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# Regulation of Fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen

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**Abstract**

During neoplastic growth and metastasis, the immune system responds to the tumor by developing both cellular and humoral immune responses. In spite of this active response, tumor cells escape immune surveillance. We previously showed that FasL expression by breast tumor plays a central role in the induction of apoptosis of infiltrating Fas-immune cells providing the mechanism for tumor immune privilege. In the present study, we showed that FasL in breast tissue is functionally active, and estrogen and tamoxifen regulate its expression. We identified an estrogen recognizing element like-motif in the promoter region of the FasL gene, suggesting direct estrogen effects on FasL expression. This was confirmed by an increase in FasL expression in both RNA and protein levels in hormone sensitive breast cancer cells treated with estradiol. This effect is receptor mediated since tamoxifen blocked the estrogenic effect. Interestingly, tamoxifen also inhibited FasL expression in estrogen-depleted conditions. Moreover, an increase in FasL in breast cancer cells induces apoptosis in Fas bearing T cells and, tamoxifen blocks the induction of apoptosis. These studies provide evidence that tamoxifen inhibits FasL expression, allowing the killing of cancer cells by activated lymphocytes. This partially explains the protective effect of tamoxifen against breast cancer. 

*Keywords:* FasL; Estrogen; Tamoxifen; Breast cancer

## 1. Introduction

Tumor growth and metastatic spread are not random mechanical phenomena. They are regulated by the interaction between breast cells, stroma, immune cells and surrounding tissue. This interaction is mediated in part by steroid hormones, growth factors and various cytokines that influence the behavior and phenotypic expression of breast cells [1].

Estrogen and progesterone have major roles in the normal physiology of the breast by regulating cell proliferation [2–4]. In addition, estrogens have been shown to influence the breast by regulating production of locally acting hormones, growth factors and cytokines [5]. The latter represent a system of signals that organize and coordinate cellular proliferation, migration and the interaction with other cell types such as the immune system [6]. At basal physiological levels, these factors provide a homeostatic environment, but at elevated levels, the balance shifts toward rapid cell division and transformation that can lead to neoplastic proliferation. In addition, growth factors and hormones acting through paracrine and autocrine mechanisms play important roles in other aspects of neoplastic transformation [7,8]. This report addresses these local actions in breast cancer and how they affect the 'escape' of transforming or neoplastic cells from immune surveillance. The 'immune escape' which contributes to successful tumor growth and metastasis may be due to the inability of the immune system to react normally to reject the tumor. This could be a consequence of non-recognition or non-reactivity of tumor antigens, in-

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duced by anergy, tolerance or immunosuppression [9–11]. We and others have shown that the escape of tumor cells from immune surveillance is an active process and is mediated by the Fas–FasL system [12–14].

The Fas–Fas ligand (Fas–FasL) system is a primary mechanism for the induction of apoptosis in cells and tissues [15]. The Fas–FasL interaction is the mechanism for peripheral clonal deletion and control of T-cell expansion during immune responses and for killing by cytotoxic T-cells [16]. Fas, also called APO-1 or CD95, is a type I membrane protein of 45 kDa that belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family [15]. FasL, a type II membrane protein of 37 kDa, also belongs to the TNF superfamily [15,17]. Fas is normally expressed in various tissues such as thymus, liver, heart and kidney [18], and its expression by T and B cells is enhanced after lymphocyte activation. In contrast, FasL expression was reported originally to be restricted to activated T cells [15,17].

More recently, FasL expression has been reported in non-immune cells, mainly in cells from immune-privileged tissues, suggesting that the Fas–FasL system may play an important role in the mechanism underlying immune privileged status [19]. These data suggest that FasL expression by tumor cells may contribute to creating an immune privileged site and immunosuppression. Thus, FasL expression has been detected in stromal cells of the retina, Sertoli cells in the testis [20,21] and in the human placenta, mediating trophoblast invasion/proliferation [12,22]. This appears to be true for cancer as well. FasL has been shown to be expressed in melanomas [23], myeloma [24], colon cancer [25], choriocarcinoma [12] and breast cancer [26]. Furthermore, it has been shown that cancer cells induce apoptosis in Fas-sensitive, but not in Fas-insensitive lymphoma cells [27]. Despite all this information, the factors regulating FasL expression on cancer cells, including those in breast cancer, have not been elucidated.

Currently the main indication for the use of anti-estrogens in mammary carcinoma arises from the observation that estrogen is a mitogen in breast cancer and approximately one-third of patients will respond to endocrine therapy. The main therapeutic anti-estrogen that has been used is tamoxifen. Since estrogen is known to regulate many aspects of the immune response, including the production and secretion of cytokines [28,29], and the Fas–FasL system could be an important mechanism for the anti tumor effect of tamoxifen, we tested the hypothesis that, estrogen regulates FasL expression in breast tissue and that tamoxifen acting as an anti-estrogen down regulates FasL, preventing tumor escape from immune surveillance. We used an in vitro system to show by reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot analysis and RNase protection assay that the expression of FasL in hormone sensitive breast cancer cells is regulated by estrogen. Furthermore, we show that FasL present in the cancer cells is active since it induces apoptosis in Fas positive T cells but not in Fas resistant immune cells. In demonstrating that tamoxifen inhibits FasL expression, we found that this occurred independent of the presence or absence of estrogen.

## 2. Material and methods

### 2.1. Chemicals

Dulbeccos Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Life Technologies (Grand Island, NY). 17-β estradiol and tamoxifen were purchased from Sigma (St. Louis, MO). The tamoxifen was proven pure by high performance liquid chromatography (HPLC).

### 2.2. Cell culture

The human breast cancer cell line MCF-7 and the ductal breast carcinoma cell line T47D, were purchased from ATCC (Rockville, MD), and cultured in DMEM media containing antibiotics-antimycotics (1% vol/vol) and fetal bovine serum (10% vol/vol) at 37°C in a humidified chamber (5% $CO_2$ in air). Cells were passed by standard methods of trypsinization, plated in six-well dishes and allowed to replicate to 80% confluence. Afterward cell cultures were treated in 'estrogen-depleted conditions' consisting of serum-free and phenol red-free DMEM-F12 media for 24 h before the treatment with sex hormones/anti hormones was initiated.

### 2.3. Preparation of total RNA and protein samples for Western blot analysis

Total RNA and protein were prepared from MCF-7 and T47D cells using TRIzol® reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. The TRIzol® method allowed us to extract RNA and protein from the same cells. This is an advantage since we were able to study the same samples at both the mRNA and protein level.

### 2.4. RT-PCR analysis

RT-PCR was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, NJ) according to the manufacturer's directions. cDNA synthesis was performed with $pd(N)_6$ 0.2 μg and 5 μg total RNA. The primers used for amplification of FasL have previously been described [27] and have the following sequence: upstream, 5′-ATAGGATCCATGTTTCTGCTCTTC-

CACCTACAGAAGGA-3″; downstream, 5′-ATAGAATTCTGACCAAGAGAGAGCTCAGATACGTTGAC-3″. Each PCR cycle consisted of denaturation at 95°C, 30 s; annealing at 52°C, 30 s; and elongation at 72°C, 1 min, for a total of 35 cycles. The PCR products were analyzed in TBE 1.2% agarose gel with ethidium bromide.

The FasL signal was measured by a densitometer and standardized against the actin signal using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA). The linearity of the system was determined using serial dilutions of cDNA and the regression of dilution factor on amplified cDNA was linear ($y = 2881.125x - 785.75$) and the correlation coefficient was $r = 0.994$ (Fig. 1A).

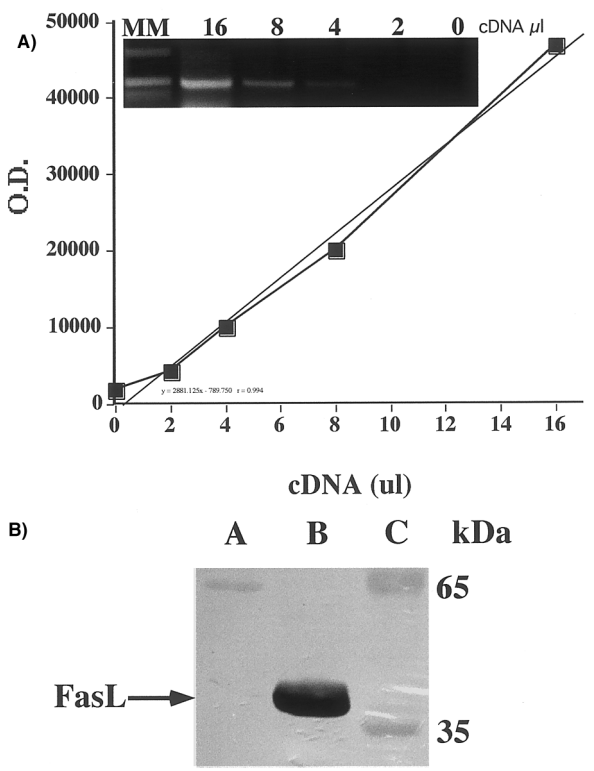


Fig. 1. A: RT-PCR linearity. The linearity of the PCR system was determined using serial dilutions of cDNA. The FasL signal (inset) obtained after RT-PCR was densitometered and standardized against the beta-actin signal using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA). The insert is a representative gel of the RT-PCR. Values are arbitrary units provided by the computer software according to a gray scale. B: Specificity of the monoclonal antibody for FasL, clone 33. Western blot analysis of Jurkat cell lysates was probed with the monoclonal antibody for FasL (clone 33, Transduction Technologies). Clone 33 recognize a 37-kDA protein in PMA (10 ng/ml) stimulated Jurkat cells (lane B) but not in the unstimulated Jurkat cells (Lane A). C = molecular markers.

### 2.5. Western blot analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immuno-blotting was performed after blocking non-specific binding by the membranes with 5%-powdered milk. The blots were incubated first with the primary antibody (FasL monoclonal antibody, clone 33, Transduction Laboratories, Lexington, KY at 1:1000 dilution) for 1 h. After washing, the membranes were incubated with the second antibody, peroxidase labeled horse anti-mouse gamma globulin (Vector, Burlingame, CA) for another hour. Finally, the blots were developed with TMB Peroxidase substrate kit (Vector, Burlingame, CA). The specificity of clone 33 for FasL was previously demonstrated [30]. Here we further confirmed its specificity by using Jurkat cells stimulated with concavalin A (Fig. 1B). Jurkat cells were previously shown to produce FasL upon concavalin A or anti-CD3 stimulation [31].

### 2.6. RNase protection assay (RPA)

The synthesis and labeling of the templates was done using the RiboQuant system (PharMingen, San Diego, CA) according to the manufacturer's instructions. In brief the Multiprobe, hAPO-3, which contains templates for FasL, Fas and the housekeeping genes L-32 and GAPDH was labeled with [$-^{32}$P]UTP using T7 RNA polymerase. Ten micrograms of total RNA was hybridize for 16 h at 56°C. mRNA probe hybrids were treated with RNase + Proteinase K and extracted with phenol-chloroform. Protected hybrids were resolved on a 5% acrylamide/bis gel, dried under vacuum and exposed to Kodak film for 24–72 h at −70°C. Densitometry was performed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA).

### 2.7. Co-culture DNA fragmentation assay (the JAM test)

Target Jurkat cell (Fas-positive) death resulting from the co-culture with effector MCF-7 tumor cells (FasL-positive) was quantified by measurement of target cell DNA fragmentation using the JAM assay [32]. Adherent MCF-7 breast cancer cells were seeded into the wells of a flat 96-well microtiter plate at a cell number appropriate to give the required E/T ratios. Target Jurkat cells' DNA was labeled by prior incubation with 10 Ci/ml of [$^3$H]TdR at 37°C for 24 h. Labeled Jurkat cells were washed and added to the seeded effector cells in a final volume of 200 µl/well. After co-culture at 37°C for 8–24 h, the cells were removed from the wells and filtrated onto glass fiber filters using an automatic

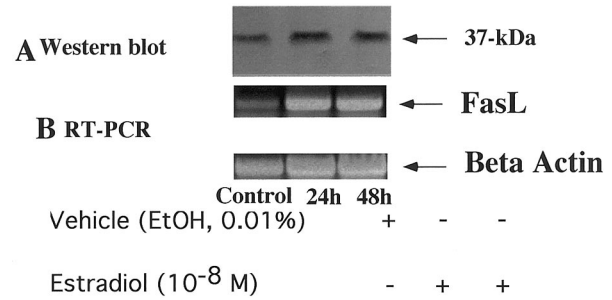


Fig. 2. Effect of estrogen on FasL expression. MCF-7 cells grown in phenol free DMEM and serum free conditions for 24 h were treated with 17-β-estradiol ($10^{-8}$ M) for 24 and 48 h. Control cells received 0.01% Ethanol in the same phenol and serum free media. A: Western blot analysis. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with FasL monoclonal antibody (clone 33). The secondary antibody (peroxidase labeled horse anti-mouse) was developed with TMB Peroxidase substrate kit. The FasL signal was standardized to the amount of protein loaded by staining the membrane with Ponceau Red and analyzed with a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA). The figure is representative of three independent experiments. B: RT-PCR for FasL was performed with total RNA extracted from the same in vitro monolayer cultures of MCF-7 cells as described for the Western blot. β-Actin housekeeping gene was amplified to verify that the same amount of cDNA was loaded in each lane. Each experiment was performed at least three times.

96-well filtration unit. The cells were then lysed with hypotonic buffer and their DNA was washed through the filter by four washes with D/D water. The radioactivity of intact chromosomal DNA retained on each filter was measured by liquid scintillation counting. Specific cell killing was calculated using the following equation according to Matzinger [32]:

$$\%\ \text{Specific Killing} = (S - E/S)*100$$

where $E$ (experimental) is cpm of retained (complete) Jurkat cell DNA in the presence of MCF7 effector cells and $S$ (spontaneous) is cpm of retained DNA in the absence of effector cells.

## 3. Results

### 3.1. Effect of estrogen on FasL expression

Previously, we showed that breast tumor cells express FasL and induce apoptosis of immune cells. This allows the tumor cells to escape from immune surveillance [26]. To examine the role of estrogen in the immune-surveillance mechanism, we used the human breast cancer cell lines MCF-7 and T47D, and monitored FasL mRNA and protein expression by RT-PCR, RPA and Western blot analysis. Breast cancer cells were treated with 17-β-estradiol ($10^{-8}$ M) for 24 and 48 h and the relative levels of FasL mRNA were quantified. Fig. 2B shows a representative gel for analysis of PCR products. We found that the treatment of MCF-7 or T47D cells with estradiol resulted in an increase in FasL mRNA levels at 24 and 48 h (Fig. 2B). A similar effect was found at the protein level: Western blot analysis tested with a monoclonal (clone 33) or a polyclonal antibody (clone N-20) for FasL showed an increase in the expression of FasL following treatment with estradiol (Fig. 2A).

### 3.2. Estrogen modulation of FasL expression in human breast cancer cells

To further characterize the observed effect of estrogen on FasL expression, MCF-7 cells were treated with 17-β-estradiol ($10^{-8}$ M) for time intervals of 0, 3, 6, 18 and 24 h after which the experiment was terminated. Effects of estrogen treatment on FasL mRNA expression were detected by RPA and RT-PCR. This effect presented a biphasic pattern, showing a strong band as early as 3 h of incubation and diminishes thereafter. A secondary increase in the FasL band is seen at 24 h (Fig. 3). Quantitative densitometry of the gels shows maximal FasL mRNA levels after 3 h of estrogen stimulation, followed by a 40% decrease from 6 to 12 h, with a definite but smaller increase at 24 h. At the protein level, increase of FasL expression was present only after 24 h of incubation with estradiol, remaining high up to 48 h (Fig. 2A and Fig. 3). Similar results were found with T47D cells (data not shown).

### 3.3. Effect of tamoxifen on estrogen-stimulated FasL expression

To determine if the increase of FasL expression was indeed estrogen receptor-mediated, we examined the effect of tamoxifen on estradiol-treated MCF-7 and T47D cells. As expected, and as shown in Fig. 4, the addition of tamoxifen ($10^{-7}$ M) inhibited the stimulatory effect of estrogen, both at the protein (Fig. 4A) and at the mRNA level (Fig. 4B). This supports the idea that estrogen's increase of FasL expression is a receptor-mediated action. However, when both cancer cells were treated with tamoxifen alone we found inhibition of FasL mRNA and protein expression, suggesting an estrogen-independent effect of tamoxifen (Fig. 4 lane 3).

### 3.4. Inhibitory effect of tamoxifen on FasL expression

We further characterized the inhibitory effect of tamoxifen using RT-PCR and Western blot analysis. MCF-7 cells were incubated with increasing concentrations of tamoxifen for 24 h. RNA was analyzed by RT-PCR. As shown in Fig. 5, treatment with tamoxifen inhibited FasL mRNA expression in a dose-dependent

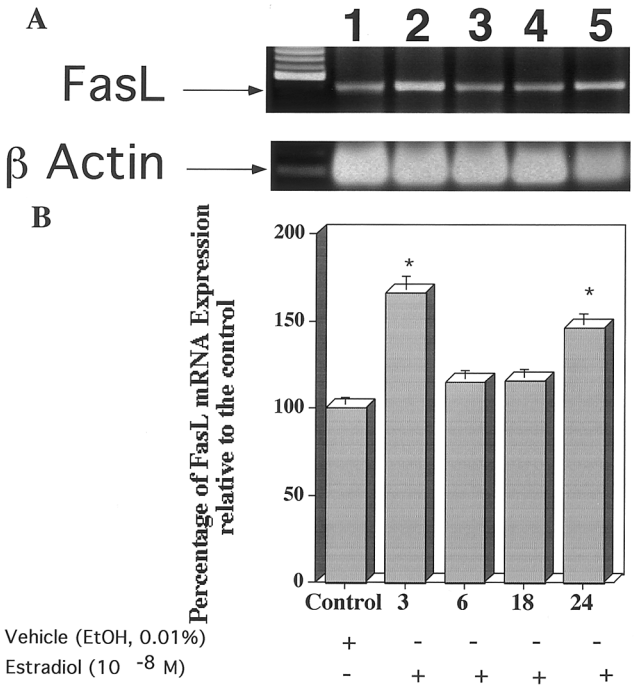


Fig. 3. Effect of estrogen on FasL mRNA expression, time response. MCF-7 cells were treated with 17-β-estradiol ($10^{-8}$ M) for 3, 6, 18 and 24 h. RPA assay and RT-PCR were performed using 5 μg total RNA. The intensity of the products was calculated using a digital imaging and analysis system as described in Section 2. A: Representative PCR gel stained with ethidium bromide. Each experiment was repeated at least three times. B: Quantification of the FasL signal. Estrogen increases FasL mRNA expression after 3 h (lane 2), followed by a decline (lanes 3 and 4) and increases again after 24 h (line 5). $^{*}P < 0.01$ control vs. treated cells. The intensity of the products is given in the $y$-axis as the percentage expression relative to the control. Beta actin expression was used as internal control for each individual sample. Error bars represent standard error of the mean (S.E.M.). Statistical significance was determined by ANOVA.

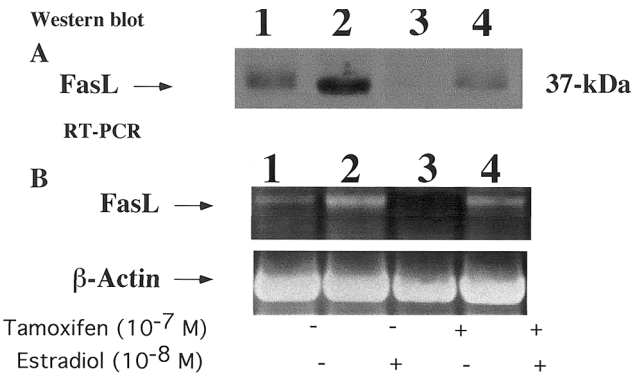


Fig. 4. Effect of estrogen and tamoxifen on FasL mRNA expression. MCF 7 or T47D cells were treated with estradiol ($10^{-8}$ M), tamoxifen ($10^{-7}$ M) or estrogen plus tamoxifen. Twenty micrograms of protein from each sample was subjected to 10% SDS-PAGE and transferred to nitrocellulose. Immuno-blot analysis was performed as described in Section 2. A: Representative Western blot for T47D cells. As shown in Fig. 2A for MCF-7 cells, estrogen also increases the expression of FasL in T47D cells. The administration of tamoxifen to the culture blocked the stimulatory effect of estrogen (Lane 4) and inhibited FasL expression when administrated in estrogen depleted conditions (Lane 3). B: Representative RT-PCR of T47D cell treated with estrogen and tamoxifen. Five micrograms of total RNA from the same samples described in A were reverse transcribed followed by PCR with specific primers for FasL and beta actin. The products were separated by electrophoresis in a 1.5-% agarose gel and stained with Ethidium bromide. The same effect of estrogen and tamoxifen described at the protein level was also found at the mRNA level.

manner (Fig. 5A). Quantification of the signal with a desitometer, shows significant decrease, 53% ($P > 0.001$; $n$: 5) and 44% ($P > 0.001$; $n$: 5) at concentrations of tamoxifen of $10^{-6}$ and $10^{-8}$ M, respectively. Similarly, using Western blots we found a decrease at the protein level (Fig. 4 and data not shown). When we treated cells with tamoxifen for different periods of time, and tested for FasL mRNA expression, we found a 20% decrease in the signal as early as 3 h of incubation reaching a peak of inhibition at 12 and 24 h (Fig. 6).

### 3.5. RPA for FasL mRNA

We further tested the direct effect of tamoxifen on FasL mRNA expression by RPA using a series of apoptosis gene templates, each of distinct length and each representing a sequence in a distinct mRNA species. The advantages of the multiple probe-RPA approach are its sensitivity and its capacity to simultaneously quantify several mRNA species in a

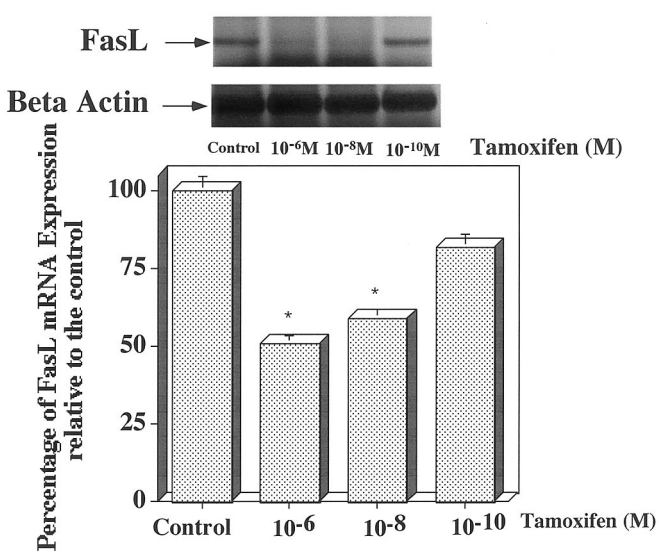


Fig. 5. Effect of tamoxifen on FasL mRNA expression. MCF-7 cells were treated with tamoxifen at concentrations of $10^{-6}$, $10^{-8}$, and $10^{-10}$ M for 24 h. Total RNA was analyzed for FasL expression by RT-PCR as described before. A: Representative gel analysis from MCF-7 cells treated with tamoxifen at different doses. B: The intensity of the signal was analyzed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA) and standardized to the beta-actin signal. The $y$-axis represents the relative percentage mRNA expression relative to the control. Error bars represent standard error of the mean (S.E.M.). Statistical significance was determined by ANOVA. $^{*}P > 0.001$.

single sample of total RNA. Thus, MCF-7 cells were treated with different concentrations of tamoxifen for 24 h. Total RNA was isolated and analyzed by RPA. As shown in Fig. 7, tamoxifen inhibited FasL mRNA in a dose-dependent manner, but had no effect on Fas associated death domain (FADD) and death receptor 3 (DR3) mRNA expression. It is worth to note that tamoxifen at the concentration of $10^{-8}$ M had some

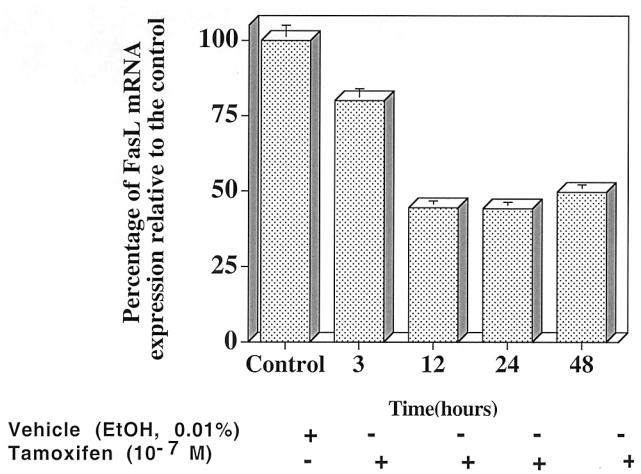


Fig. 6. Time-response effect of Tamoxifen on FasL mRNA expression in human breast cancer cell lines. The breast cancer cell lines MCF-7 and T47D cells were treated for 3, 12, 24 and 48 h with tamoxifen at a concentration of $10^{-7}$ M. Total RNA was extracted and RT-PCR was performed to study FasL expression. The intensity of the signal was analyzed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA) and standardized to the beta-actin signal. The $y$-axis represents the relative percentage mRNA expression relative to the control. Error bars represent standard error of the mean (S.E.M.). Statistical significance was determined by ANOVA. $^{*}P > 0.001$. Each experiment was repeated at least three times for each cell line.

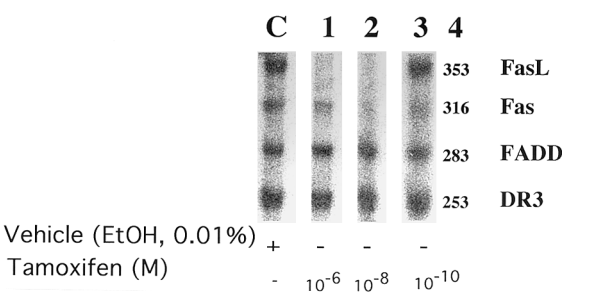


Fig. 7. RNase Protection assay (RPA). In order to confirm the specificity of the tamoxifen effect on FasL expression, similar samples as described in Fig. 5 were analyzed by RPA. MCF-7 and T47D cell cultures were treated with tamoxifen at concentration of $10^{-6}$ M (Lane 1), $10^{-8}$ M (Lane 2) and $10^{-10}$ M (Lane 3) for 24 h. The figure shows a representative RPA assay performed using 10 μg total RNA of MCF-7 cells. Note the inhibitory effect of tamoxifen on FasL mRNA expression at concentrations of $10^{-6}$ (Lane 1) and $10^{-8}$ (lane 2) but not on Fas, FDDA and DR3 mRNA. Lane 4 indicates the size of the protected product. Control (C) = MCF-7 cells without any treatment.

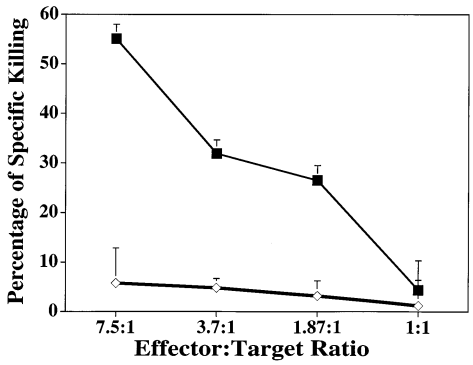


Fig. 8. MCF-7-induction of apoptosis in Fas-bearing cells. [$^{3}$H]Thymidine labeled target cells were co-cultured with MCF-7 cells at different E:T ratios for 24 h. Induction of apoptosis was quantified using the JAM assay. Co-culture of untreated MCF-7 cells. Co-culture of MCF-7 cells-tamoxifen-treated ($10^{-7}$ M) and Jurkat cells for 24 h.

inhibitory effect on Fas mRNA expression by MCF-7 cells, however, this effect was not consistent and was not statistical significant.

### *3.5.1. Fas-positive Jurkat cells are killed by MCF-7 cells*

In order to ascertain whether the FasL present on the breast cancer cells is functional we established a co-culture system in which MCF-7 cells attached to 96-well plates were incubated with labeled Fas-positive Jurkat cells. As shown in Fig. 8, 55% of Jurkat cells underwent apoptosis after 24 h of incubation with MCF-7 cells at a ratio of 7.5:1. Cell killing was proportionate to the effector:target ratio (E:T) and statistically significant killing (20%) occurred even when there was only a ~2:1 MCF-7/Jurkat cell ratio. No apoptosis was induced in Fas-resistant Ramos cells (data not shown).

### *3.5.2. Tamoxifen treatment protects Jurkat cells from being killed by breast cancer cells*

To test our hypothesis that tamoxifen inhibits FasL expression, and therefore blocks the protective effect of FasL in tumor cells, we treated MCF-7 cells with tamoxifen ($1 \times 10^{-7}$ M for 24 h) prior and during co-culturing them with labeled (Fas-positive) Jurkat cells. The JAM assay showed that treatment with tamoxifen protected Jurkat cells from killing by the MCF-7 cells (Fig. 8).

### *3.5.3. Tamoxifen protective effect is ER dependent*

To confirm that the effect of tamoxifen on FasL expression is ER-dependent we carried out similar experiments as with MCF-7 cells but instead used the Jar choriocarcinoma cell line. Jar cells express FasL but are negative for estrogen receptors. Under these circumstances, tamoxifen did not have any effect on FasL expression and did not inhibit the induction of apoptosis in Jurkat cells (Fig. 9).

### 3.6. Presence of an estrogen responsive elements-like motif (ERE) in the promoter region of the FasL gene

The molecular basis for selective transcriptional activation by estrogen is the result of the estrogen-receptor complex interacting with specific nucleotide sequences termed estrogen responsive elements (ERE). To determine if the effect of estrogen on FasL expression could be mediated by the classical ER-ERE pathway, we looked for the presence of ERE at the FasL gene using a computerized gene homology program from the National Institutes of Health. Motifs resembling the consensus ERE were found only at the promoter region of the FasL gene located at nucleotides 543–552 (Table 1). The FasL ERE consist of two palindromic arms separated by 3-bp. One of the arms GGTCA has perfect homology to the canonical ERE while the second arm has two mismatches (bold). A second pathway for transcriptional regulation by the estrogen receptors αand β is through the AP-1 enhancer element. Accordingly, a complete AP-1 sequence TTAGTCAG, was identified at nucleotides 234–241 of the FasL promoter region.

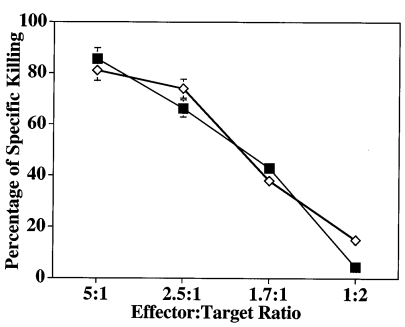


Fig. 9. Induction of apoptosis in Jurkat cells by FasL expressing Jar cells. [$^3$H]Thymidine labeled Jurkat target cells were co-cultured with choriocarcinoma cell line, Jar cells at different E:T ratios for 24 h. Induction of apoptosis was quantified using the JAM assay. No effect on Fas-positive induced apoptosis was found after pre-treatment of Jar cells with tamoxifen ($10^{-7}$ M). Co-culture of untreated Jar cells and Jurkat cells. Co-culture of Jar cells treated with tamoxifen ($10^{-7}$ M) and Jurkat cells.

Table 1
Sequences matching the Xenopus vitellogenin A2 gene ERE and the human FasL promoter region gene by using a sequence analysis program[a]

| | |
|---|---|
| Vitellogenin | 5′ AGGTCANNNTGACC 3′ |
| Human FasL | 5′ AGGTCAGGGTAAAT 3′ |

[a] The sequence in the FasL gene consisted of two pentamers (underlined) with close homology to the canonical ERE. In both genes the two pentamer sequences are separated by three nucleotides. Mismatches are in bold.

## 4. Discussion

Numerous in vivo and in vitro studies have shown the induction of lymphocyte apoptosis by FasL-bearing tumor cells. These implicate the Fas/FasL system as a mechanism by which tumors escape immune surveillance [19,33]. In the present study, using two human breast tumor cell lines we demonstrated the regulation of FasL expression by estrogen and tamoxifen.

Until recently, T cells were thought to be the major source of active FasL molecules and its role was mainly related to the process of acquisition of peripheral self-tolerance [34]. Further studies revealed that in addition, cells of the testis, retina and trophoblast also express FasL resulting in the establishment of classical immune privileges sites [12,20,22,30,35]. More recently, T cell-derived neoplastic cells [27], ovarian carcinoma cells [36], neuroblastoma cells [37], choriocarcinoma [12] and breast tumor cells [26] have also been shown to express FasL.

These observations indicated that FasL-induced suppression of tumor-specific Fas-bearing T cells might also be one of the mechanisms by which neoplastic cells escape from immune surveillance. Recently, we described the presence of FasL in breast tumor cells as well as in apparently normal epithelium of human breast glands that are located in the same quadrant as the tumor; however, FasL was absent in normal tissue far away from the tumor. We therefore proposed that FasL expression might be associated with early changes in the processes of neoplastic transformation [26]. We now have evidence indicating that this may be due to local estrogen formation by migrating macrophage [38].

In the present study we analyzed FasL expression in the breast cancer cell lines MCF-7 and T47D cells treated with estrogen: 17-β-estradiol, at concentration of $1 \times 10^{-8}$ M induced a three-fold increase in FasL mRNA levels after 24 and 48 h (Fig. 2B). This effect extended to the protein level as shown by Western blot analysis (Fig. 2A). The effect of estrogen on FasL mRNA expression by both cell lines showed a biphasic pattern, with an early increase after 3 h of incubation followed by a decrease after 6 and 12 h and a second moderate increase at 24 h. Such biphasic effect has been reported for other estrogen regulated genes [39] and could be explained by the conformation of the ERE. The hormone-activated estrogen receptor complex binds to specific ERE located in the promoter region of estrogen-regulated genes. Therefore, we looked for the presence of ERE-like motifs (GGTCANNNTGACC) in the human FasL gene using the NIH Entrez computer program. Indeed, an ERE-like motif was found in the promoter region of the FasL gene (nucleotides 543–552) [40], having the characteristic of the ERE, that is a 13-bp palindromic element consisting two 5-bp arms separated by a 3-bp spacer (Table 1). The FasL

ERE has one arm of the palindromic element sequences with perfect nucleotide homology to the described ERE [41] and a second incomplete set (Table 1). The two arms of the palindrome are separated by the exact spacing (3 bp), which is essential for estrogen receptor action [42] (Table 1).

Structural and functional analysis of estrogen regulated genes have shown that most EREs are imperfectly palindromic and that these changes could be related to the affinity to bind the receptor and the efficiency to regulate gene transcription [43–45]. More over, these differences may have an effect on the sensitivity to partial agonist activities, i.e. tamoxifen and raloxifene. In other words, it is not only the promoter context but also the sequence of the binding site itself, which can allow distinction between receptor activated by agonist and that activated by antagonist [44].

The presence of an ERE in the promoter region of the FasL gene suggest that estrogen's effects described in the present study could be mediated by ER-ERE regulation. The functionality of these recognizing elements is being addressed.

In the rat uterus, it has been shown for example that the *fos* gene in the presence of continuous estrogenic stimuli after a first increase in transcription, the gene become refractory to the hormone. During this time, the concentration of transcriptionaly active ER-complexes increases within the first 2–3 h and then decreases, to a level that is approximately 1/4 of the peak level. Comparable phenomenon we can see with FasL expression. The decrease in FasL mRNA after 6 h of estrogen could be as a result of the dissociation of the weakly bound receptor from the FasL-ERE, with the consequent reduction of transcription of the gene. A similar observation was made with the human pS2 gene ERE, also imperfectly palindrome are less sensitive to the receptor in transcription experiments [43]. Our in vitro studies have shown that the FasL ERE, contrary to the canonical consensus palindromic ERE of the *Xenopus laevis* vitellogenin A2 gene requires about ten times more receptor (HEO) to activate transcription (unpublished data).

We then went on to demonstrate that this regulatory effect is ER-mediated by using the estrogen antagonist, tamoxifen which, at concentration of $10^{-7}$ M was able to block the estrogen-induced increase on FasL expression (Figs. 4 and 5).

Tamoxifen has been used as an anti-estrogen for treatment of hormone-dependent breast tumors and more recently as primary prophylaxis against breast cancer. The modulation of breast cancer proliferation by tamoxifen has been reported to be mediated mainly by its anti-estrogenic activity, which includes the decrease of c-erbB-2 and c-myc RNA levels, cellular production of factors such as TNF $\alpha$ and $\beta$, cyclin D and A, and CD36 [46,47].

All of this notwithstanding, we believe that the inhibitory effect of tamoxifen on FasL expression described in this study could also explain prophylactic actions of tamoxifen on the breast. That is, as pointed out above, the early expression of FasL in breast tissue undergoing neoplastic changes provides the growing cancer cell with a defense mechanism against immune surveillance. If FasL expression is inhibited, for example by tamoxifen, the apoptotic signal to immune cells responding to the presence of the tumor is blocked, allowing the immune system to remove the tumor cells. This scenario is supported by our co-culture experiments in which treatment of MCF-7 cells with tamoxifen inhibited the induction of apoptosis of Fas bearing T cells (Fig. 8) but not in Fas insensitive Ramos cells (data not shown). The observation that tamoxifen inhibits almost completely the induction of apoptosis in Fas bearing cells while decreases 30–50% the cell expression of FasL is intriguing and rather difficult to understand. It is possible that tamoxifen could have also effect in the transport of de novo synthesized FasL to the membrane and/or depleting the protein from the cytoplasm. Another explanation could be a direct effect of tamoxifen on a different apoptotic pathway such as p53 or c-myc [48].

The mechanism of action of tamoxifen in FasL expression seems to be mediated by ER but independent of the presence of estrogen. Jar cells, a human choriocarcinoma cell line, express high levels of FasL and induce apoptosis in activated immune cells [12]. However, using similar conditions as with MCF-7 cells, we find no effect of either estrogen or tamoxifen on FasL expression by Jar cells (Fig. 9). Thus since Jar cells are ER negative, we may conclude that the effect of estrogen and tamoxifen on human breast cancer cells is clearly ER mediated.

Of interest, when tamoxifen was added to T47D or MCF-7 cells in estrogen depleted media, FasL expression was almost completely inhibited (Fig. 4, line 3). In repeating this study, we further characterize this effect and found it to be dose- and time-dependent. Thus, as early as 3 h after treatment, tamoxifen decreased FasL mRNA levels by 25%, in relation to the control (Fig. 6).

In explanation, a possible mechanism by which tamoxifen exerted its direct inhibitory effect on the FasL gene could involve the ER/AP-1 pathway. We have found the presence of a perfect AP-1 motif (TTAGTCAG) to be located at the 5′-flanking region of the human FasL gene (nucleotides 234–241). Fos–Jun heterodimers and Jun–Jun homodimers are the principal components of the AP-1 transcription factor family, which interact with genes containing AP-1 specific sequences at the promoter region. Several lines of evidences have suggested that nuclear receptors, such as the glucocorticoid receptor [49,50], retinoic acid recep-

tor [51,52] and ER [53–55], can modify the effect of Fos–Jun complex. Furthermore, it has been shown that most of the modifications by these receptors result in negative effects on the AP-1 enhancer activity [56], possible because interactions with overlapping binding sites or inhibition of the activity through direct protein–protein interaction [57]. More recently, Paech et al. [58] have reported that the tamoxifen-ER complex binds at the AP-1 site and constitutes an alternative ER-regulatory pathway.

In addition, the fact that the FasL ERE is an imperfect palendomic sequence could also explain the inhibitory effect of tamoxifen on FasL expression. As we pointed previously, the physiological distinction between receptor activated by agonist and that activated by antagonist it is not only the promoter context but in the sequence of the binding site itself [44].

In conclusion, it is important to consider that the progression of a tumor is not only dependent on its proliferative rate but also in how it interacts with other cells and systems of the body, specifically the immune system. In recent years a growing number of reports have made evident the relevant role played by immune cells and their products in the regulation of the microenvironment normal and tumor tissues [38]. Alterations in local cell kinetics are followed by activation of several factors, allowing further proliferation, and calling for immune rejection. However, the presence or administration of agents that prevent the expression immune-regulatory factors such as FasL may frustrate this.

This study provides new evidence for the interaction of sex hormones, cancer cells and the immune system. The recognition that these factors influence growth and dissemination of breast cancer will provide new targets for therapeutic and preventive intervention.

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