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ORIGINAL ARTICLE

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Tamoxifen induces apoptosis in Fas$^+$ tumor cells by upregulating the expression of Fas ligand

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Abstract *Purpose*: Tamoxifen (TAM), a nonsteroidal anticancer agent, is used in the treatment of breast cancer. In the current study, we investigated whether TAM induces apoptosis in tumor cells by altering the expression of Fas and Fas ligand (FasL). *Methods*: Several tumor cell lines were used to test the ability of TAM to induce apoptosis, which was studied using the TUNEL assay. The effect of TAM on the expression of Fas and FasL was analyzed using a flow cytometer. *Results*: TAM was found to suppress the growth of an estrogen receptor-positive human mammary tumor cell line (T-47D) by inducing apoptosis. Interestingly, TAM also induced apoptosis in an estrogen receptor-negative murine T cell lymphoma cell line, EL-4. The ability of TAM to induce apoptosis in T-47D and EL-4 tumor cells correlated with the increased expression of FasL but not Fas on the tumor cells. Similar to TAM, a metalloproteinase (MP) inhibitor, which is known to increase the expression of membrane-bound FasL, was found to induce apoptosis in both T-47D and EL-4 tumor cells by increasing the expression of FasL but not Fas. Furthermore, both TAM and the MP inhibitor failed to induce apoptosis in L1210 tumor cell lines that failed to express FasL. *Conclusions*: The current study demonstrates that TAM can induce apoptosis in Fas$^+$ tumor cells by upregulating FasL.

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Introduction

Fas ligand (FasL) is a membrane protein belonging to the tumor necrosis factor family and is expressed in both transmembrane and soluble forms. It induces apoptosis by crosslinking with the Fas receptor [1]. FasL is normally expressed on activated cells of the immune system and is used for killing cells infected with virus or cancer cells that express Fas [2]. Recent studies have demonstrated that tumor cells can also express FasL and therefore kill the immune cells that express Fas. This phenomenon has attracted considerable attention and is believed to play a major role in the ability of tumor cells to evade the immune system [3, 4].

In the current study, the hypothesis that some of the anticancer drugs used clinically would have a direct effect on the expression of Fas and/or FasL by tumor cells was tested. This is important because alterations in the expression of Fas or FasL could have a significant impact on the ability of tumor cells to grow, metastasize and cause significant damage to the host. Furthermore, alterations in the expression of Fas and FasL by tumor cells could also influence their susceptibility to immune cell-induced lysis. To this end, we studied tamoxifen (TAM), a nonsteroidal anticancer agent used in the treatment of breast cancer [5]. TAM acts as an estrogen antagonist and is the endocrine treatment of choice against tumors positive for the estrogen receptor (ER). However, TAM is not effective in a small number of patients, and some individuals treated with TAM develop drug resistance after prolonged treatment. TAM has also been shown to exhibit estrogen-like properties and thereby facilitate the growth of human endometrial carcinoma [6].

One of the important factors facilitating metastasis of cancer includes the ability of tumor cells to produce metalloproteinase (MP) thereby causing degradation of

the extracellular matrix. Thus, synthetic MP inhibitors have been shown to have significant antitumor activity in preclinical models [7]. Most of the current research on MP inhibitors has focused on the ability of these compounds to inhibit tumor cell invasion. MP is also used by cells to cleave the FasL that is expressed on cell membranes, thereby converting it into a soluble, less-toxic form [8]. It is likely, therefore, that MP inhibitors can increase the membrane expression of FasL. Whether this would, in turn, lead to induction of apoptosis in tumor cells, has not been previously investigated.

In the current study, the effect of TAM and MP inhibitors on the expression of Fas and FasL was investigated using both human and murine tumor cell lines. The results demonstrate that these anticancer agents upregulate the expression of FasL thereby inducing apoptosis. Furthermore, this effect of increasing the expression of FasL could represent a mechanism by which TAM may facilitate the growth of certain types of tumors in vivo.

Materials and methods

Cell lines and culture

T-47D cells (HTB133; ATCC, Manassas, Va.), a human breast cancer cell line that expresses the ER [9], were grown in minimum essential medium supplemented with 10% fetal calf serum, 2 m*M* glutamine, penicillin/streptomycin, 0.01 mg/ml bovine insulin, 0.1 m*M* non-essential amino acids and 1 m*M* sodium pyruvate. EL-4 cells (TIB39; ATCC), a murine T cell lymphoma cell line that fails to express ER [10], were grown in RPMI medium supplemented with 10% fetal calf serum, 10 m*M* HEPES, 1 m*M* glutamine and gentamicin sulfate. L1210 (CCL 219; ATCC) Fas^+ and Fas^- tumor cell lines were grown similarly in RPMI medium containing 5% fetal calf serum [11]. Medium, supplements and trypan blue dye were purchased from GIBCO BRL (Gaithersburg, Md.) with the exception of fetal bovine serum which was obtained from Biofluids (Rockville, Md.).

Cell counts

To study the effect of TAM and MP inhibitor on cell growth, cells were cultured for 18 h in medium containing drugs or vehicle control. The cells were harvested and counted in the presence of trypan blue dye to determine the number of viable cells.

Detection of apoptosis

Apoptosis was detected using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, referred to as TdT-mediated nick end-labeling (the TUNEL technique), for labeling DNA strand breaks [4]. After incubation with the anticancer agents as described below, the tumor cells were studied for the induction of apoptosis by first fixing the cells with 4% formaldehyde for 30 min at room temperature. The cells were then washed with phosphate-buffered saline (PBS), permeabilized on ice for 2 min and incubated with FITC-dUTP and TdT for 1 h at 37°C. A total of 5000 cells were analyzed per sample using a flow cytometer (Coulter Epics V, Hialeah, Fl.).

Detection of Fas

The expression of Fas on EL-4 cells was determined using hamster anti-mouse Fas antibodies followed by FITC-conjugated anti-hamster antibodies (Pharmingen, San Diego, Calif.). To detect Fas on T-47D cells, FITC-conjugated anti-human Fas antibodies were used. The adherent cells were dislodged using trypsin-EDTA and counted using trypan blue dye exclusion. The cells were stained with the above-mentioned antibodies by incubation on ice for 30 min, washed with PBS, and fixed in 1% paraformaldehyde until flow cytometric analysis was performed.

Detection of FasL

FasL was detected on T-47D cells using biotin-conjugated anti-human FasL antibodies and PE-streptavidin (Pharmingen, San Diego, Calif.). The T-47D cells were harvested, washed and counted. Biotin-conjugated anti-FasL antibodies were added as recommended by the manufacturer and the samples were kept on ice for 30 min. The cells were washed and PE-streptavidin was added for detection. The samples were kept on ice for 20 min and then washed. Cells (5000 per sample) were analyzed using a flow cytometer. To detect FasL on EL-4 tumor cells, the cells were incubated with anti-mouse FasL antibodies followed by biotin-conjugated anti-mouse IgG and PE- streptavidin. The staining was performed as described above.

Use of TAM and MP Inhibitor

TAM (10 μ*M*; Sigma Chemical Company, St. Louis, Mo.) and MP inhibitor (GM6001, 1 m*M*; AMS Scientific, Calif.) were added to tumor cell cultures and incubated overnight. The cells were harvested, washed and studied for induction of apoptosis and expression of Fas and FasL, as described above.

Statistical analysis

All experiments were repeated at least twice with consistent results. To depict flow cytometry data, a representative experiment is shown. Statistical analysis of cell viability was performed using Student's *t*-test and *P*-values < 0.05 were considered statistically significant.

Results

Both TAM and MP inhibitor reduce growth of T-47D cells

T-47D cells were cultured with TAM or MP inhibitor for 18 h and cell viability was measured. As shown in Fig. 1, treatment with either MP inhibitor or TAM caused a significant decrease ($P < 0.05$) in the viable cell counts.

TAM increases the expression of FasL and induces apoptosis in T-47D cells

Next, we investigated the effect of TAM on induction of FasL and apoptosis. As shown in Fig. 2, TAM-treated T-47D tumor cells demonstrated significant levels of apoptosis when compared to cells treated with vehicle alone. Furthermore, 74.6% of TAM-treated T-47D tumor cells showed higher levels of FasL expression when compared to cells treated with the vehicle. Also, TAM caused a slight increase in the expression of Fas on

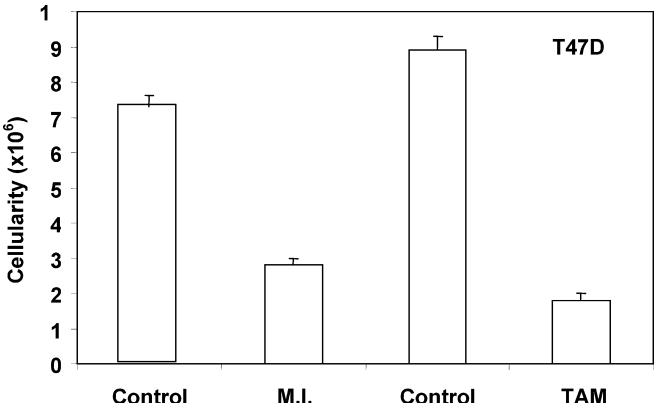

Fig. 1 Effect of MP inhibitor (*M.I.*) and tamoxifen (*TAM*) on growth of T-47D tumor cells. T-47D tumor cells were cultured in the presence of MP inhibitor (1 m*M*) or TAM (10 μ*M*) or in the presence of medium containing the vehicle (*control*) used for dissolving the anticancer agents. After an 18 h incubation, the cells were harvested and viable cells were counted in the presence of trypan blue dye. The *bars* represent mean cellularity ±SE of duplicate cultures

T-47D tumor cells (Fig. 2). These results demonstrate that TAM might induce apoptosis in T-47D tumor cells by upregulating the expression of FasL. It should be noted that about 25.4% and about 78% of T-47D tumor cells were $FasL^+$ and Fas^+, respectively, prior to any treatment. Also, when Fas and FasL were analyzed, a significant proportion of the cells had in fact died (Fig. 1). Thus, the expression of these molecules may have been on cells that survived or those that were in the process of undergoing apoptosis.

MP inhibitor upregulates expression of FasL and triggers apoptosis in T-47D cells

To test whether increased expression of FasL by T-47D tumor cells would trigger apoptosis, the effect of MP inhibitor on apoptosis was tested. MP inhibitors have been shown to increase the expression of FasL on the membrane by preventing the cleavage of FasL by MP [7]. As shown in Fig. 3, T-47D tumor cells treated with MP inhibitor exhibited high levels of apoptosis (85.2%). Furthermore, T-47D tumor cells treated with MP inhibitor demonstrated increased levels of FasL when compared to cells treated with vehicle alone. In contrast, expression of Fas was not significantly altered following treatment with MP inhibitor. These results demonstrated that MP inhibitor, which upregulates the expression of FasL, also induces apoptosis in the T-47D tumor cell line.

Both TAM and MP inhibitor reduce viability of EL-4 cells

We next addressed the effect of TAM and MP inhibitor on a murine tumor cell line, EL-4, that fails to express

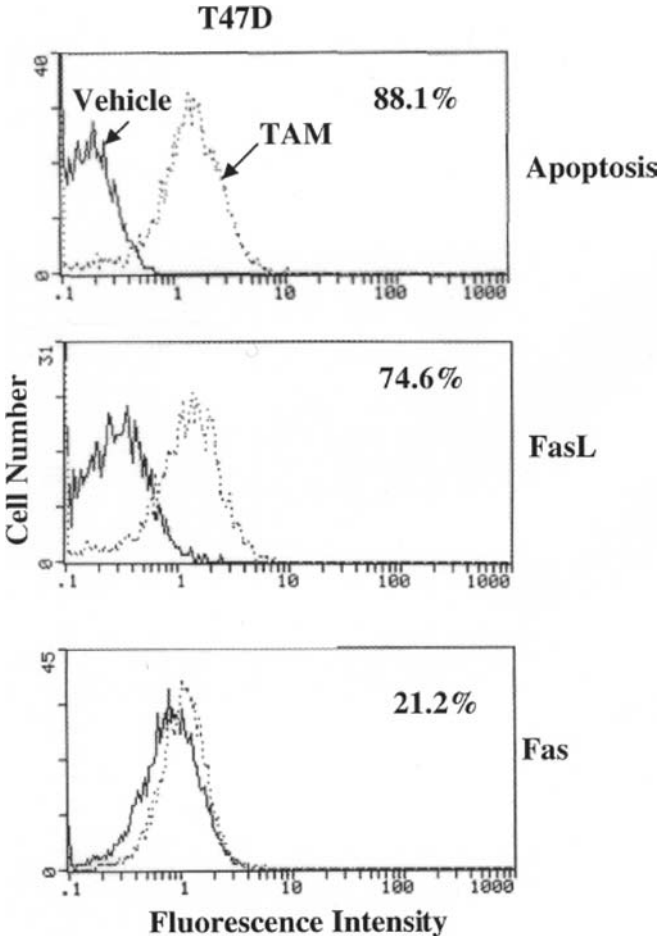

Fig. 2 Effect of tamoxifen (*TAM*) on apoptosis and expression of Fas and FasL. T-47D tumor cells were cultured as described in Fig. 1 with TAM or the vehicle, after 18 h, the cells were harvested and stained with FITC-dUTP to detect apoptosis or the expression of Fas and FasL using a flow cytometer. The *bold histograms* represent cells treated with the vehicle and the *broken histograms* represent cells treated with TAM. The percent positive cells are shown in each panel

ER. As shown in Fig. 4, EL-4 tumor cells cultured with TAM showed a marked decrease in viable cell counts when compared to vehicle controls ($P < 0.05$). Similar results were also obtained with EL-4 cells treated with MP inhibitor. These results demonstrate that both MP inhibitor and TAM were able to decrease the cell viability of EL-4 cells.

TAM induces apoptosis in EL-4 cells by upregulating FasL expression

EL-4 tumor cells treated with TAM showed significant levels of apoptosis when compared to cells treated with the vehicle alone (Fig. 5). Interestingly, TAM also caused increased expression of FasL in EL-4 tumor cells but failed to induce increased expression of Fas. These results together demonstrate that TAM was acting independently of the ERs to upregulate FasL expression.

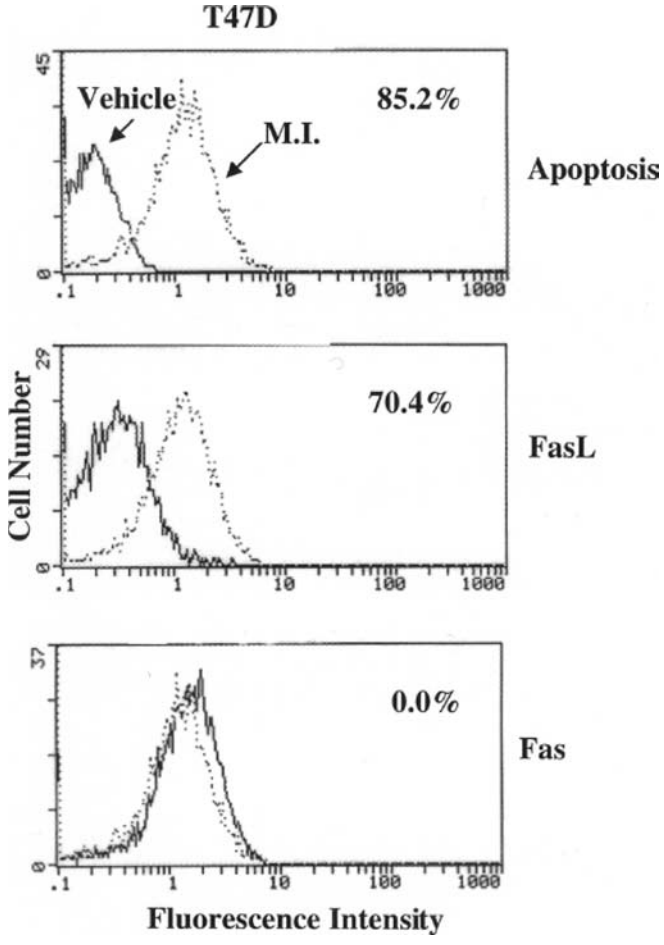

Fig. 3 Effect of MP inhibitor (*M.I.*) on the expression of Fas and FasL and apoptosis induction in T-47D tumor cells. The experiment was carried out as described in Fig. 2 except that MP inhibitor was added at a concentration of 1 m*M*

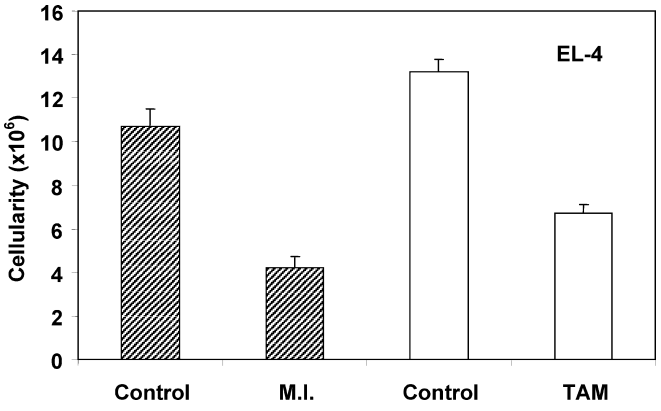

Fig. 4 Effect of MP inhibitor (*M.I.*) and tamoxifen (*TAM*) on the growth of EL-4 tumor cells. EL-4 tumor cells were grown in the presence of MP inhibitor (1 m*M*) or TAM (10 μ*M*), and the cells were harvested after 18 h. The cellularity was determined by trypan blue dye exclusion. The bars represent the means ± SE of duplicate cultures

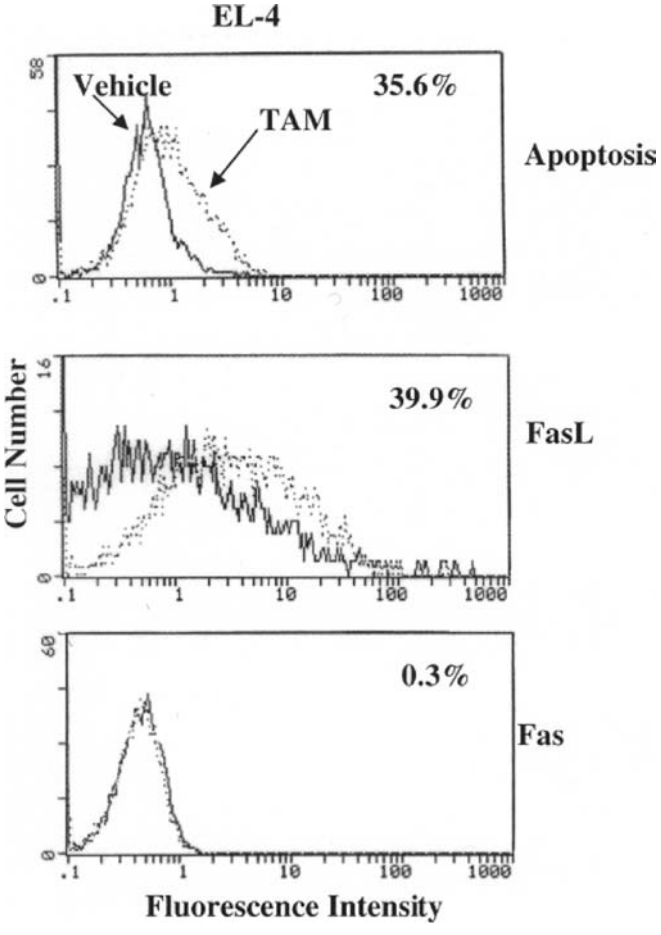

Fig. 5 Effect of tamoxifen (*TAM*) on induction of apoptosis and expression of Fas and FasL in EL-4 tumor cells. These experiments were carried out in a similar manner to that described in Fig. 2 except that EL-4 tumor cells were used

MP inhibitor upregulates expression of FasL and induces apoptosis

EL-4 tumor cells treated with MP inhibitor also expressed significantly increased levels of FasL but not Fas (Fig. 6). The upregulation of FasL correlated with increased induction of apoptosis in EL-4 cells when compared to vehicle controls. These results also demonstrate that MP inhibitors may prevent the cleavage of FasL from the membrane into a soluble form, thereby increasing the levels of expression on the membrane. Furthermore, such an increase in the expression of FasL on the membrane may trigger, through an autocrine pathway, apoptosis in EL-4 tumor cells.

Neither MP inhibitor nor TAM induce apoptosis in L1210 tumor cells that fail to express FasL

To further corroborate that the MP inhibitor and TAM were inducing apoptosis following upregulation of FasL, we included a $FasL^-$ tumor cell line, L1210 [11]. Both Fas^+ and Fas^- L1210 transfectants were stained with anti-FasL antibodies to ensure that these cell lines lacked FasL and to determine the specificity of the anti-

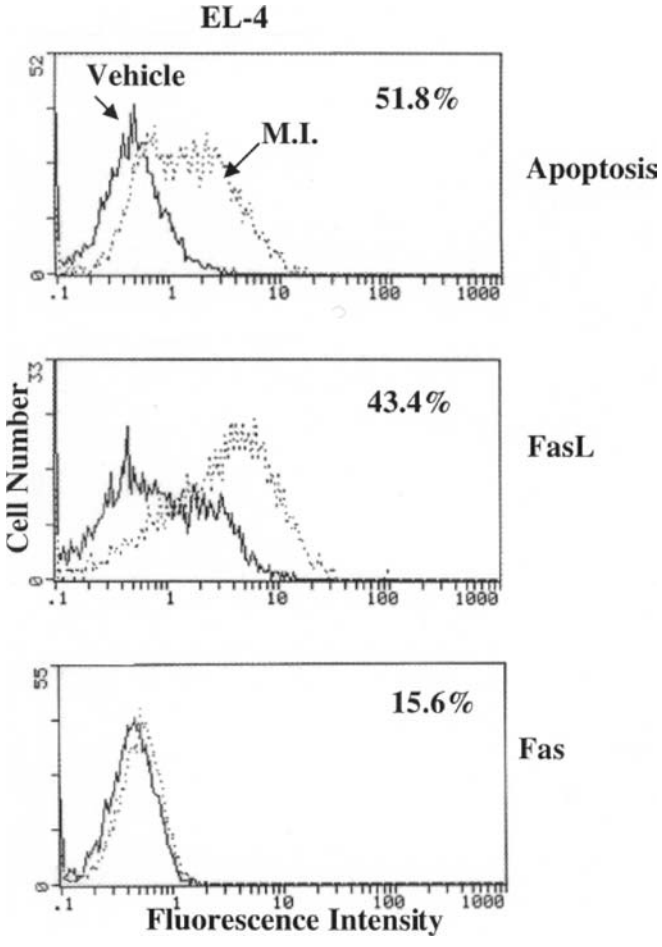

Fig. 6 Effect of MP inhibitor (*M.I.*) on induction of apoptosis and expression of Fas and FasL in EL-4 tumor cells. This experiment was carried out as described in Fig. 4 except that EL-4 tumor cells were used

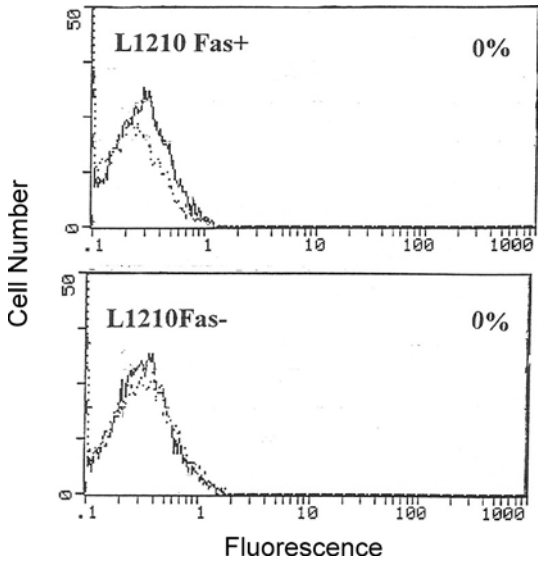

Fig. 7 L1210 tumor cells fail to express FasL. L1210 Fas^+ and Fas^- tumor cells were stained with antibodies against FasL. *Broken histograms* represent cells treated with anti-mouse FasL antibodies followed by biotin-conjugated anti-mouse IgG and PE-streptavidin and the *bold histograms* represent cells treated with biotin-conjugated anti-mouse IgG and PE-streptavidin

FasL antibodies used in the study. As shown in Fig. 7, L1210 cell lines failed to express FasL. Next, we tested the effect of TAM or MP inhibitor on apoptosis induction in L1210 cells. The L1210 cell lines were resistant to apoptosis induction (Fig. 8). These results demonstrate that the anticancer drugs used in this study were not effective against a tumor cell line that was deficient in FasL.

Discussion

TAM is a nonsteroidal anticancer agent used in the treatment of breast cancer [5]. Clinical evidence has shown that a small percentage of patients do not respond to TAM. This may be due to the estrogen antagonistic and agonistic effect of TAM in vivo. Furthermore, it has also been demonstrated that TAM-resistant clones develop during treatment of breast cancer patients [5]. The exact mechanism by which TAM treatment leads to drug resistance or inability to cure cancer is not clear. Recent studies have demonstrated that tumor cells expressing FasL can kill Fas-bearing immune cells of the host [3, 4]. Upon activation, T cells have been shown to express increased levels of FasL and Fas. It has been shown that FasL can act as an autocrine factor inducing cell death in activated T cells [12]. Such a suicide by the T cells is called activation-induced cell death. In the current study, it was noted that TAM increased the expression of FasL. Thus, FasL may act in an autocrine fashion to kill the tumor cells that express Fas. This may be one of the mechanisms used by TAM to induce apoptosis in tumor cells that express both Fas and FasL. In this context it was interesting to note that tumor cells that lacked FasL (L1210) failed to exhibit susceptibility to killing by TAM. However, if the tumor cells were to express only FasL but not Fas, the upregulation of FasL induced by TAM may allow the tumor cells to be more metastatic by enhancing their ability to kill immune cells of the host and thereby spread rapidly. This may be one of the factors involved in the lack of effectiveness of TAM in certain patients.

It has also been shown that TAM may facilitate the growth of certain types of tumors such as endometrial cancer [6]. Recently it has been demonstrated that TAM inhibits the growth of human cholangiocarcinoma cells in culture by inducing apoptosis [13]. It was observed that TAM induces apoptosis in only Fas^+ but not Fas^- tumor cells. Also, TAM has been shown to act independently of the antiestrogenic mechanism because the tumor cell line used failed to express ERs [13]. The current study supports this observation by demonstrating, in a murine T-cell lymphoma model (EL-4), that TAM can induce apoptosis in an ER-negative tumor cell line. Furthermore, our studies demonstrate that the

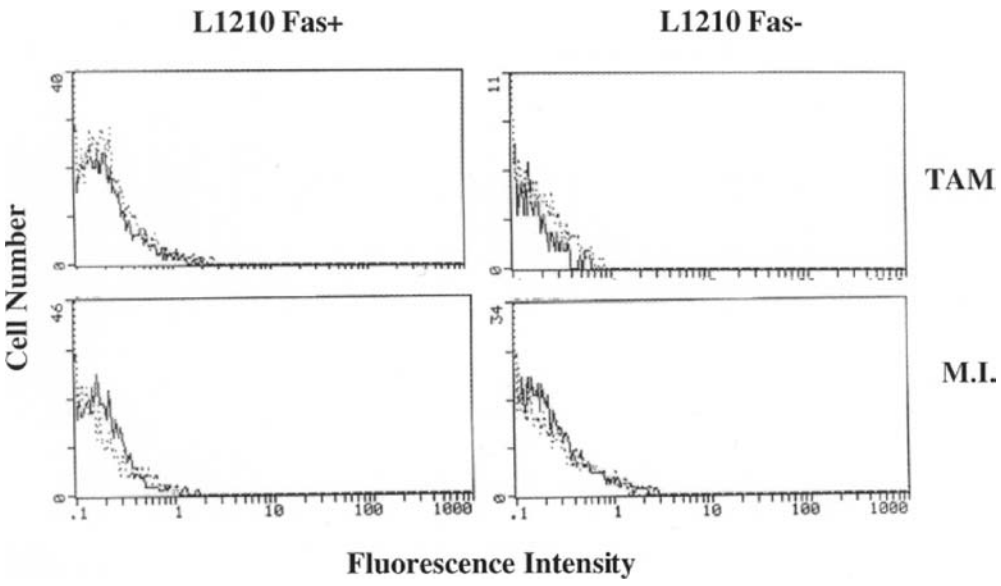

Fig. 8 Tamoxifen (*TAM*) and MP inhibitor (*M.I.*) fail to induce apoptosis in FasL$^-$ L1210 tumor cells. L1210 Fas$^+$ and Fas$^-$ tumor cells were cultured with TAM or MP inhibitor and the cells were analyzed for apoptosis. *Bold histograms* represent cells treated with the vehicle and *broken histograms* represent cells treated with TAM or MP inhibitor

reason why Fas$^+$ tumor cell lines may be sensitive to TAM could be because it upregulates the expression of FasL thereby triggering autocrine-mediated cell death. Recently Mor et al. [14] have demonstrated that TAM inhibits expression of FasL on MCF-7 and T-47D tumor cells. In these studies, however, the doses of TAM used were very low (10^{-6}–10^{-10}) compared to those used in our studies. Also, these authors found that TAM has no effect on FasL expression in Jar cells, a human choriocarcinoma cell line. Thus, the study concluded that the effect of TAM on human breast cancer cells is mediated through ER [14].

In contrast to the above studies, the doses of TAM used in our study were markedly higher, however, they are comparable to those used in other studies in which TAM was found to induce apoptosis in different cell types [13, 15, 16, 17]. Although TAM primarily acts through inhibition of ER, it is also known to act via non-ER-mediated mechanisms (for review, see reference 18). These include modulation of signaling proteins such as protein kinase C, calmodulin, transforming growth factor-beta, and the protooncogene c-*myc*. TAM-induced apoptotic signaling may involve caspases and mitogen-activated protein kinases, oxidative stress, mitochondrial permeability transition, and ceramide generation [18]. Thus, the mode of action of TAM may at least in part depend on the dose used [13]. The current study, as well as those by others [13], indicate that at higher doses, TAM can induce apoptosis involving Fas-FasL interactions independently of the ER.

Some tumor cells have been shown to express both Fas and FasL, including the T-47D and EL-4 tumor cell lines tested in the current study. However, it is not clear why this does not induce self-killing through autocrine or paracrine production of FasL. One of the mechanisms involved in failure to undergo apoptosis through autocrine or paracrine pathways includes the fact that FasL expressed on the membrane undergoes MP-mediated processing in the extracellular domain resulting in the release of soluble FasL [19]. Furthermore, the ability of soluble FasL to induce apoptosis is significantly less than that of membrane-bound FasL [8]. These findings suggest that tumor cells may produce MP to cleave the FasL into a less-toxic form such that they do not kill each other. In the current study, it was observed that MP inhibitor increased the expression of FasL on the tumor cells. Interestingly, MP inhibitor also caused increased apoptosis in T-47D and EL-4 cells. These results support the hypothesis that tumors escape self-killing by cleaving FasL. It is also interesting to note that MP inhibitor was not able to induce apoptosis in a tumor cell line that failed to express FasL (the L1210 cell line). MP inhibitors have recently been shown to inhibit tumor growth in an animal model [7]. Thus, further in vivo studies should provide useful information on the possible use of MP inhibitors or a combination of MP inhibitor and TAM in the treatment of cancer.

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