasL, Fas, cyclin B1, cdc2, and β-actin expression. (**c**) L1210Fas were first labeled with ^{3}H-thymidine, followed by co-incubation with A1.1 cells (1 : 2) on anti-CD3 coated plate, in the presence of different concentrations of FR901228. Unfragmented DNA was determined by liquid scintillation counting at 18 h. (**d**) CTLL-2 cells were treated with FR901228 at 10 ng/ml for different times. Total RNA was isolated for FAsL Northern blot hybridization. (**e**) Parental 3T3 cells and the myc transfectants were treated with or without FR901228 at 10 ng/ml for 24 h. The expression of FasL detected by RT–PCR followed by Southern blotting analysis. The expression of β-actin was used as a control

growth and differentiation (Green and Scott, 1994; Roy *et al*., 1993). It is conceivable that c-Myc is influencing the transcription of genes critical to the initiation of apoptosis (Mercep *et al*., 1989). Our data suggest that c-*myc* is a mandatory component sustaining FasL expression.

Materials and methods

Cells and reagents

A1.1 (Fotedar *et al*., 1985) and CTLL-2 cells were maintained as described (Shi *et al*., 1990). FR901228 was obtained from Fujisawa Pharmaceutical Company Ltd. (Osaka, Japan). Antibodies to c-Myc and to Grb-2 were from Upstate Biotechnology Inc. (Lake Placid, NY). Antisense phosphothioate oligodeoxynucleotides to murine c-*myc* (5′-CACGTTGAGGGGCAT) and sense control (5′-ATGCCCCTCAACGTG) were from Integrated DNA Technologies, Inc. (Coralville, IA. Murine 3T3 fibroblasts overexpressing c-*myc* were kind gifts of Dr Linda Penn (University of Toronto).

DNA fragmentation assay

DNA fragmentation assay was carried out essentially as described (Shi *et al*., 1995). Briefly, cells (5×10^5) were lysed by 30 μl of lysis buffer (80 mM EDTA, 200 mM Tris, pH 8.0, 1.6% sodium lauryl sarcosinate and 1 mg/ml proteinase K). After incubation at 50°C for 4 h, RNase A was added to the concentration of 0.2 mg/ml and the lysate was further incubated for another 30 min. The resulting DNA samples were then fractionated by agarose gel electrophoresis.

IL-2 ELISA

IL-2 in culture supernatants was detected with the Cytoscreen Immunoassay Kit (BioSource, Camarillo, CA) according to the manufacturer recommended protocol. The amount of IL-2 was determined by reression curves obtained with recombinant murine IL-2 provided in the kit.

Functional analysis of FasL expression

Activation-induced FasL expression was assessed by its ability to cause DNA fragmentation in Fas expressing L1210 cells (L1210Fas) (Brunner *et al*., 1996), which were labeled with ^{3}H-thymidine at 5 μCi/ml for 2 h. Labeled cells (2×10^4) were incubated with 4×10^4 T cell hybridomas in 96-well plates coated with anti-CD3. Cells were then harvested onto glass fiber filters and the c.p.m. of unfragmented DNA was determined on a Matrix 9600 Direct Beta Counter (Packard, Downers Grove, IL). DNA fragmentation was calculated as follows: % = $100 \times (1 - \text{c.p.m. of experimental group/c.p.m. of control group})$.

MTT assay

After treatments as indicated, 10 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (5 mg/ml in H_2O) was introduced and cells were incubated at 37°C for 4 h, followed by addition of 100 μl acid-isopropanol (0.04 N HCl). After the reduced crystals were dissolved, samples were scanned by an ELISA reader with a 595 nM filter (Mosmann, 1983).

DNA content analysis

Cells were fixed with 70% ethanol for 30 min at 4°C. The fixed cells were then incubated in PBS containing propidium iodide (50 μg/ml) and RNase A (0.1 mg/ml) at room temperature for 30 min. DNA content was determined by flow cytometry for FL2 intensity.

Western blotting

Equal numbers of cells (5×10^6/sample) were lysed for Western analysis with PVDF membrane (Schleicher & Schuell, Inc., Keene, NH) according to manufacturer's instruction. The immunoreactive proteins were detected with an ECL kit (Amersham, Arlington Heights, IL).

Northern blotting

Total RNA was fractionated on agarose/formaldehyde gel and blotted onto Nytran membrane (Schleicher & Schuell). The cDNA probes (mouse Fas and FasL from Dr Shigekazu Nagata, Osaka Bioscience Institute, Japan; c-*myc* from Dr. Peter Wirth, NIH) were randomly primed with [^{32}P]-dCTP. Hybridization was carried out at 42°C in $5 \times$SSC, 2.5 mM EDTA, 0.1% SDS, $5 \times$Denhardt's solution (Denhardt, 1966), 2 mM sodium pyrophosphate, 50 mM sodium phosphate and 50% formamide. The membrane was washed and subjected to autoradiography.

RT–PCR analysis

Murine 3T3 cells were treated with or without FR901228 for 24 h. Total RNA samples were prepared with the Tri-Reagent kit (Molecular Research Center, Inc., Cincinnati, OH) according to a manufacturer recommended protocol. For the first strand cDNA synthesis, 2 mg of total RNA was primed with oligo[dT] in reverse transcription with the cDNA Cycle Kit (Invitrogen, Co., San Diego, CA). Subsequently, FasL cDNA was amplified by 30 cycles of PCR reactions with primers of 5′-ACTCCGTGAGTT-CACCAACCAAGCC-3′ and 5′-ATGTCCCTCAGTCA-CAGACCTCCTTC-3′ (Midland Certified Reagent Company, Midland, TX). As a control, β-actin cDNA was amplified from the same first strand cDNA reactions, with primers of 5′-GTGGGCCGCTCTAGGCACCA-3′ and 5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′ (Stratagene, La Jolla, CA). PCR amplifications were carried out with the cycling parameters recommended by the respective manufacturers, on a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT).

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