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Award Number: DAMD17-98-1-8359

TITLE: Macrophages, Estrogen and the Microenvironment in Breast
Cancer

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REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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DTIC QUALITY INSPECTED 4

20001019 034

REPORT DOCUMENTATION PAGE

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 98 - 31 Aug 99)	
4. TITLE AND SUBTITLE Macrophages, Estrogen and the Microenvironment in Breast Cancer			5. FUNDING NUMBERS DAMD17-98-1-8359
6. AUTHOR(S) Frederick Naftolin, M.D., Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Yale University New Haven, Connecticut 06520-8047 E-MAIL: frederick.naftolin@yale.edu			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (<i>Maximum 200 Words</i>) Experimental evidence suggests interaction between the endocrine and immune systems. Functional interactions are likely since immune cells produce hormones and neuropeptides, and endocrine glands can produce a variety of cytokines. In spite of this, the monocytes/macrophages that are normally present in reproductive organs, including the breast; have mainly been studied from an immunological point of view. We propose to expand this horizon with the novel hypothesis that differentiated local macrophages in breast tissue constitute an <i>in situ</i> source of estrogen acting in an autocrine or paracrine manner to regulate breast cell division and differentiation. The scope of this study is to evaluate immune and non-immune regulatory mechanism on breast cancer cells			
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 82
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

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MACROPHAGES, ESTROGEN AND THE MICROENVIRONMENT IN BREAST CANCER.

P.I. F. Naftolin, MD, PhD

Co-Investigator: Gil Mor, MD, Ph.D.

INTRODUCTION

Experimental evidence suggests interaction between the endocrine and immune systems. Functional interactions are likely since immune cells produce hormones and neuropeptides, and endocrine glands can produce a variety of cytokines. In spite of this, the monocytes/macrophages that are normally present in reproductive organs, including the breast; have mainly been studied from an immunological point of view. We propose to expand this horizon with the **novel hypothesis that differentiated local macrophages in breast tissue constitute an *in situ* source of estrogen acting in an autocrine or paracrine manner to regulate breast cell division and differentiation.** The scope of this study is to evaluate immune and non-immune regulatory mechanism on breast cancer cells

BODY

Macrophages, estrogen and the microenvironment of breast cancer

Estrogen is a breast promotor and has in many ways been associated with the incidence and cause of breast cancer. Estrogen sources include ovarian and extraglandular sites, and the breast tissue itself. In addition to the circulating estrogens, which source primarily maintain benign and breast cancer tissue estrogen concentrations remains unclear. While macrophages may comprise up to 50% of the mass of breast carcinomas, previous studies neglected to study them as possible sources of estrogen. We present new evidence that breast macrophages constitute an important *in situ* source of estradiol and that the amount produced is sufficient to mediate cellular proliferation via inducing growth factors and cytokines or by direct action. We utilized immunohistochemistry and RT-PCR to study cell-specific aromatase expression in: (i) 29 breast tissue biopsies, (ii) human monocytes/macrophages and (iii) a myeloid cell line (THP-1) capable of differentiating into macrophages. Use of a breast cancer cell line (MCF-7) provided biologic confirmation of the role of aromatization in cell proliferation. We demonstrated considerable amounts of immunoreactive-aromatase (irARO) in breast tissue macrophages and a positive correlation between the proportion of irARO present in macrophages and lesion severity. Using *in vitro* techniques, we demonstrated that monocytes and THP-1 cells require differentiation into macrophages to produce

aromatase in amounts approaching placental levels. Estrogen production by macrophages in breast tissue appears to be sufficient to stimulate the proliferation of adjacent epithelial cells and to autoregulate cytokine production. The amount of estrogen produced by THP-1 cells stimulated MCF-7 cells to proliferate, an effect blocked by aromatase inhibitors. These findings represent a new dimension of cellular regulation in breast tissue with major biologic implications that are amenable to pharmacological manipulation.

This work has been published in the journal of "Steroid Biochemistry and Molecular Biology" :67: 403-411, 1998 (See Appendix I)

The Fas/Fas ligand system: a mechanism for immune evasion in human breast carcinomas

Breast tumors are frequently associated with a predominantly lymphocytic infiltrate, which constitutes a cellular immune response against the tumor. In spite of this massive infiltrate, the immune response appears to be inefficient and the tumor is able to evade it. *We propose that in breast cancer, tumor escape from immunological surveillance results from the induction of apoptosis of Fas bearing activated lymphocytes by FasL bearing breast cancer cells.*

To test our proposal we studied the expression of FasL by human breast carcinomas and the MCF-7 breast cancer cell line by RT-PCR, immunohistochemistry

and Western blot analysis. Moreover, we describe the presence of apoptosis and Fas expression in the lymphocytic population surrounding the tumor. Strong FasL membranous and cytoplasmic staining was detected in ductal carcinomas and hyperplastic breast tissue, but it was absent from normal breast tissue. No staining was found in normal glands in the non-tumor quadrants, however the normal appearing ducts surrounding the carcinoma (tumor quadrant) showed intense immunoreactivity. Apoptosis was found predominantly among the lymphocytic population, as well as in the blood vessels and fibro-fatty tissue close to the tumor. Further characterization of apoptotic cells demonstrated that they were CD3+ cells.

Our results suggest that breast tumors may elude immunological surveillance by inducing, via the Fas/FasL system, the apoptosis of activated lymphocytes. [Recent data have demonstrated FasL RNA in other tumor types, which will be the subject of further scientific articles and funding applications]. Up-regulation of FasL expression in hyperplastic and normal breast ducts close to the tumor also suggests a possible role in early neoplastic transformation and proliferation

This work has been published in the journal "Breast Cancer Research and Treatment"

:54: 245-253, 1999 (See Appendix 2)

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

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 have identified an estrogen recognizing element like-motif in the promotor 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.

This work has been submitted for published in the journal Cancer Research, 1999 (See Appendix 3)

Macrophage derived growth factors modulate Fas ligand expression in cultured breast cancer cells.

Fas/Fas ligand (FasL) interactions play a significant role in the immune privileged status of certain cell populations. Several macrophage-derived cytokines and growth factors appear to modulate this system. When a FasL expressing cell binds a Fas-bearing immune cell, it triggers its death by apoptosis. During this project, we demonstrated that macrophage-conditioned media induced FasL expression by breast cancer cells in a dose-dependent manner. To elucidate which macrophage product was responsible for the up-regulation of FasL, MCF-7 cell cultures were treated with the macrophage products PDGF, TGF- β_1 , and bFGF (see below). The first two, known to be elevated in breast tissue from women with breast cancer, induced a dose-dependent up-regulation of FasL expression, which was specifically inhibited by specific antibodies to PDGF or TGF- β_1 . Interestingly, bFGF -which is not elevated in breast cancer- did not induce any response. These results suggest that the pro-inflammatory nature of breast microenvironment induces the FasL expression by glandular epithelial cells, and signals Fas-mediated cell death of activated immune cells. This could be a mechanism for cancer cells to escape immune surveillance, grow and metastasize.

The present work is in preparation for publication. We summarize the results obtained so far.

Results:

a. Effect of macrophage-conditioned media(CM) on FasL mRNA expression

To evaluate the effect of activated macrophage secreted soluble products on FasL expression, normal mouse breast epithelial cells HC-11 cells, which normally do not express FasL, were incubated with CM obtained from differentiated macrophages/THP-1 cells. FasL expression was analyzed after a 24-hour incubation using RT-PCR analysis. As shown in figure 1, CM induced a marked increase in FasL expression detected at both the mRNA and protein level.

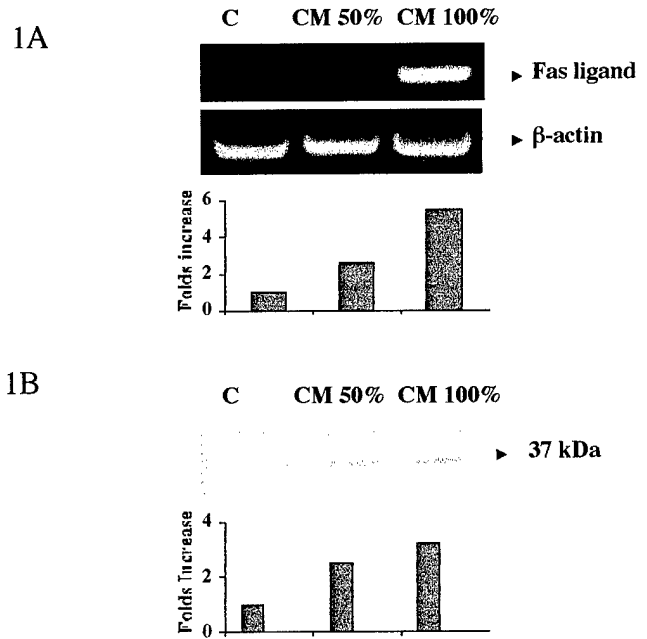


Fig 1 Effect of CM on normal epithelial breast cells. **1A**. RT-PCR. **1B** Western Blot analysis

b. PDGF, TGF-β₁, and bFGF modulation of FasL mRNA expression in breast cells

To characterize the macrophage-secreted soluble growth factors which could mediate the observed effect of CM on FasL expression, HC-11 cultures were treated with PDGF, TGF-β₁, and bFGF and the expression of FasL was analyzed both, at the RNA and protein levels.

- Time-course experiments:

MCF-7 cells were treated with PDGF (10 ng/ml), TGF-β₁ (1 ng/ml) and bFGF (10 ng/ml), and incubated for time intervals of 0, 3, 6, 12, and 24 hours, after which the

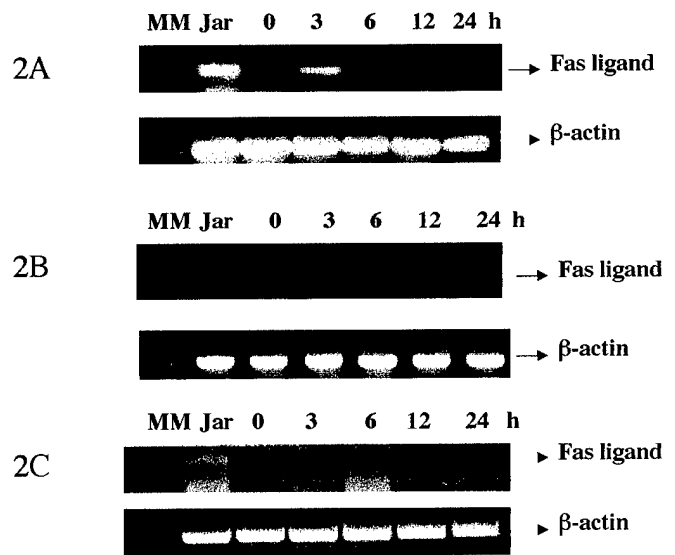


Fig. 2 Effect of cytokines on normal epithelial breast cells. **2A**. RT-PCR of cells treated with PDGF. **2B** RT-PCR of cells treated with TGF-β₁. **2C** RT-PCR of cells treated with bFGF

experiment was terminated. Treatment with PDGF or TGF- β_1 induced an early increase in the transcription of FasL observed at 3 hours, and which gradually decreased at 6 and 12 hours. Interestingly, no up-regulation of FasL expression was observed with bFGF, (Fig. 2).

c. Effect of CM, PDGF, and TGF β_1 on FasL expression at the protein level

The FasL protein expression was examined by Western blot analysis of whole cell lysates. As demonstrated in figure 3, FasL was present in cell lysates obtained from HC-11 cultures treated for 24 hours with either CM, PDGF or TGF- β_1 , confirming the previous mRNA findings.

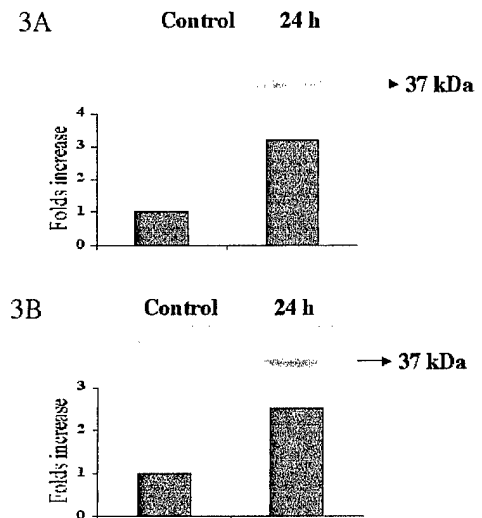


Fig 3. Effect of cytokines on normal epithelial breast cells. **3A.** WB analysis of cells treated with PDGF. **3B** Western Blot analysis of cells treated with TGF- β_1

KEY RESEARCH ACCOMPLISHMENT

We have shown, for the first time, the presence of an immune regulatory mechanism, the Fas/Fas Ligand system, present in human breast tissue. This system responds to factors produced by macrophages and is sensitive to the regulation by sex hormones, mainly estrogen.

RELEVANCE OF THE STUDY:

- Our results suggest the breast tumors may elude immunological surveillance by inducing via the Fas/FasL system, the apoptosis of activated lymphocytes.

- Macrophages present at the tumor site increase, through cytokines and estrogen, the expression of FasL in the tumor, and further contribute to the "immune escape" of the tumor.
- We have described the regulation of FasL expression by cancer cells by estrogen. Moreover, 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.

REPORTABLE OUTCOMES

Manuscripts:

- Mor, G., Yue, W., Santen, R.J., Gutierrez, L., Eliza, M., Bernstein, L., Harada, N., Wang, J., Lysiak, J., Diano, S., Naftolin, F. (1998). Macrophages, estrogen and the microenvironment of breast cancer. Journal of Steroid Biochemistry and Molecular Biology: 67: 403-411
- Diano, S.; Horvath, T.; Mor, G.; Register, T.; Adams, M.; Harada, N.; Naftolin, F. (1998) Aromatase and estrogen receptor immunoreactivity in the coronary arteries of monkeys and humans. Menopause: 6 (1) 21-28
- Gutierrez, L., Eliza, M., Niven-Fairchild, T., Naftolin, F., Mor, G. (1999). Fas/Fas-Ligand system induced apoptosis in human breast carcinoma: A mechanism for immune evasion. Breast Cancer Research and Treatment. 54 (3):245-253
- Mor, G., Kohen, F., Brown, S., Nilsen, J., Brown, W., Naftolin, F. (1999). Regulation of Fas Ligand expression in breast cancer cells by estrogen: Functional differences between estradiol and tamoxifen. Cancer Research: Submitted

Degrees Obtained:

Mariel Eliza (class 99), presented her thesis for the Medical degree based on this work and received Honors.

CONCLUSION

The findings summarized in this report represents a new dimension of cellular regulation in breast tissue with major biological implications, amenable to pharmacological manipulation. We have shown not only that immune cells are a source of sex hormones and growth factors affecting breast cancer cells' growth and proliferation but also we provide evidences that tumor cells employ immune regulatory mechanism to "escape" from immune surveillance.

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Appendixes

We provide three Appendixes.



PERGAMON

Macrophages, Estrogen and the Microenvironment of Breast Cancer

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Estrogen is a major mitogenic stimulus to established breast cancer. Estrogen sources include ovarian, extraglandular sites and breast tissue. Which source primarily maintains benign and breast cancer tissue estrogen concentrations remains unclear. While macrophages may comprise up to 50% of the mass of breast carcinomas, previous studies neglected to study them as possible sources of estrogen. We present evidence that breast macrophages constitute an *in situ* source of estradiol and that the amount produced is sufficient to mediate cellular proliferation. We utilized immunohistochemistry and RT-PCR to study cell-specific aromatase expression in (i) 29 breast biopsies, (ii) human monocytes/macrophages and (iii) a myeloid cell line (THP-1) capable of differentiating into macrophages. Use of a breast cancer cell line (MCF-7) provided biologic confirmation of the role of aromatization in cell proliferation. We demonstrated considerable amounts of immunoreactive-aromatase (irARO) in breast tissue macrophages and a positive correlation between the proportion of irARO present in macrophages and lesion severity. Using *in vitro* techniques, we demonstrated that monocytes and THP-1 cells require differentiation into macrophages to produce aromatase in amounts approaching placental levels. The amount of estrogen produced by THP-1 cells stimulated MCF-7 cells to proliferate, an effect blocked by aromatase inhibitors. Estrogen production by macrophages in breast tissue appears sufficient to stimulate the proliferation of adjacent epithelial cells and to autoregulate cytokine production. These findings represent a new dimension of cellular regulation in breast tissue with major biologic implications, amenable to pharmacological manipulation. © 1999 Elsevier Science Ltd. All rights reserved.

J. Steroid Biochem. Molec. Biol., Vol. 67, No. 5-6, pp. 403-411, 1998

INTRODUCTION

Complex interactions between epithelial, stromal, endothelial and lymphoid cell components influence breast tissue proliferation. These dynamic cell-cell interactions require a well-developed array of intercellular communication signals. So far, most attention has been directed to the stromal and epithelial components of the breast and their products, whereas the potential role(s) and relative importance of migratory white blood cell types have received relatively limited attention.

Macrophages are a major component of the normal breast's stroma and comprise a substantial cellular component of the cell mass of breast carcinomas [1,2]. Although they constitute a stable presence in normal breast tissue, a marked influx of macrophages from the circulation characterizes breast disease. For example, macrophages are found in large numbers during the first phase of tumor growth [3]. A salient feature of these cells is their ability to communicate with each other via a complex network of extracellular signals, including many cytokines and their soluble receptors. These macrophage factors are thought to be produced largely within the tumor and to act locally in an autocrine and/or paracrine manner [4]. Thus, contrary to their expected

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 Received 29 Jun. 1998; accepted 16 Sep. 1998.

cytotoxicity towards tumor cells, tumor associated macrophages (TAM) may promote tumor growth through their secretion of growth factors [2].

Despite the low estrogen plasma levels characteristic of menopause, breast tumors excised from postmenopausal women contain estradiol concentrations similar to those from premenopausal patients [5]. Increased uptake from the circulating plasma estradiol produced in extraglandular sites via aromatase provides one explanation for the maintenance of high tissue estradiol levels [6]. *In situ* estrogen synthesis from plasma androgens via local estrogen synthetase aromatase (ARO) provides a more plausible explanation. Previous data from others and us have demonstrated aromatase in both epithelial and stromal breast cells. The activity in isolated and cultured stromal cells from breast has been shown to respond to known enhancers of aromatase with up to a 30,000 fold stimulation of aromatase expression [7]. Furthermore, recent evidence indicates that breast tumors produce factors able to enhance the activities of the enzymes involved in estrogen synthesis, thereby promoting an (estrogenic) environment favorable for tumor growth [8]. Many of these factors are macrophage products [9–11] and their expression has been shown to be regulated by estrogen [12, 13]. Thus, macrophages could be a major source of tissue estrogen, for example, during the climacteric.

Our prior studies and those of others neglected to examine the macrophage cells in breast tissue [14, 15]. Consequently, in the present study we investigated the relationship between TAM and local estradiol production in the breast using a variety of immunohistochemical, biochemical, molecular and biologic methods. We found that these cells may not only be the target for estrogen action, but may constitute a local source of estrogen to furnish both autocrine and paracrine regulation of the breast microenvironment.

MATERIALS AND METHODS

Clinical material-human breast tissue specimens

Human breast tissue from biopsies of normal breast ($N = 8$), ductal hyperplasia ($N = 9$) and *in situ* breast carcinoma ($N = 12$) were obtained from the Department of Pathology, Yale University School of Medicine. A signed written consent was obtained from each patient and the use of tissues was approved by the Yale University Human Investigation Committee.

Cells and chemicals

THP-1 cell line was purchased from the American Type Culture Collection (Rockville, MD). MCF-7 cells were kindly provided by Dr Bruggemeier (Ohio State University, Columbus, OH). RPMI 1640 medium and FBS were purchased from Life Technologies

(Grand Island, NY). DMEM and penicillin-streptomycin solution were obtained from Gibco BRL (Gaithersburg, MD). Testosterone and estradiol were purchased from Steraloids (Wilton, NH). [$1\text{-}^3\text{H}$] Androstenedione (24.5 Ci/mmol) and [^3H] thymidine (20 Ci/mmol) were purchased from NEN Dupont (Boston, MA). Letrozole and ICI 182,780 were gifts from Novartis and ICI Pharmaceuticals, respectively. All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Immunocytochemistry

The peroxidase-antiperoxidase immunocytochemical method described by Naftolin *et al.* was used [14]. This technique employ the polyclonal antibody of Dr Harada directed against highly purified aromatase [16].

Double staining of breast cancer tissue sections for ARO and CD68 was carried out to determine the identity of ARO+ cells morphologically resembling macrophages. For this, slides were incubated with CD68 and ARO as described above, but using SAP and DAB substrate for color development of CD68 (brown color) and Alkaline Phosphatase substrate Blue Kit (Vector) for ARO.

Induction of differentiation of THP-1 cells

THP-1 cells were induced into macrophage-like phenotype by incubation with PMA at a concentration of 6 ng/ml.

Aromatase (tritiated water release) assay

Aromatase activity was measured using the tritiated water release assay as previously described by Lipton [17]. Aromatase activity was expressed as fmol of estrone produced/mg of protein/h. Cell monolayers or pellets were lysed with 0.1 N sodium hydroxide to determine their protein content. The protein concentration of the homogenate was measured as described by Lowry *et al.* [18].

Preparation of conditioned media

THP-1 cells were seeded in 6-well plates at the density of 1×10^6 cells/well in phenol red-free RPMI-1640 with 5% DCC-FBS. The culture media was supplemented with PMA (6 ng/ml) or PMA plus testosterone (10^{-8} M) with or without the aromatase inhibitor, letrozole (10^{-7} M). The cells were cultured at 37°C in a CO₂ incubator for 24 h. The media was then removed from the plates, centrifuged and stored at 4°C for <48 h. These media were designated as THP-1-conditioned media (CM). Nonconditioned media consisted of fresh phenol red-free RPMI-1640 with 5% DCC-FBS. Supplementation of the non-conditioned media was carried out as for the THP-1-CM.

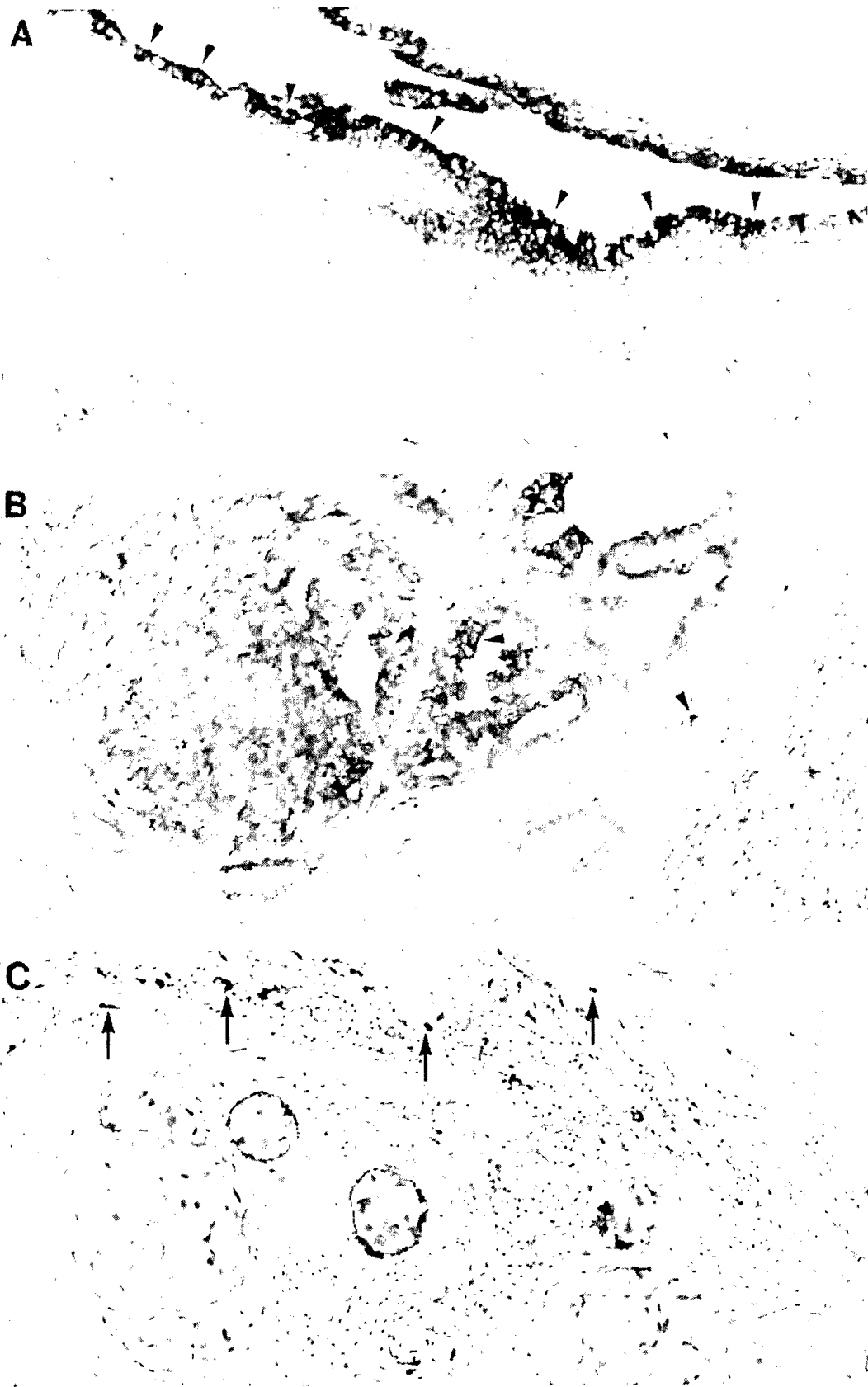


Fig. 1. Aromatase immunocytochemistry. (A) Section of fibrocystic breast tissue with normal ductal epithelium stained for irARO. Staining is localized to the breast ductal epithelium. irARO positive cells are absent from the stroma and fibrous tissue. Magnification, $\times 400$. (B) Breast tissue section showing atypical ductal hyperplasia stained for irARO. Staining is mainly localized to the breast ductal epithelium; note, however, the presence of several irARO positive cells in the stroma. Magnification, $\times 400$. (C) Section of an intraductal *in situ* breast carcinoma stained for irARO. Notice increased staining of the nonepithelial compartment of the lesion. Close inspection of the morphological characteristics of these irARO positive cells suggests they are macrophages (arrows, see Fig. 2). Magnification, $\times 400$.

[³H] Thymidine incorporation proliferation assay

MCF-7 cells were seeded in 12-well plates at a density of 0.15 million cells/well in regular DMEM and incubated for 18 h with the THP-1 CM or non-conditioned media, followed by a 2 h incubation with [³H] thymidine (1 μCi/well). DNA extraction was carried out using the phenol-chloroform procedure. Two aliquots were made from the DNA solution: one for spectrophotometric measurement and the other for scintillation counting. Incorporated [³H] thymidine was expressed as dpm/μg of DNA.

Human peripheral monocyte isolation/cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood (taken at random throughout the cycle) from healthy females (without exogenous hormone treatment) and male volunteer donors between the ages of 24–32. PBMC were separated by fractionation on Ficoll Lymphocyte Separation Media (specific gravity 1.077). The interface (containing lymphocytes and monocytes) was removed and washed in an equal volume of PBS by centrifugation for 10 min at 1500 rpm. The pellet was then resuspended in 80 ul of phosphate buffered saline (PBS) with 5 mM EDTA and 0.5% BSA per 10⁷ cells. A pure monocytic population was obtained as previously described [19] using the MiniMACS separation system from Miltenvi Biotec Inc. [20]. The positive fraction, containing the monocytes, was isolated for further studies.

RT-PCR and hybridization for ARO mRNA

Total RNA was prepared from frozen normal human placentas, breast tissue and from 2 × 10⁶ monocytes and macrophages, using TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Macrophages were differentiated in culture for 48–72 h prior to RNA extraction. RT-PCR was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, NJ) according to the manufacturer's protocol. cDNA synthesis was carried out with 0.2 μg of pd(N)₆ and 10 μg of total RNA. The primers used for amplification of ARO mRNA have been recently described [21]. The PCR product was then separated by electrophoresis and transferred to blotting membrane by capillary elution. Southern hybridization was performed with the ECL 3'-oligolabelling and detections system (Amersham, U.K.) according to the instructions of the manufacturer.

RESULTS

Immunohistochemical localization of ARO in normal and malignant breast tissue

We initially compared the degree of breast aromatase immunostaining in biopsies from normal breast,

ductal hyperplasia with accompanying fibrocystic disease and ductal *in situ* breast carcinoma. Localization of ARO in these breast tissues involved an affinity-purified antiserum antibody specific against ARO [16]. irARO was detected in stromal cells and tumor epithelial cells, as previously described [14]. The breast ductal epithelium exhibited variable staining for irARO; the intensity of irARO staining appeared to be inversely related to the degree of dysplasia, but this was not substantiated by formal morphometric analysis [Fig. 1(a)–(c)]. Conversely, staining of the nonepithelial compartment (stroma and fibrous tissue) was more intense in the more advanced lesions. irARO was also localized to the local inflammatory cells, more specifically to cells morphologically resembling macrophages [arrows, Fig. 1(c)]. To establish that these cells were macrophages, tissues were doubled-stained with a monoclonal antibody recognizing the tissue macrophage-specific antigen, CD68 and an antibody recognizing ARO. CD68 and ARO-positive cells were localized to the local inflammatory cells around and within the tumor [Fig. 2(a) and (b)]. Colocalization of ARO and CD68 immunoreactivity identified the cells expressing irARO as macrophages [Fig. 2(c)].

irARO, ARO and estrogen production by PMA-differentiated THP-1 myeloid leukemic cells and their effect on the proliferation of MCF-7 breast cancer cells

To study irARO expression in THP-1 myeloid leukemia cells and to show whether there is a biological effect of THP-1 cell-formed estrogen on nearby cells,

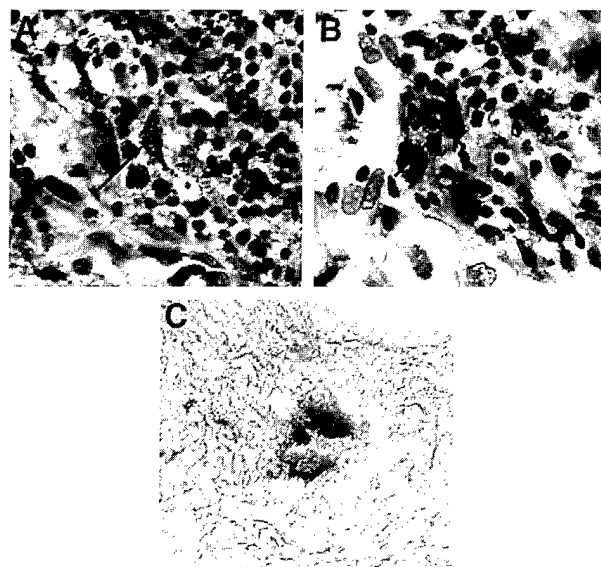


Fig. 2. Staining of breast cancer tissue sections for ARO and CD68. Immunostaining was carried out to establish the identity of ARO+ cells morphologically resembling macrophages, as shown in Fig. 1(B) and (C). For this, serial slides were incubated with anti-ARO (A) and anti-CD68 antibodies (B). Magnification, ×400. (C) A high magnification view of TAMs showing colocalization of irARO and CD68 immunoreactivity. Magnification, ×1000.

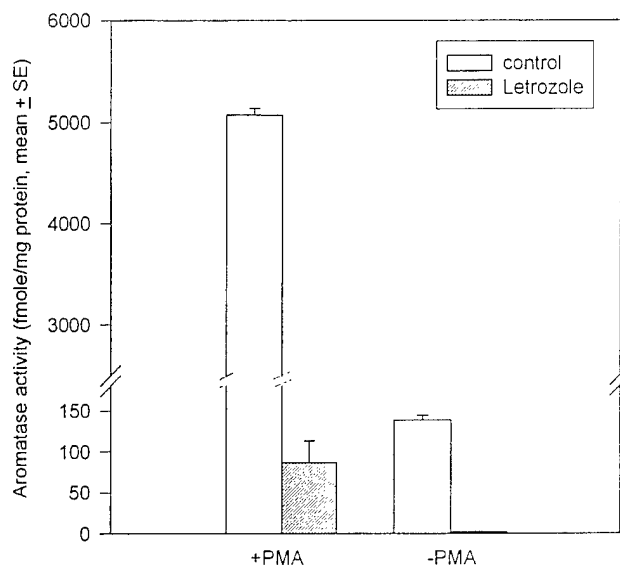


Fig. 3. Aromatase activity in THP-1 cells. ARO activity, as measured by the [^3H] water release method, is expressed as the amount of estrone produced by THP-1 cells cultured with or without PMA (6 ng/ml) for 24 h. PMA treatment resulted in a 30-fold increase in ARO activity in relation to basal ARO activity in the nontreated cells ($p < 0.001$). Both basal and PMA-induced ARO activity were suppressed by the ARO inhibitor letrozole (10^{-7} M).

THP-1 cells were induced to differentiate in culture, using PMA. Using the $^3\text{H}_2\text{O}$ release method, undifferentiated THP-1 cultures incubated in the presence of ^3H testosterone (10^{-8} M) were found to produce 0.14 pmol estrone/mg of protein/h, while differentiated cells produced 5.1 pmol estrone/mg of protein/h (Fig. 3). Addition of the aromatase inhibitor, letrozole (10^{-7} M), during the incubation period significantly inhibited both basal and poststimulation aromatase activity levels (Fig. 3). Using the thymidine incorporation assay, THP-1 CM from the differentiated cell cultures was found to stimulate the *in vitro* growth (as evidenced by increased levels of thymidine incorporation) of MCF-7 breast cancer cells. Our ability to block THP-1 CM-induced MCF-7 cell growth by adding the aromatase inhibitor letrozol, confirms that the effect on proliferation was in fact due to estrogen (Fig. 4).

Immunohistochemical localization of ARO in peripheral human monocytes (PHM)

We studied whether expression of ARO depends on differentiation and whether this phenomenon is tissue-specific. To establish an *in vitro* model, we began by testing the ability of cultured PHM to express ARO. PHM were isolated using anti-CD-14-coated magnetic beads and cultured. In culture, PHM differentiated from typical monocytes [Fig. 5(a)] to the macrophage phenotype [Fig. 5(b)]. While irARO was absent in PHM at the beginning of culture [Fig. 5(c)], PHM cultured for >48 h were

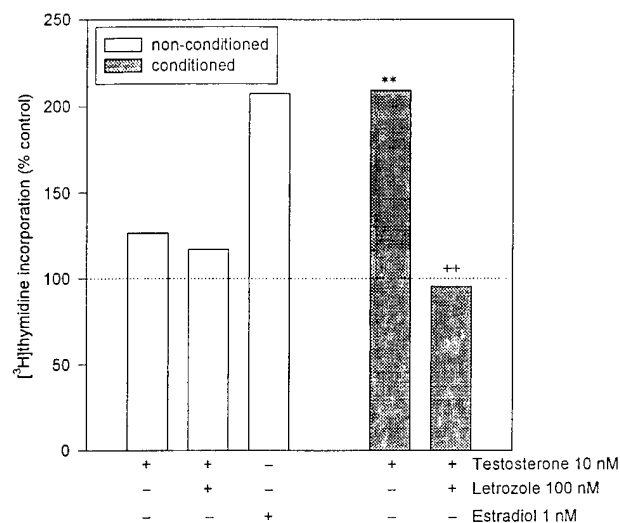


Fig. 4. Effect of THP-1 CM on MCF-7 cell proliferation. MCF-7 cells were treated with THP-1 CM or non-CM for 20 h. [^3H] thymidine incorporation into DNA is expressed as a percent of the levels of incorporation by MCF-7 cells in untreated cultures. Culture of MCF-7 in the presence of THP-1 CM resulted in a doubling of [^3H] thymidine incorporation. A similar proliferative effect was observed when MCF-7 cells were incubated in regular medium supplemented with 10^{-9} M of estradiol (E_2), as a control for the E_2 group. The corresponding medium containing PMA (6 ng/ml) was used as control for the other treatment groups. All experiments were repeated at least three times. The standard errors for all means were <10% of the mean. ** $p < 0.01$ compared with PMA control; ++ $p < 0.01$ compared with PMA + T. (Student's *t*-test).

positive for irARO [Fig. 5(d)]. Similar results were obtained for the THP-1 cells [Fig. 5(e) and (f)].

Expression of ARO mRNA

To evaluate the expression of ARO mRNA, total RNA from human placentas, monocytes and *in vitro* differentiated macrophages was tested using RT-PCR. As illustrated in Fig. 6, ARO mRNA was identified in normal placenta (Fig. 6, lane 1). A similar size product (272 bp) was detected in the differentiated macrophages and THP-1 cells (Fig. 6, lanes 2 and 5). ARO mRNA was absent from the monocytes, lymphocytes and undifferentiated THP-1 cells (Fig. 6, lanes 3, 4 and 6).

DISCUSSION

Despite low estrogen plasma levels after menopause, breast tumors excised from postmenopausal women contain estradiol concentrations similar to those from premenopausal patients [22]; a possible explanation is *in situ* estrogen synthesis from plasma androgens via local ARO [23]. The present study further substantiates previous work establishing aromatase expression in breast tissue and suggests that a significant portion of the tissue estrogen may arise from TAMs. Therefore the study achieves its

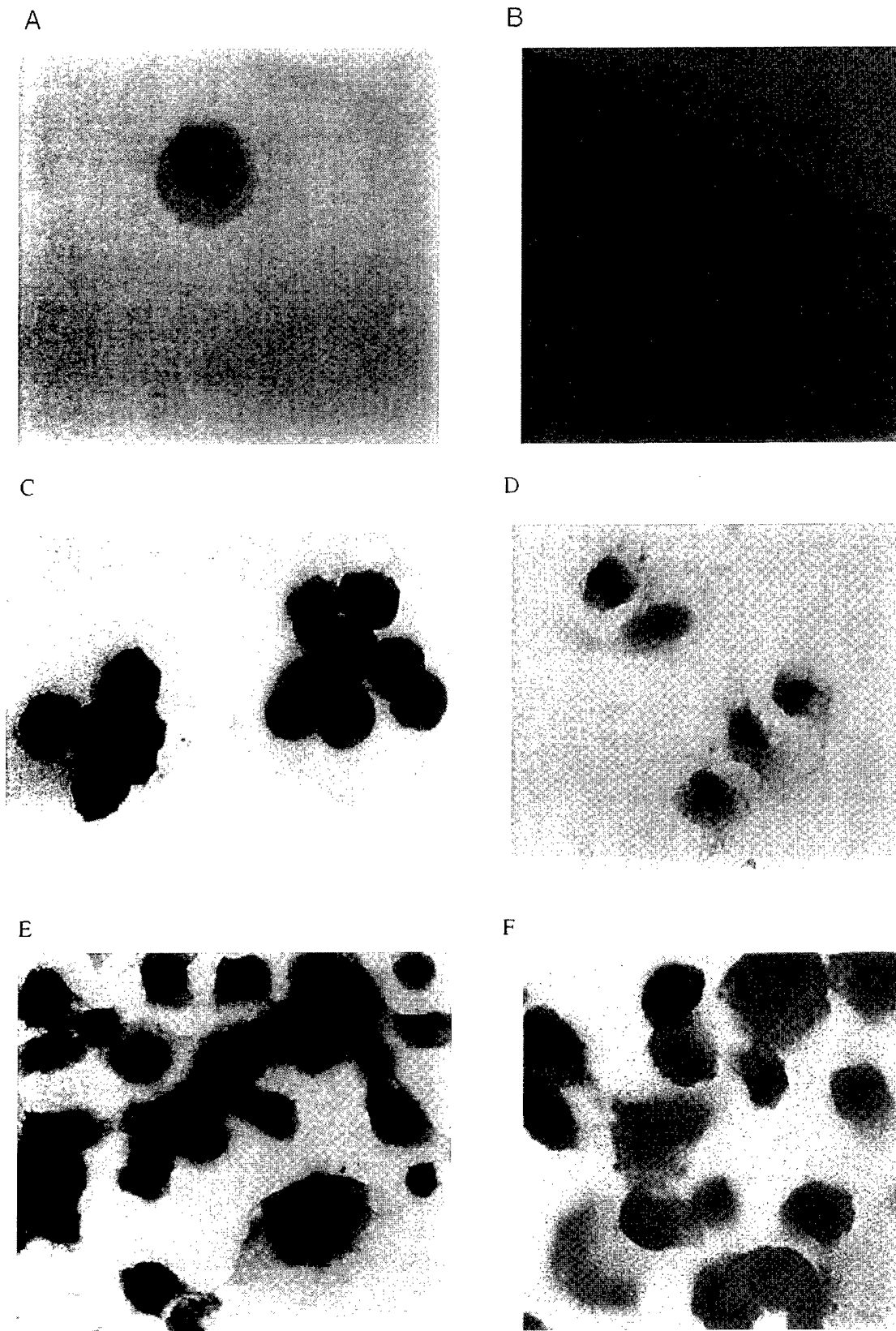


Fig. 5. Light micrograph of monocytes/macrophages. Note the differences in morphology between the two cell lines. (A) Freshly isolated monocytes are round with a horseshoe-shaped nucleus and a nuclear-cytoplasmic ratio of approximately one. (B) In contrast, after 48 h in culture, the macrophage-like morphology with pseudopods and a nuclear-cytoplasmic ratio <1 are evident. Magnification, $\times 1000$. (C)–(D) Human monocytes stained for irARO immediately or after >48 h in culture. While no irARO was detected in freshly isolated monocytes (C), positive irARO was found in cells cultured >48 h, whose morphology resembles that of tissue macrophages (D). Magnification, $\times 1000$. (E) PMA/differentiated THP-1 cells stained with anti-aromatase antibody show strong cytoplasmic irARO. (F) No staining is detected when the first antibody is omitted.



Fig. 6. ARO mRNA expression. To evaluate the expression of ARO mRNA, total RNA from human placentas, monocytes and *in vitro* differentiated macrophages was tested using RT-PCR. Hybridizable amplification products of ARO mRNA was identified in normal placenta (lane 1). A similar size product (272 bp) was detected in the differentiated macrophages and THP-1 cells (lanes 2 and 5). ARO mRNA was absent from peripheral monocytes, lymphocytes and undifferentiated THP-1 cells (lanes 3, 4 and 6).

objective of identifying the source of local estrogen formation in breast tissues and breast cancer. It is beyond the scope of the present study to quantify the actual amount of estrogen made in specific cell types in tissue sections which were obtained for the specific purpose of identification. Similarly, further studies will be required to assess the growth kinetics of the involved cells. This study also uncovers a new level of interaction between the immune and endocrine systems, which may be applicable to other organs throughout the body: that is, estrogen production by immune cells [24, 25].

In addition to the previously described presence of irARO in breast cells, detailed examination of breast tumors stained for ARO showed positive immunoreactivity among the leukocytic population, more specifically irARO was localized to cells morphologically resembling macrophages. To establish that these cells were indeed macrophages, sections were stained with a monoclonal antibody recognizing CD68, a macrophage-specific antigen. Immunoreactive cells were localized to the leukocytes around and within the tumor; thus, positive staining for CD68 identified the cells expressing ARO as macrophages.

Although their function within the tumor is not clear, up to 80% of the leukocytes in some breast carcinomas are macrophages [26, 27]. Tissue macrophages are recruited from peripheral monocytes, which are known to express estrogen receptors (ER) [19]. Upon entering the tissues, monocytes differentiate into macrophages capable of performing tissue-specific functions. Under the effect of local factors they may lose their ability to produce some proteins while becoming able to express the ones required for their new role [28]. Our *in vitro* studies suggest that the aromatase complex is one such product, since freshly isolated monocytes are irARO-negative, while cultured monocytes which have acquired the tissue macrophage phenotype become irARO-positive. Since ARO is not constitutively expressed by circulating monocytes, it must be induced, e.g. by differentiation. Whether this irARO expression is specific to the macrophages in breast tissue and the factors inducing it is presently under investigation.

Our *in vitro* studies using the THP-1 cells (a human promyeloid leukemia cell line that can be induced to differentiate by vitamin D₃ or PMA) support this theory of aromatase expression following differentiation of monocytes into tissue macrophages. Undifferentiated THP-1 cells were found to be negative for irARO, while PMA stimulated cells did not only acquire the morphology of tissue macrophages, but showed both strong cytoplasmic irARO and CD68 immunoreactivity.

Jakob *et al.* [29] have previously reported the expression of aromatase mRNA and enhancement of aromatization of testosterone in vitamin D₃ and PMA-treated THP-1 cells. In the present study, we compared and measured aromatase activity in PMA-treated THP-1 cells using the tritiated water release assay. This differentiation resulted in a dramatic increase in aromatase activity (more than 30-fold) compared to basal activity levels. In addition, a 98–99% decrease in both basal and poststimulation aromatase activity levels was achieved by culturing the THP-1 cells in the presence of the aromatase inhibitor, letrozole.

The biological significance of aromatase expression in PMA-differentiated THP-1 cells was examined by studying the effect of THP-1 CM on cultured MCF-7 cells. Incubation of MCF-7 cells for 20 h in testosterone (10⁻⁸ M) containing THP-1 CM resulted in a 2-fold increase in [³H] thymidine incorporation. In the presence of regular media containing an identical concentration of testosterone no stimulation or changes in [³H] thymidine incorporation by MCF-7 were observed. Furthermore, the stimulatory effect of testosterone (10⁻⁸ M) containing THP-1 CM on MCF-7 cells was abolished in the presence of the ARO inhibitor letrozole during the incubation period. The degree of stimulation, as reflected by the amount of [³H] thymidine incorporation by MCF-7 cells incubated with THP-1 CM, was similar to that induced by the addition of estradiol (10⁻⁹ M) to the cultures. Likewise, the amount of estradiol synthesized by 1 × 10⁶ THP-1 cells during a 24 h period was similar to previously reported estradiol concentrations produced by vitamin D₃ or PMA stimulated THP-1 cells [29]. These studies clearly demonstrate that growth stimulation of MCF-7 cells cultured in THP-1 CM is mediated by estrogen resulting from the aromatization of testosterone by THP-1 cells.

The presence of irARO in tissue macrophages indicates that these cells may be a major source of *in situ* estrogen formation in both normal and diseased breast tissues. These *in vitro* studies also suggest that macrophage-derived estrogen could contribute to the development and progression of breast tumors. Thus, *in situ* production of estrogen by the macrophages may constitute a new level of cellular regulation in the normal and malignant breast tissue. As mobile

regulatory cells, macrophages are free to reach regions of the breast where they could cooperate with factors that induce neoplasia or other pathologic changes. This characteristic mobility is concordant with reports of increased tissue estrogen levels in the breast quadrants where the tumor is located [30].

Although much is known about the general role of estrogen in the breast, the biological role of locally formed estrogen is not completely understood. Based on our studies with THP-1 CM and MCF-7 cells, we believe that estrogen production by macrophages in the breast may affect adjacent cells and autoregulate cytokine production from the macrophages, themselves (Fig. 7). This is an area of great importance, owing to the pleiotropic properties of many cytokines, such as IL-1, IL-6 and TNF- α . These cytokines have been shown to be involved in the pathogenesis of most age-related diseases, i.e. atherosclerosis, fibrosis, osteoporosis and others [31–34]. Recent studies have elucidated the role of cytokines such as IL-6, TNF α , IL-1 and IGF II in the regulation of estrogen synthesis [8]. We believe that the regulatory effect of these cytokines may not only be restricted to the breast cells, but to the macrophages as well, in what could potentially constitute a regulatory loop between the different cell components of the breast microen-

vironment. In addition, chemokine-attracting factors, such as MCP-1, have been shown to attract monocytes into a variety of tissues, including the breast [35,36]. This is a self-regulating system [37], which implies a feedback loop between TAMs and local estrogen synthesis. Of course, the presence of this estrogen regulated influx of 'inflammatory cells' does not obviate the presence of a more traditional immune response.

The concept of local estrogen and its role in the breast microenvironment has wide implications, for example in the confusing area of the role of estrogen replacement therapy (ERT) and the pathogenesis of breast cancer. So far, the possible role of exogenous estrogen in breast carcinogenesis has been addressed without regards to local estrogen production and secretion. The effect of local estrogen formation could be powerful without overtly affecting circulating estrogen, conversely, the amount of circulating estrogen levels would have to achieve extremely high levels in order to compete with the effects of local estrogen synthesis. The results of this study lead us to believe that the determination of the source, amount and role of *in situ* estrogen formation in breast disease could resolve apparent inconsistencies in theories that only consider the circulating estrogen levels to explain the

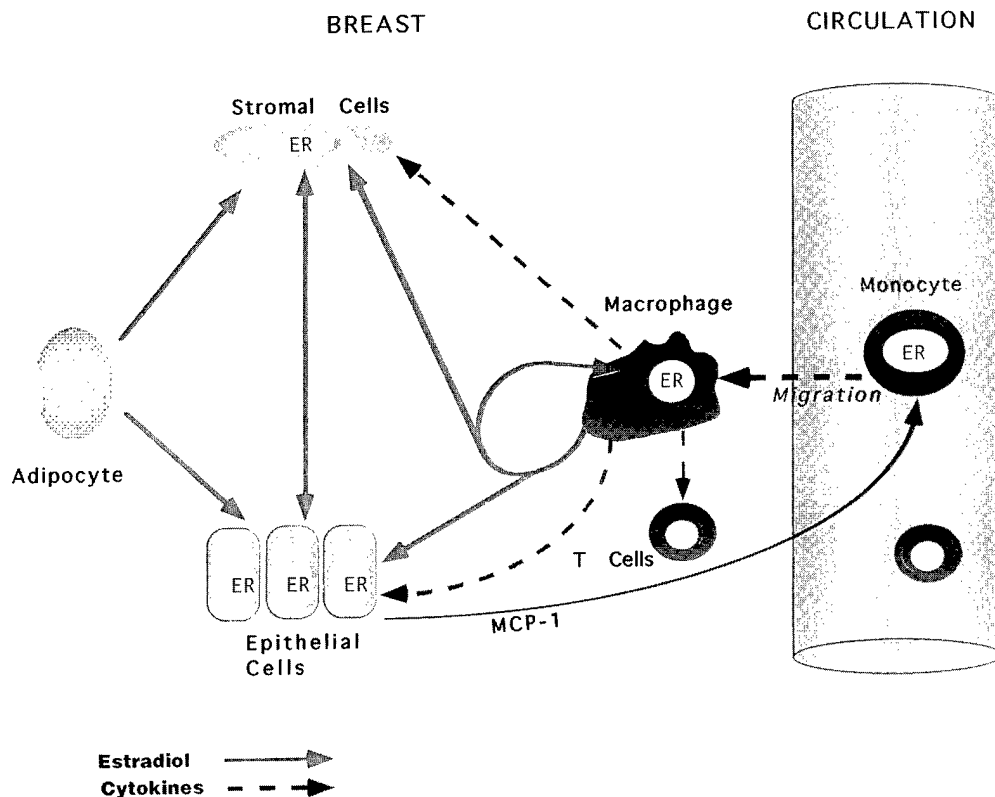


Fig. 7. Sources and effects of local estrogen formation in breast tissue. The life cycle of the peripheral monocytes includes entry into the tissues, to become macrophages. In the breast there is also a nonimmune interaction between the macrophages and nearby cells which includes autocrine and paracrine effects of macrophage-derived estrogen. There is also an inverse relationship between the secretion of chemokines (e.g. MCP-1) and local estradiol, which closes this regulatory loop. ER, estrogen receptors; MCP-1, monocyte chemoattractant protein-1.

role of physiologic ERT in breast cancer pathogenesis [38]. Similarly, understanding how and how much estrogen-producing macrophages contribute to the microenvironment of the breast could help explain the success of antihormone treatments and open new therapeutic possibilities for breast cancer.

Acknowledgements—We are indebted to Dr Steve Gonias for his assistance with the THP-1 cell experiments and to Dr Maria Luisa Carcangiu for helpful discussions and tissue review. We appreciate helpful discussions with Dr Brinda Witte. M. E. is a Medical Student Research Fellow sponsored by the NIH Cancer Education Grant.

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Report

The Fas/Fas-ligand system: a mechanism for immune evasion in human breast carcinomas

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Key words: apoptosis, breast carcinoma, immunology, tolerance/suppression, tumor immunology

Summary

Breast tumors are frequently associated with a predominantly lymphocytic infiltrate, which constitutes an immune response against the tumor. In spite of this massive infiltrate, the immune response appears to be inefficient and the tumor is able to evade it. We propose that in breast cancer, tumor escape from immunological surveillance results from the induction of apoptosis of Fas-bearing activated lymphocytes by FasL-bearing breast cancer cells.

To test this proposal we studied the expression of FasL by human breast carcinomas and the MCF-7 breast cancer cell line by RT-PCR, immunohistochemistry, and Western Blot. Moreover, we describe the presence of apoptosis and Fas expression in the lymphocytic population surrounding the tumor. Strong membranous and cytoplasmic staining was detected in ductal carcinomas and hyperplastic breast tissue, but it was absent from normal breast tissue. No staining was found in normal glands in the non-tumor quadrants; however, the normal appearing ducts surrounding the carcinoma (tumor quadrant) showed intense immunoreactivity. Apoptosis was found predominantly among the lymphocytic population, as well as in the blood vessels and fibro-fatty tissue close to the tumor. Further characterization of apoptotic cells demonstrated that they were CD3+ cells.

Our results suggest the breast tumors may elude immunological surveillance by inducing, via the Fas/FasL system, the apoptosis of activated lymphocytes. Recent data have demonstrated FasL RNA in other tumor types. Upregulation of FasL expression in hyperplastic and normal breast ducts close to the tumor also suggests a possible role in early neoplastic transformation and proliferation.

Abbreviations: Con A: concanavalin A; FasL: Fas ligand; RT-PCR: reverse transcription-polymerase chain reaction.

Introduction

An important question in tumor immunology is why neoplasms expressing tumor-specific antigens are not eliminated as 'foreign' by the immune system. This process of evasion, also called 'tumor escape', has been suggested to result from the inability of the immune system to react to the tumor, because of either non-recognition of tumor antigens or non-reactivity secondary to insufficient co-stimulation, anergy, tolerance, or immunosuppression [1, 2].

Local immune-suppression due to factors derived from the tumor is a feature of many cancer types.

In 1993, for example, O'Mahony et al. described the non-necrotic cell death (apoptosis) of activated lymphocytes associated with esophageal squamous cell carcinoma [3]. Further studies have identified tumor-derived immunomodulatory molecules, such as cytokines, amino sugars, and gangliosides [2, 4], as responsible for such a state which may result in tumor evasion.

Recent reports showing the expression of FasL in Sertoli cells of the testis and ocular tissues [5, 6], and more recently in the placental trophoblast [7], have provided new insights into the concepts of tolerance and immune-privilege. When grafted under the

kidney capsule, Sertoli cells expressing FasL were protected for longer periods against rejection. In the eye, constitutive FasL expression was shown to control the proliferation of Fas+ lymphoid cells entering this organ. Recently, we described the expression of FasL in the human placenta and its neoplastic form, the choriocarcinoma, and the presence of apoptotic cells within the surrounding inflammatory infiltrate [8, 9]. Moreover, FasL has been found to be expressed in several tumor cells [10, 11], suggesting that this system may constitute one of the mechanisms by which tumor cells successfully escape immune surveillance.

The Apo-1/CD95 (Fas) and CD95-ligand (FasL) are cell surface molecules that induce the programmed cell death or apoptosis of lymphoid cells and play an important role in maintaining an appropriate immune response [12, 13]. Following antigen-induced activation, T cells express Fas and can then be triggered to undergo apoptosis in response to FasL expressed by other activated T cells. This system is involved in the deletion of autoreactive lymphocytes and elimination of excess lymphocytes during and after an immune response to foreign antigens. Both activated T and B cells express high levels of Fas and are highly susceptible to apoptosis upon binding of Fas by its ligand [14].

Breast cancer is the most common and dreaded malignancy affecting women. It has an unpredictable course and a sustained risk of metastasis that spans 20 years or more after diagnosis and initial treatment. Unfortunately, the incidence of breast cancer is increasing, and it is estimated that one in nine women will develop the disease during her lifetime. Much is known about the hormonal and genetic aspects of the disease; however, very little is known about why the tumor, in spite of the presence of a massive infiltrate of leukocytes, is not rejected but rather continues growing and even metastasizes.

In the present report, we studied the expression of FasL by human breast carcinomas and the MCF-7 breast cancer cell line. We also studied the presence of apoptosis and Fas expression in the lymphocytic population surrounding the tumor. Our results suggest that breast tumors may elude immunological surveillance by inducing, via the Fas/FasL system, the apoptosis of the activated lymphocytes that would have otherwise mediated tumor rejection.

Materials and methods

Clinical material – human breast tissue specimens

Human breast tissue from mammoplasty surgery ($n = 12$), ductal invasive breast carcinoma ($n = 18$), and normal breast tissue from breast reductions ($n = 7$) were obtained from the Department of Pathology, Yale University School of Medicine. A signed written consent was obtained from each patient and the use of tissues was approved by the Yale University Human Investigation Committee. Following surgical excision, breast tissue specimens were frozen in liquid nitrogen for RNA extraction or fixed in 10% buffered formalin and paraffin-embedded for immunohistochemistry. Tissue blocks were stored at room temperature until 5- μ m sections were cut.

Cells and chemicals

The human T cell line Jurkat and the breast carcinoma cell line MCF-7 were purchased from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium and FBS were purchased from Life Technologies (Grand Island, NY). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture conditions

Jurkat cells were maintained in RPMI-1640 nutrient medium supplemented with 10% FBS, penicillin (100 unit/ml) and streptomycin (100 μ g/ml), pH 7.15, and incubated at 37°C in a 5% CO₂ atmosphere. MCF-7 cells were cultured in phenol red DMEM supplemented with 5% FBS.

Immunohistochemistry for FasL, Fas, CD45, and CD3

Detection of FasL and Fas expression was performed using a rabbit polyclonal IgG containing anti-human FasL or Fas, respectively (Q-20 and N-20, Santa Cruz Biotechnology, Santa Cruz, CA). Detection of CD3 and CD45 positive cells was performed using mouse anti-human monoclonal antibodies (DAKO, San Diego, CA). Deparaffinized and rehydrated 5- μ m thick sections were blocked for endogenous peroxidase activity with 0.3% H₂O₂ in methanol, washed in PBS, and pre-incubated with 10% normal goat or horse serum in PBS-5% BSA for 30 min. One microgram per milliliter of FasL and Fas antibodies were applied. For CD3 and CD45 detection, two

drops of the already diluted anti-CD3 antibody and CD45 respectively were applied. Slides were then incubated for 1 h at room temperature in a humidified chamber. After washing, sections were incubated for 1 h with a biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA) for Fas and FasL or a biotinylated horse anti-mouse for CD45. Streptavidin-peroxidase incubation for 30 min and color development with diaminobenzidine or amino-ethyl-carbazole (AEC) were then carried out. Following incubation with the first antibody, slides for CD3 detection were immersed directly in diaminobenzidine.

In situ 3' end labeling of DNA for cell death detection

The presence in breast tissue sections of single strand DNA breaks indicating apoptosis was assessed using the TUNEL technique (*In situ* Cell Death Detection Kit, Fluorescent, Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Briefly, following deparaffinization and re-hydration of previously stained sections with anti-CD3, slides were incubated with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.4) for 30 min at 37°C. Samples were then treated with terminal deoxynucleotidyl transferase enzyme and fluorescein-labeled nucleotides for 60 min at 37°C in the dark. After washing with PBS, the slides were cover-slipped and analyzed under the fluorescence microscope. For Fas/apoptosis co-localization, slides already stained for Fas were treated as described above. An alkaline phosphatase-antifluorescein antibody was added and a nitro-blue-tetrazolium alkaline phosphatase kit (Vector) was used for color development.

RT-PCR for FasL mRNA

Total RNA was prepared from frozen normal breast, breast carcinomas, and 2×10^6 Jurkat cells using TRIZOL reagent (GIBCO BRL, Grand Island, NY), according to the manufacturer's instructions. Prior to RNA extraction, Jurkat cells were stimulated in culture for 24 h with 5 µg of ConA. RT-PCR was performed using the RT-PCR kit from Pharmacia Bio Tech (Piscataway, NJ), according to the manufacturer's protocol. cDNA synthesis was carried out with 0.2 µg of pd(N)₆ and 5 µg of total RNA. The primers used for amplification of FasL have been recently described [15] and have the following sequence; upstream: 5'-ATAGGATCCATGTTTCTGCTCCTTCCACCTACA-GAAGGA-3; downstream: 5'-ATAGAATTCTGACC-

AAGAGAGAGCTCAGATACGTTGAC-3. Each PCR cycle consisted of: a denaturation step at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min for 10 cycles, followed by 35 cycles modified by a cumulative 5 s increase of extension time per cycle. The PCR products were analyzed on a TAE % agarose gel with ethidium-bromide.

Cloning and sequencing of FasL RT-PCR products

The RT-PCR products were cloned into PCR-Script Amp SK(+) plasmid vector (Stratagene, La Jolla, California) according to the manufacturer's instructions. Briefly, cDNA amplified with Pfu polymerase was purified by ethanol precipitation. Blunt-ended ligation was done at room temperature for 1 h. The ligation product was added to Epicurian Coli XL1-Blue MRF' Kan supercompetent cells for transformation and the cells were grown on LB plates with 50 µg/ml ampicillin, x-gal, and IPTG. Positive colonies were identified according to the method described by Sandhu et al. [16]. The cloned FasL RT-PCR product was sequenced at Yale's Keck DNA Sequencing facility using a fluorescent dideoxy chain termination system (Applied Biosystems, Inc.).

Western Blot analysis

Proteins were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed after blocking the membranes with 5% powdered milk. The primary antibodies (FasL monoclonal antibody, clone 33 (Transduction Laboratories, Lexington, KY), and FasL polyclonal antibody N-20 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 dilution. The secondary antibody (peroxidase-labeled horse anti-mouse, or goat anti-rabbit, Vector, Burlingame, CA), were developed with TMB Peroxidase substrate kit (Vector, Burlingame, CA).

Results

Immunohistochemical localization of FasL in malignant and normal human breast tissue

Paraffin sections from normal breast tissue ($n = 7$) and breast tumors ($n = 18$) were studied for FasL expression using a specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). In each case, intense immunoreactivity of tumor cells was detected,

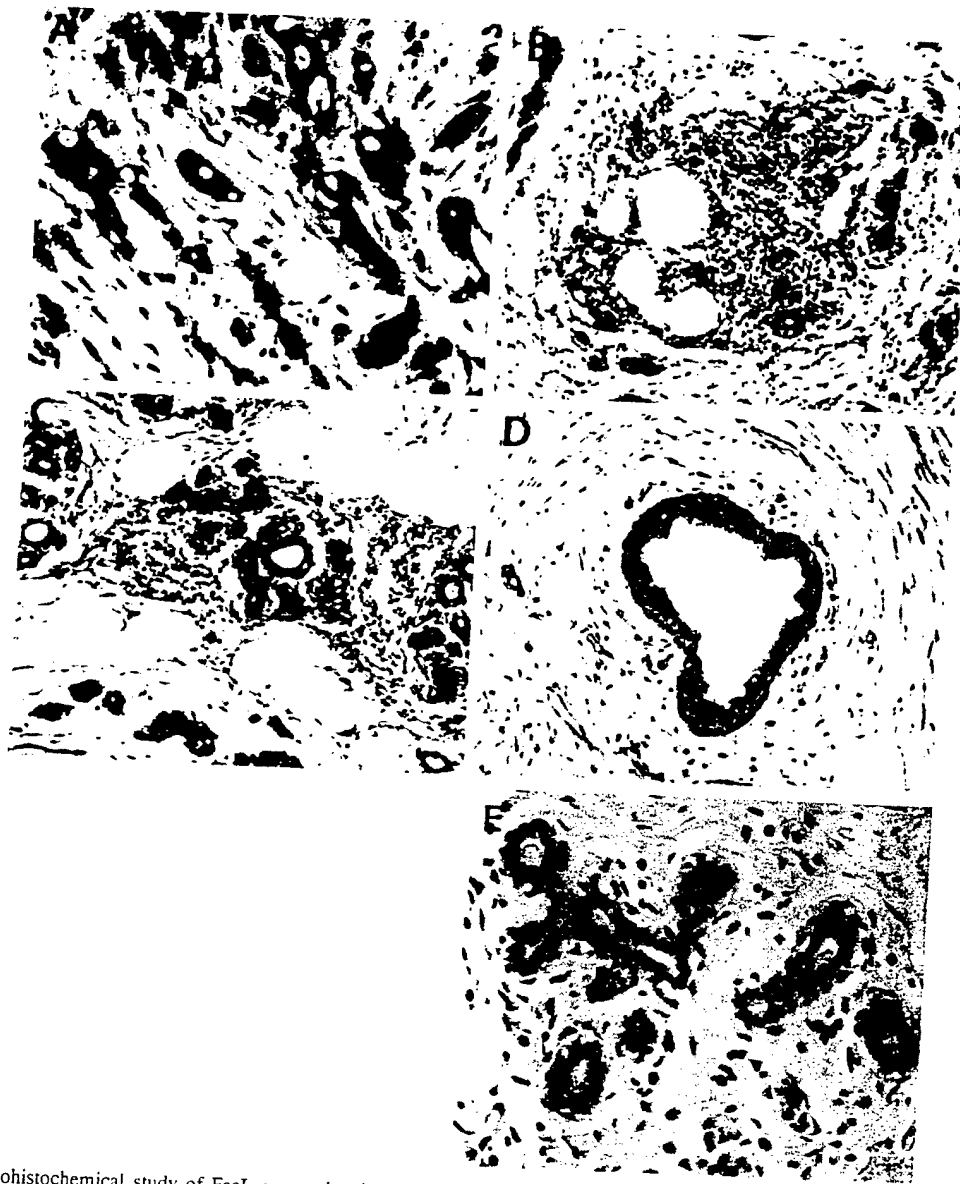


Figure 1. Immunohistochemical study of FasL expression in breast tissue: Paraffin sections of breast tumor were stained with anti-human FasL antibody. A. Invasive ductal carcinoma, showing strong cytoplasmic immunoreactivity, as well as few fibroblasts and macrophages within the stroma (magnification: 400 \times). B. FasL-positive tumor cells surrounded by an intense lymphocytic infiltrate. C. Apparently normal breast glands surrounding the tumor (T) exhibit positive immunoreactivity for FasL. Note the intense lymphocytic infiltrate. The ductal carcinoma as well shows strong FasL immunoreactivity (magnification: 400 \times). D. Paraffin section from hyperplastic duct obtained from non-tumor breast tissue: positive cells are found predominantly in the myoepithelium of the glands. The stroma is FasL-negative with the exception of some immunoreactivity present in blood vessels and fibroblasts near the ducts (magnification: 400 \times). E. Normal breast tissue obtained from a different quadrant of the tumor: No staining is evident.

including both cytoplasmic and surface distribution. The staining was stronger in cells at the periphery of the tumor, mainly in areas of tumor invasion (Figure 1A). A high number of FasL-positive tumor cells surrounded by an intense lymphocytic infiltrate was also noted (Figure 1B). The lymphocytic cell population was predominantly FasL-negative, but FasL-

positive lymphocytes were found in the area of the tumor (Figure 3C). Interestingly, FasL-positive immunoreactivity was found in normal appearing ducts and hyperplastic breast located in the same quadrant of the tumor, and was always surrounded by a strong inflammatory infiltrate (Figure 1C). In contrast, no immunoreactivity was found in the normal ducts in

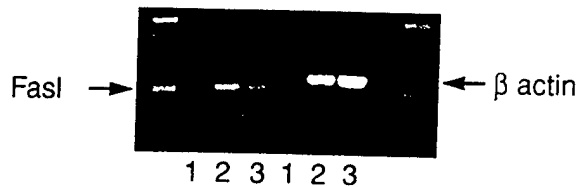


Figure 2. Expression of FasL mRNA in human breast tissue. FasL and β -actin mRNA were detected by RT-PCR in: the breast cancer cell line MCF-7 (lane 1), breast tumor (lane 2), and hypertrophic breast tissue (mammoplasty) (lane 3). The PCR product is consistent with the known FasL sequence.

quadrants located farther away from the tumor (Figure 1E). FasL expression in the hyperplastic breast tissue samples obtained after mammoplastic surgery showed a different pattern of immunoreactivity: positive cells were found in the basal epithelial layer of the glands while the luminal epithelial layer was predominantly negative (Figure 1D).

Expression of FasL mRNA in the MCF-7 breast carcinoma cell line, non-tumor breast tissue, and breast carcinoma

To confirm the results obtained from the immunohistochemical studies, we evaluated the expression of FasL mRNA in human breast tissue and the MCF-7 human breast cancer cell line. Total RNA was extracted and tested using RT-PCR. As illustrated in Figure 2, FasL RNA was identified in MCF-7 cells (Figure 2, lane 1), tissue from breast carcinomas (Figure 2, lane 2), and tissue from mammoplastic surgery (Figure 2, lane 3). The FasL RT-PCR products were cloned and their sequence determined. The sequence of the 521-bp product matched that previously published for the human FasL [17].

In situ cell death detection – terminal deoxy (d)-UTP nick end-labeling (TUNEL)

To determine the possible role of FasL expression by tumor cells in the induction of apoptosis of lymphocytes, we studied the presence of apoptosis in breast carcinomas. Apoptotic cells were detected by staining of DNA fragments with the TUNEL technique. TUNEL-stained nuclei were mainly found among the inflammatory infiltrate directly contacting the tumor (Figure 3A). No apoptosis, or very scattered apoptotic nuclei, was detected within the tumor. The lymphocytic infiltrate surrounding normal glands near the tumor displayed numerous apoptotic cells (Figure 3D). Further characterization of these cells with

anti-CD45 and anti-CD3 antibodies identified these apoptotic cells as leukocytes and T cells respectively (Figures 3B and 3E). We were also able to detect apoptotic cells surrounding the blood vessels and in the fibro-adipose tissue (data not shown). On close inspection, apoptotic cells were detected inside the lumen, around the periphery of the blood vessels, and migrating through the walls of the vessels.

Co-localization of Fas and apoptosis

The induction of apoptosis by the FasL is mediated by the Fas receptor present in the target cell. The staining of breast tumor sections with an anti-Fas antibody (Santa Cruz, Santa Cruz, CA) revealed numerous Fas-positive cells. These Fas-immunoreactive cells were localized mainly to the inflammatory infiltrate around the tumor, as well as near normal appearing glands, vessels, and fibro-fatty tissue. In all the cases studied, the cells showed membranous staining (Figures 3C and 3F). The tumor component was Fas-negative, with only scattered immunoreactive intratumor leukocytes. Double staining for Fas and TUNEL revealed that these Fas-positive cells showed apoptotic features (Figure 3C; inset).

Western blot analysis

The specificity of the antibodies used for immunocytochemistry was evaluated using Western Blot analysis. Whole cell lysates from MCF-7 cell cultures and breast tissues, from which we had previously obtained mRNA, were electrophoresed in a polyacrylamide gel as described in 'Material and methods'. As seen in Figure 4, Clone N-20 and clone 33 recognize the same 37-kDa protein in a dose-dependent manner.

Discussion

Breast tumors are frequently associated with a predominantly lymphocytic infiltrate that constitutes an immune response against the tumor. Despite this massive infiltrate, the immune response appears to be inefficient and tumor cells evade it and metastasize.

Blockade of the responsiveness of T cells to tumor-specific antigens had major implications for tumor immunology. Apoptosis is one way by which the immune system generates tolerance towards antigens, and the Fas/FasL system is one mediator of apoptosis. In this study, we demonstrate FasL expression

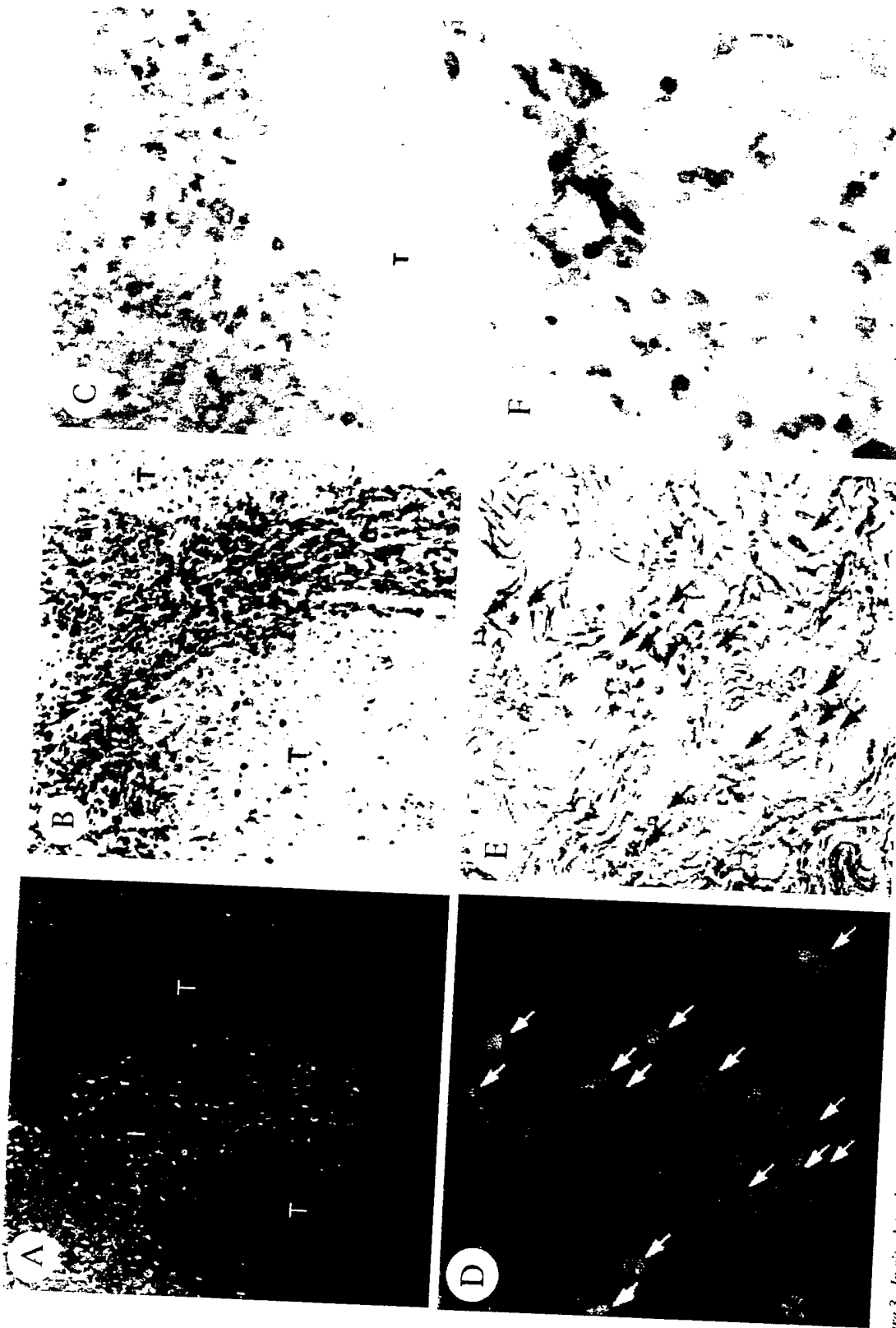


Figure 3. *In situ* detection of apoptotic cells in breast tissue. Apoptotic cells were detected by staining of DNA fragments with the TUNEL assay. Apoptotic cells are present in the vicinity of breast tumor. TUNEL negative areas correspond to tumor sheets (T) (magnification: 1000x). B. CD45 staining of breast tumor sections showing the distribution of the leukocytic infiltrate (I) and its relation to the tumor mass (T) (magnification: 1000x). D and E. Co-localization of apoptotic cells and CD3 antigen. Paraffin sections from the breast tissue were stained first with the anti-CD3 antibody and then with the TUNEL assay. Fluorescent microscopy reveals apoptotic cells in contact with a normal duct and within the fibroconnective tissue (white arrows) (D). Staining of the same section for CD3 (interface) demonstrates that the apoptotic cells are immunopositive (black arrows) (E). C and F. Fas expression is found in the leukocyte infiltrate (I) as well as in the fibroadipose tissue (F). C (inset). Co-localization by double staining for Fas (pink cell membrane) and TUNEL (blue nucleus; immunohistochemistry with avidin-biotin-immunoperoxidase and alkaline phosphatase for TUNEL detection)(magnification: 1000x).

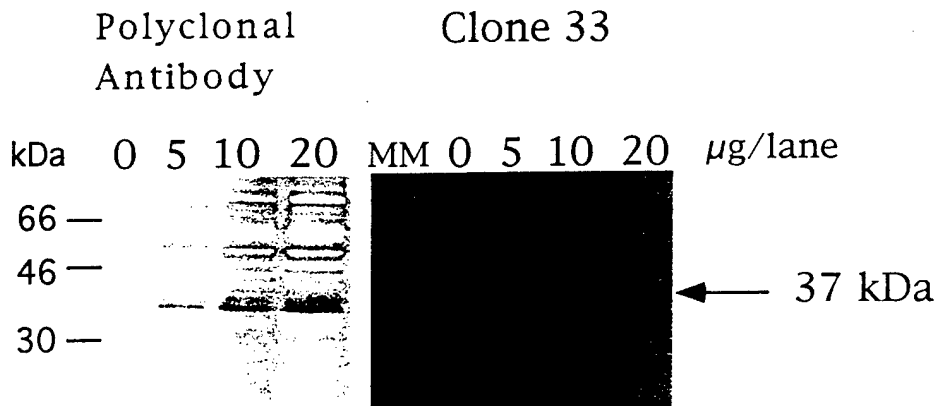


Figure 4. Immunoblot analysis of FasL protein in human breast cancer cell line. Serial dilutions: 20, 10, and 5 μ g of protein samples derived from MCF-7 cell cultures were subjected to Western blotting with a polyclonal antibody N-20 and a monoclonal antibody clone 33. Both antibodies recognize the same 37-kDa band corresponding to the FasL, in a concentration-dependent manner.

by breast tumor cells and describe their spatial relationship to Fas-bearing apoptotic lymphocytes in the proximity of the tumor.

Previously proposed mechanisms for tumor evasion of immune surveillance include: (a) aberrant regulation of antigen processing and presentation [18]; (b) release of cytotoxic substances [3], or immunomodulatory amino sugars or gangliosides [1]; and (c) changes in the immunologic profile of the cell surface [19]. Contrary to the above described mechanisms, the Fas/FasL induced apoptosis of activated lymphocytes represents an 'on-the-spot' active, cell-cell mediated, and specific immunosuppressive process which defeats the immune system by locally inducing immune tolerance. The role of FasL in local immunoregulation was demonstrated by Ferguson and Griffith [20]. These investigators showed that injection of a virus into the anterior chamber of the eye in normal mice results in an inflammatory infiltrate which undergoes apoptosis while inflammatory cells in the B6 *gld* (FasL deficient) mice do not die. These results, together with the finding of FasL expression in the eye, suggest that it is the FasL in ocular tissues that controls inflammation.

In our study, we found that breast carcinoma tissues express FasL, as determined by RT-PCR (Figure 2). To confirm that the presence of FasL mRNA in the whole breast tissue is not a product of leukocytic contamination, we tested its expression in the cloned breast carcinoma cell line MCF-7. A similar PCR product to the one found in breast cancer was detected in MCF-7 cells (Figure 2). At the protein level, two different antibodies recognized the same 37-kDa

protein corresponding to the FasL in whole lysates obtained from MCF-7 cells (Figure 4).

In breast cancer sections, FasL was localized to the cytoplasm and membrane of tumor cells and to the normal appearing glandular epithelium in the tumor quadrant. Strong immunoreactivity was also detected in the 'normal appearing' and hypertrophic glands near the tumor, but no staining was found in the tissue located farther away from the tumor quadrant. This data raises the question about these apparently 'normal' cells: are they already biochemically abnormally? Is the presence of FasL an early signal or marker of malignant transformation? Alternatively, could this unexplained expression of FasL be the result of paracrine stimulation from nearby cancer cells or other cells such as macrophages [21]?

We also found apoptotic cells among the leukocytic infiltrate localized near the tumor and glands, as well as in the blood vessels and fibro-fatty tissue close to the tumor. These apoptotic cells are CD3 immunopositive and therefore constitute apoptotic T cells. Previous findings have reported a progressive decrease in T lymphocytes consistent with the spread of the cancer [22]. Our findings of apoptotic CD3+ and Fas-positive cells in the proximity of FasL positive tumor cells may explain the mechanism behind the reported progressive decline in the number of T cells. Apoptotic cells were not detected in the normal breast tissue or among the intratumor lymphocyte population. The absence of apoptotic CD3+ lymphocytes inside the tumor could be related to the dual role of these immune cells as reported by several investigators. There is now evidence that cytokines produced by cancer infiltrat-

ing leukocytes can either down-regulate or stimulate cancer cell growth [23]. This dilemma has not been solved and the role of these intratumor lymphocytes is under investigation. We hypothesize that the apoptotic T cells could correspond to activated lymphocytes expressing Fas, [24] while the intra-tumor CD3+ cells may represent a T cell population at a different stage of activation [25].

We found two different patterns of apoptotic cell distribution in the leukocyte population: (a) a large number of apoptotic cells expressing Fas were detected among the lymphocytic infiltrate in direct contact with the tumor, as well as in the apparently normal breast surrounding the tumor; and (b) a second group of apoptotic leukocytes was also observed in the fibro-fatty tissue and blood vessels. This last group of apoptotic leukocytes could represent a population susceptible to secreted (paracrine) FasL or cytokines produced by the tumor [26].

The finding of FasL expression in 'normal' breast tissue adjacent to the tumor, as opposed to the non-tumor quadrants, is important in understanding normal and tumor microenvironment, as well as tumor progression. We hypothesize that the immunological changes detected in the tumor are also present in the surrounding, apparently normal tissue as a result of the interaction or crosstalk between the two tissues. Tumors have been shown to secrete growth factors and other products and these may affect the surrounding normal tissue. Thus, an early step in the progression towards neoplastic transformation may include the expression and upregulation of factors such as FasL. This could be of value as a 'tumor marker' of the earliest nature. In any case, FasL expression results in neoplastic cells becoming able to modify the immune response directed against them by affecting the tumor microenvironment in a way that favors the successful escape of the tumor from immune surveillance.

Acknowledgement

The present work was supported in part by a grant from the Department of the Army (DAMD17-98-1-8359).

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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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Key Words: FasL, estrogen, tamoxifen, breast cancer.

Running title: Estrogen and FasL in Breast cancer

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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 promotor 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.

words: 199

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 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, induced 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.

MATERIAL AND METHODS

Chemicals

DMEM and fetal bovine serum were purchased from Life Technologies (Grand Island, NY). 17- β estradiol and tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO). The tamoxifen was proven pure by HPLC.

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% CO₂ in air). Cells were passed by standard methods of trypsinization, plated in 6-well dishes and allowed to replicate to 80% confluence. Afterward cell cultures were treated with serum-free, phenol red-free media for 24 hours before the treatment with sex hormones/anti hormones was initiated.

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.

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'-ATAGGATCCATGTTTCTGCTCTTCCACCTACAGAAGGA-3''; downstream, 5'-ATAGAATTCTGACCAAGAGAGAGCTCAGATACGTTGAC-3''. Each PCR cycle consisted of denaturation at 95°C, 30 sec; annealing at 52°C, 30 sec; 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)

Western blot analysis

Proteins were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. Immuno-blotting was performed after blocking nonspecific 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 hour. 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).

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 [-32P] UTP using T7 RNA polymerase. Ten µg 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 24-72h at -70°C. Densitometry was performed using a digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA).

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 [3H] TdR at 37°C for 24h. 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 8-24h, the cells were removed from the wells and filtrated onto glass fiber filters using an automatic 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.

RESULTS

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 GGTC**A** 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 α and 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.

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 immunological 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 24h and 48h and the relative levels of FasL mRNA were quantified. Figure 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 48h (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).

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 hours 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 3h of incubation and diminishes thereafter. A secondary increase in the FasL band is seen at 24h (Fig. 3). Quantitative densitometry of the gels shows maximal FasL mRNA levels after 3h of estrogen stimulation, followed by a 40% decrease from 6-12h, with a definite but smaller increase at 24h. At the protein level, increase of FasL expression was present only after 24h of incubation with estradiol, remaining high up to 48h (Fig. 2A). Similar results were found with T47D cells (Data not-shown).

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 figure 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).

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 24h. RNA was analyzed by RT-PCR. As shown in figure 5, treatment with tamoxifen inhibited FasL mRNA expression in a dose-dependent 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} M and 10^{-8} M respectively. Similarly, using Western blots we found a decrease at the protein level (Fig.4 and data non-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 3h of incubation reaching a peak of inhibition at 12 and 24h (Fig. 6).

RNase protection assay (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 single sample of total RNA. Thus, MCF-7 cells were treated with different concentrations of tamoxifen for 24h. Total RNA was isolated and analyzed by RPA. As shown in Figure 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 worthy to note that tamoxifen at the concentration of 10^{-8} M had some inhibitory effect on Fas mRNA expression by MCF-7 cells, however, this effect was not consistent and was not statistically significant.

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 figure 8, 55% of Jurkat cells underwent apoptosis after 24h 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 non shown)

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 ($1 \times 10^{-7} \text{M}$ for 24h) 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)

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. (Figure 9)

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 (20); (35); (12); (22); (30). 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 macrophages (38)

Estrogen regulates breast tissue functions by modulating gene transcription (39). The hormone-activated estrogen receptor binds to specific estrogen-recognizing elements (ERE) located in the promoter region of estrogen-regulated genes. Therefore, we first 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 13bp palindromic element consisting two 5bp arms separated by a 3bp 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 (3bp) 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);(44, 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 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 suggested that estrogen could have a direct effect on FasL expression. Our hypothesis was confirmed in the present study when we analyzed FasL expression in the breast cancer cell lines MCF-7 and T47D cells treated with estrogen: 17- β -estradiol, at concentration of 1×10^{-8} M induced a three-fold increase in FasL mRNA levels after 24h and 48h (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 3h of incubation followed by decrease after 6 and 12h and a second moderate increase at 24h. Such biphasic effect has been reported for other estrogen regulated genes (46) and could be explained by the conformation of the ERE. 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 transcriptionally active ER- complexes increases within the first 2-3h 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 6h of estrogen could be as 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 *X. laevis* vitellogenin A2 gene requires about 10 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 (Fig. 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 α and β , cyclin D and A, and CD36 (47);(48).

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. 9) 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 decrease 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. We found that tamoxifen at concentrations of 10^{-6} M induce apoptosis in 20% of MCF-7 cells after 24h incubation. We are currently studying to determine that if this is Fas/FasL mediated effect.

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 3h 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 receptor (51, 52) and ER (53); (54, 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 and co-workers have reported that the tamoxifen-ER complex binds at the AP-1 site (58) and constitutes an alternative ER-regulatory pathway.

In addition, the fact that the FasL ERE is an imperfect palendromic 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.

ACKNOWLEDGEMENTS

We thank the valuable technical contribution of Tracy Niven-Fairchild. This work was supported in part by DAMD17-98-1-8359.

LEGENDS TO FIGURES

Figure 1. **A. RT-PCR linearity.** The linearity of the 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 37kDA protein in PMA (10ng/ml) stimulated Jurkat cells (lane 2) but not in the un-stimulated Jurkat cells (Lane 1). C= molecular markers.

Figure 2. **Effect of estrogen on FasL expression.** MCF-7 cells grown in phenol free DMEM and serum free conditions for 24h were treated with 17- β -estradiol (10^{-8} M) for 24 and 48h. Control cells received 0.01% Ethanol in the same phenol and serum free media. **2A** 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.).

2B. 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.

Figure 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 24h. RPA assay and RT-PCR were performed using 5 μ g total RNA. The intensity of the products was calculated using a digital imaging and analysis system as described in material and methods. **3A.** Representative PCR gel stained with ethidium bromide. Each experiment was repeated at least three times. **3B.** Quantification of the FasL signal. Estrogen increases FasL mRNA expression after 3h (lane 2), followed by a decline (lanes 3 and 4) and increases again after 24h (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 (SEM). Statistical significance was determined by ANOVA

Figure 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 μ g of protein from each sample was subjected to 10% SDS-PAGE and transferred to nitrocellulose. Immuno-blot analysis was performed as described in

Material and Methods. **4A.** Representative western blot for T47D cells. As shown in figure 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).

4B. Representative RT-PCR of T47D cell treated with estrogen and tamoxifen. 5 µg of total RNA from the same samples described in figure 4A 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.

Figure 5. Effect of tamoxifen on FasL mRNA expression. MCF-7 cells were treated with tamoxifen at concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M for 24h. Total RNA was analyzed for FasL expression by RT-PCR as described before. **5A.** Representative gel analysis from MCF-7 cells treated with tamoxifen at different doses. **5B.** 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 (SEM). Statistical significance was determined by ANOVA. * $p > .001$

Figure 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 hours 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 (SEM). 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 figure 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 hours. The figure shows a representative RPA assay performed using 10 μ g 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

Figure 8. MCF-7-induction of apoptosis in Fas-bearing cells. [³H] Thymidine labeled target cells were co-cultured with MCF-7 cells at different E:T ratio for 24h. 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 24H.

Figure 9. Induction of apoptosis in Jurkat cells by FasL expressing Jar cells. [³H] Thymidine labeled Jurkat target cells were co-cultured with choriocarcinoma cell line, Jar cells at different E:T ratio for 24h. 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.

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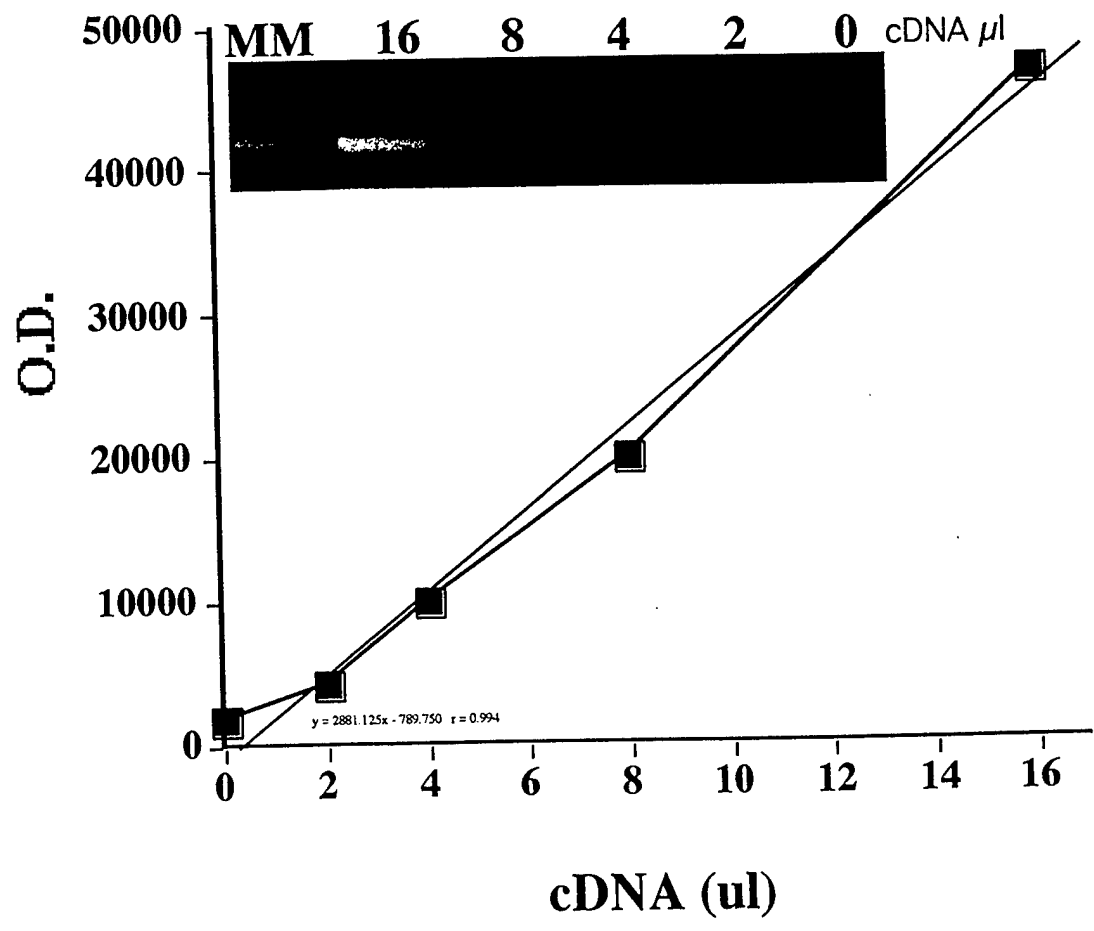
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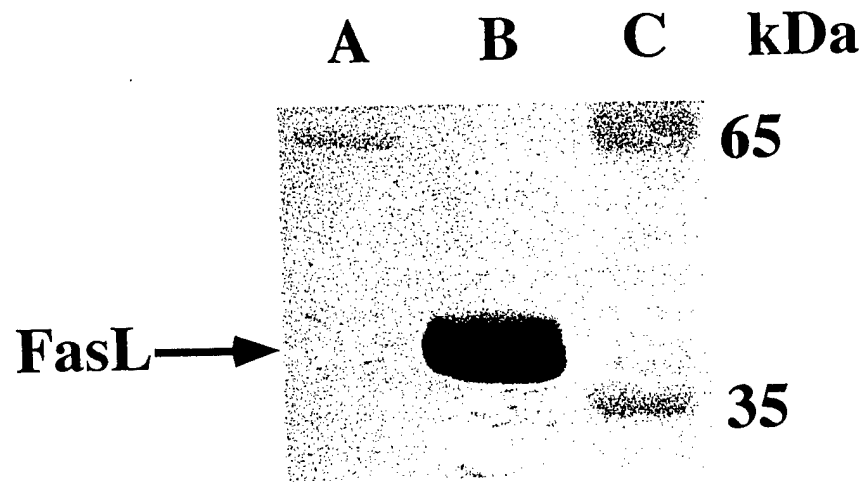
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A

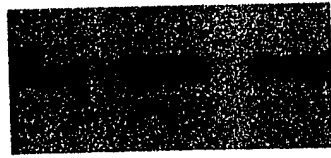


B



A

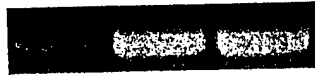
Western blot



← **37-kDa**

B

RT-PCR



← **FasL**



← **Beta Actin**

Control 24h 48h

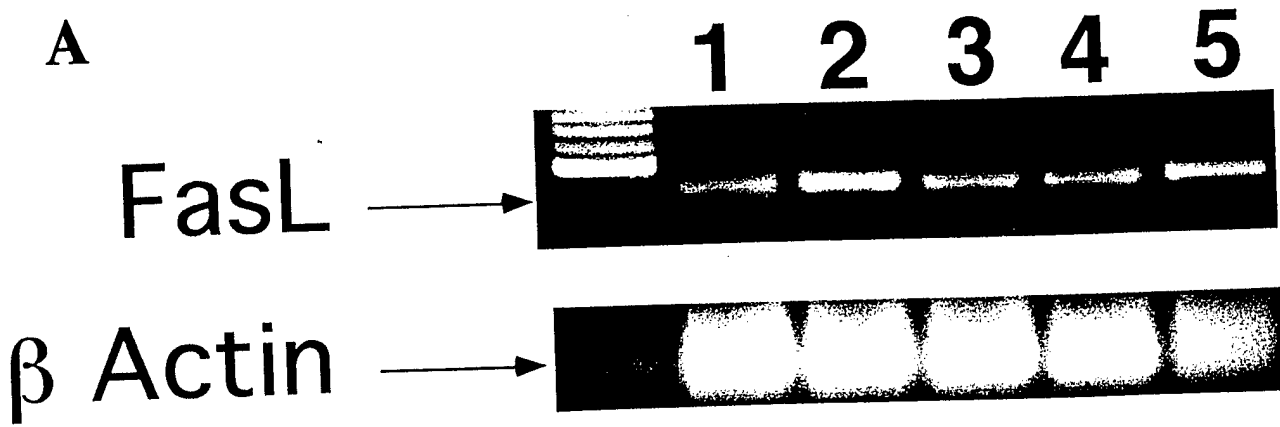
Vehicle (EtOH, 0.01%)

+ - -

Estradiol (10^{-8} M)

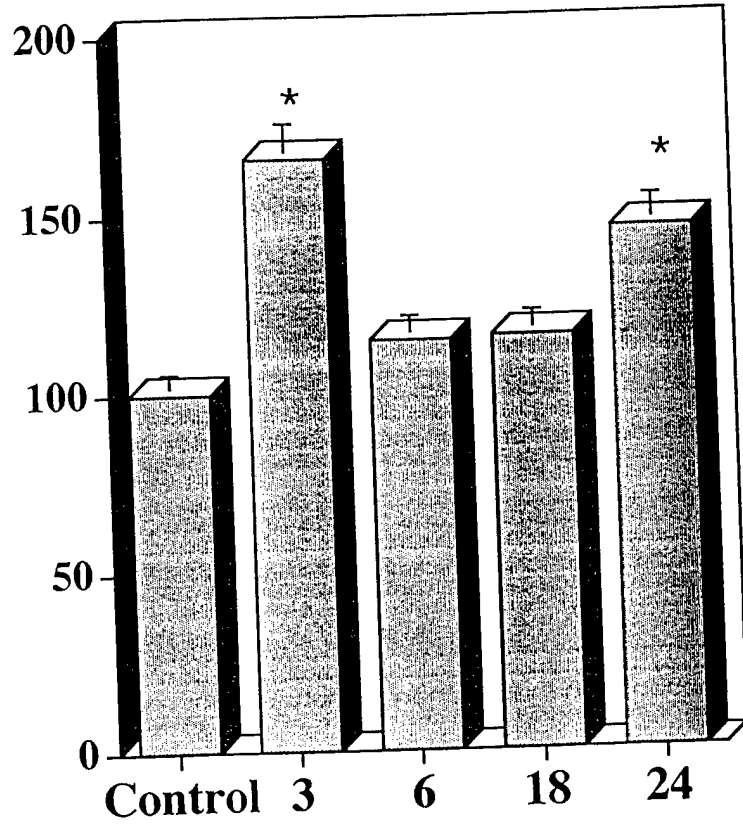
- + +

A



B

Percentage of FasL mRNA Expression
relative to the control



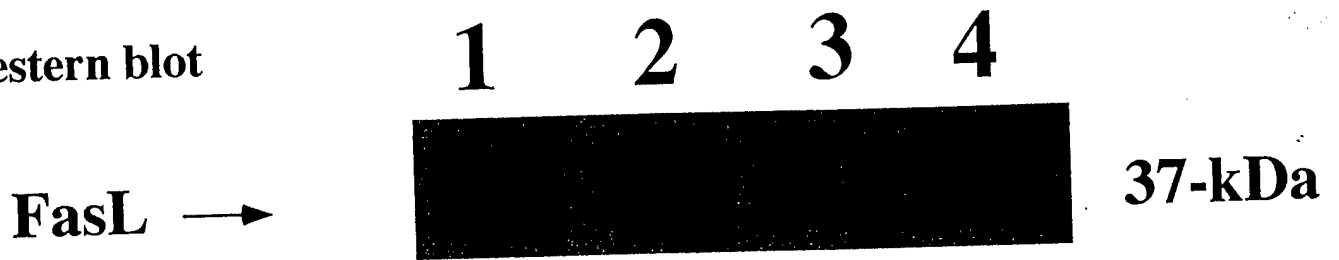
Vehicle (EtOH, 0.01%)

+ - - - -

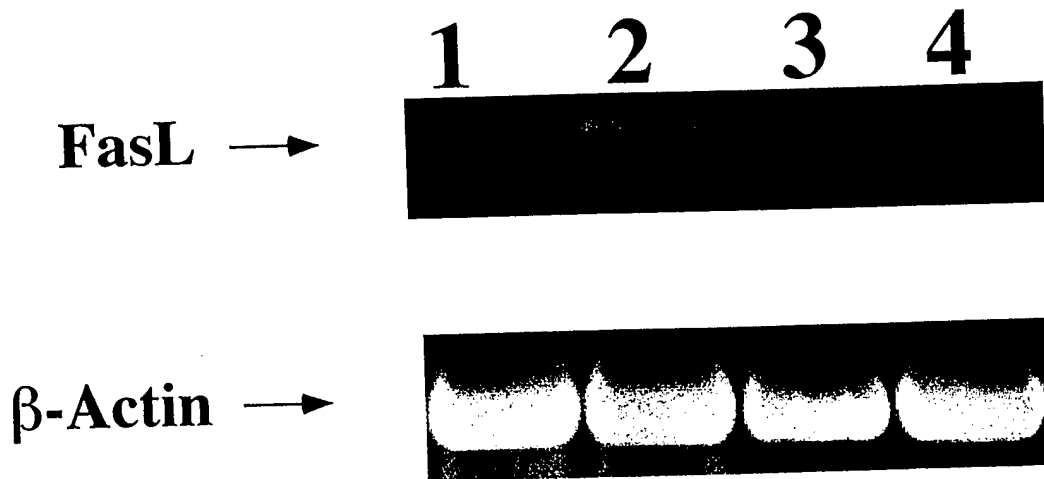
Estradiol (10^{-8} M)

- + + + +

A Western blot



B RT-PCR



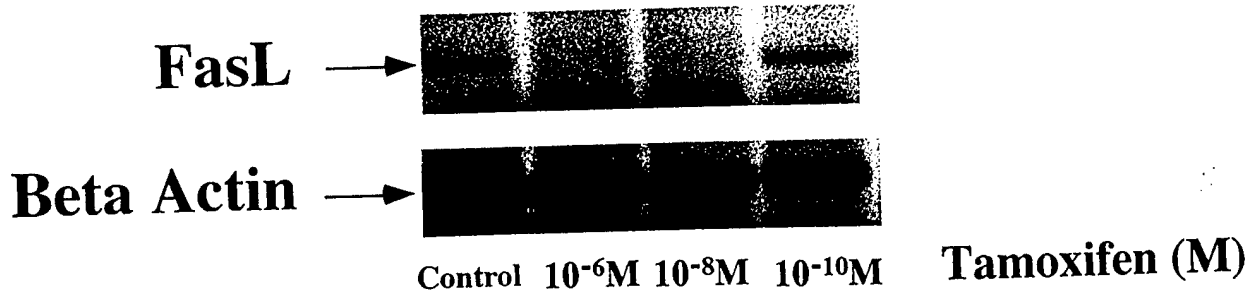
Tamoxifen (10^{-7} M)

- - + +

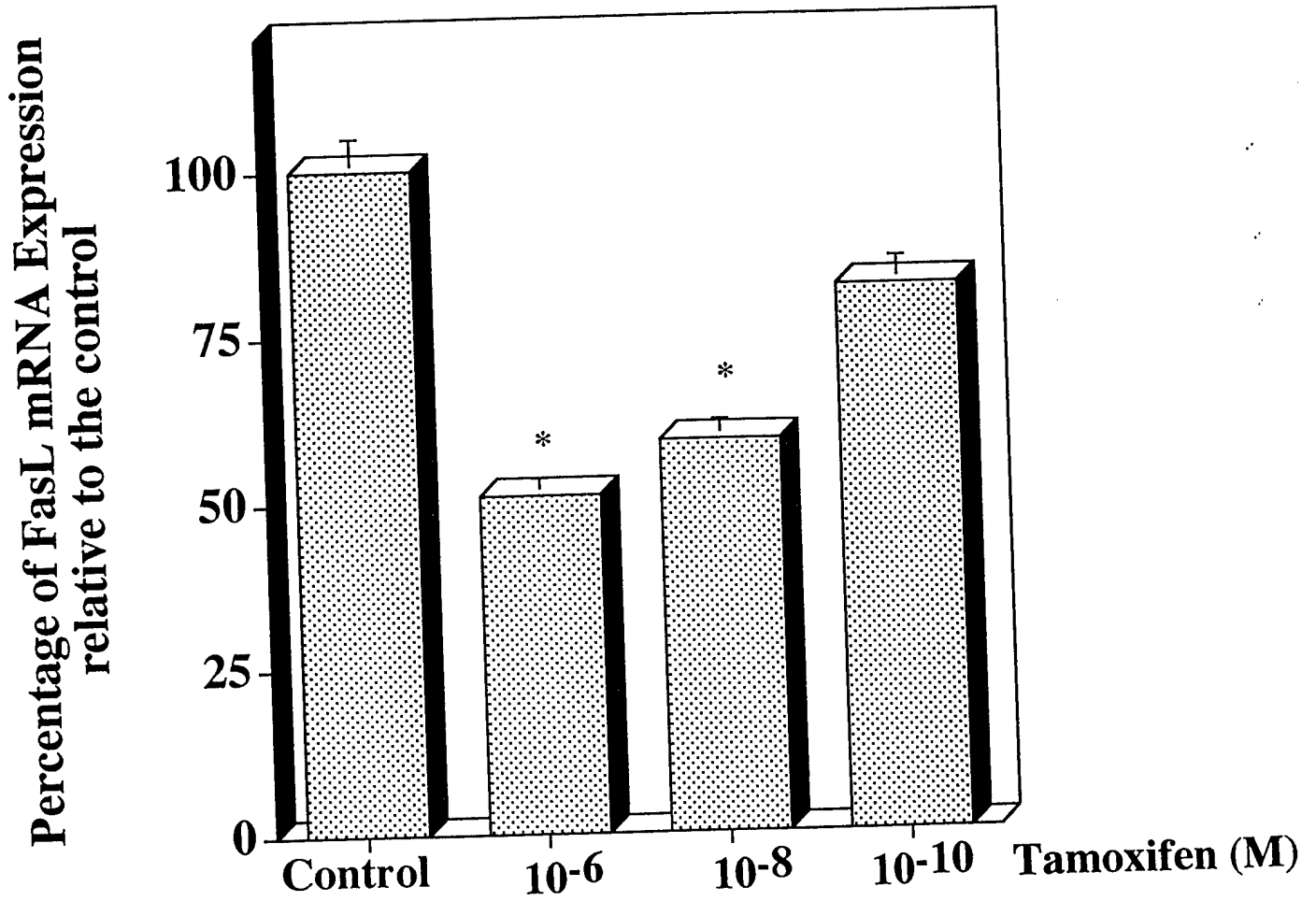
Estradiol (10^{-8} M)

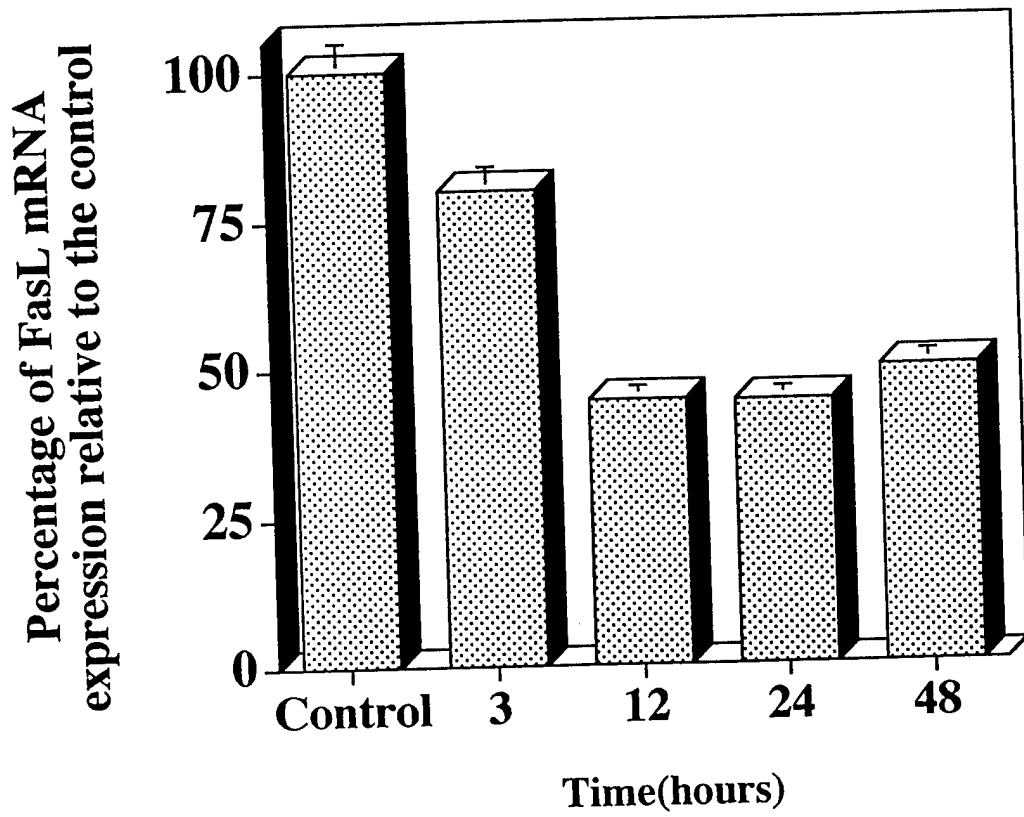
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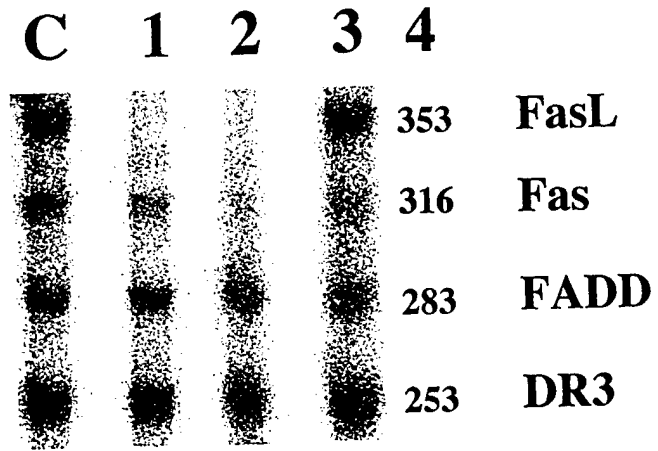


B





Vehicle (EtOH, 0.01%)	+	-	-	-	-
Tamoxifen (10^{-7} M)	-	+	+	+	+



Vehicle (EtOH, 0.01%)

Tamoxifen (M)

+	-	-	-
-	10^{-6}	10^{-8}	10^{-10}

