Effects of Type I and II Interferons on Cultured Human Breast Cells: Interaction with Estrogen Receptors and with Tamoxifen

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ABSTRACT

The combined effects of tamoxifen, a competitive inhibitor of estrogen, and type I or II interferons on the proliferation of several human breast cancer cell lines in vitro were examined. Additive antiproliferative effects were observed with interferons and tamoxifen, in two estrogen receptor negative cell lines, MCF-7 and T-47D. In MCF-7 cells neither βser interferon, γ interferon, nor α interferon were able to signifi- cantly alter estrogen receptor levels. Antigrowth activities of βser interferon and γ interferon in an estrogen receptor negative cell line, HS578T, were equivalent to those in estrogen receptor-positive cell lines. Consistent with the antiproliferative effects of interferons, high affinity βser interferon receptors and interferon inducible 2'-5'-oligoadenylate synthetase were present in both MCF-7 and HS578T cell lines. Thus steroid hormone receptor status did not influence the antiproliferative effects of interferons on breast carcinoma cells in vitro.

INTRODUCTION

IFNs have antitumor effects against a variety of human malignancies. However, the exact cellular mechanisms of these antiproliferative effects have yet to be defined. IFNs modulate a variety of intracellular proteins (1) and may interact with receptor proteins, particularly those which act as growth factors. IFNs may thus be regarded as paracrine hormones (2) sharing common second-messenger pathways of cellular modulation with other polypeptide signals (3). IFNs may also share with other hormones the ability to influence not only their own receptor expression (i.e., down-regulation) and steroid hormone receptors (5). Analysis has been presented for an effect in vitro of IFN on both insulin and epidermal growth factor receptors.

Published studies regarding the influence of IFN on steroid hormone receptor expression have been conflicting. Three studies have suggested increases in ER expression after exposure to IFN under defined conditions (8–10). Breast cancer biopsy homogenates which were ER-positive showed increased ER content in incubation in vitro with either IFN-α2a or β interferon derived IFN-α (8). Low concentrations of IFN-α2a increased ER receptor content in the ER-positive ZR-751 breast cancer cell line when added to cells plated at low density (9). A study with the CG-5 cell line, an MCF-7 variant with high expression of the ER, showed that low dose fibroblast-derived IFN-α added to cells plated at low density increased receptor content (10). In contrast, two other studies using either a variety of human breast cancer cell lines (11) or CG-5 (12), showed either no effect or decreased receptor content in response to high dose IFN-α2a, IFN-γ or fibroblast-derived IFN-β. In vivo treatment with fibroblast-derived IFN-β showed an increase in ER content in two of two and in PR content in five of six patients whose skin metastases were biopsied before and after treatment (13). In contrast, endometrial biopsy in five volunteers treated with buffy coat-derived IFN-α showed no change in ER or PR (14). Human breast cancer cells have shown sensitivity to the antiproliferative effect of all three IFN types (11, 12, 14–18). Binding of estrogen is a major factor in the growth stimulation of some breast carcinoma cells, an effect which can be blocked by TAM (19). We wished to determine the combined influence of interferons and tamoxifen on breast carcinoma growth and to clarify the influence of interferons on estrogen binding to its receptor.

We initially determined the presence of IFN receptors on both an ER-positive and -negative cell line. We then assessed the biological effects of IFN-β interferon on them. Finally, we determined the influence of IFN-α and IFN-γ on ER content and the combined antiproliferative effects of TAM with IFN-α and IFN-γ.

MATERIALS AND METHODS

MEM, DMEM with 4.5 g/liter of glucose, and RPMI 1640 medium were obtained in powder form from Sigma Chemical Company, St. Louis, MO. All media was supplemented with L-glutamine (2 mM), penicillin (10 units/ml), streptomycin (10 mg/ml) and 10% heat-inactivated fetal bovine serum. Nonessential amino acids were also added to MEM and 0.1 IU insulin/ml to RPMI (for T-47D cells). Tamoxifen, estradiol, epidermal growth factor, protease, and media supplements were obtained from Sigma Chemical Company. Fetal bovine serum came from HyClone, Logan, UT, and culture dishes, flasks and plates from Costar Company, Cambridge, MA. Recombinant IFN-β interferon (2 x 10° IU/mg protein) and IFN-α (1–2 x 10° IU/mg protein) were supplied by Tri-biotics, Inc., Alameda, CA, and IFN-γ (2 x 10° IU/mg protein) was a gift of the Biogen Corporation, Cambridge, MA. All interferons were produced by recombinant DNA technology. 125I-[2,4,6,7-3H]Estradiol (99 Ci/mMl) was obtained from New England Nuclear, Boston, MA. Scintillation vials were obtained from Research Products International, Mt. Prospect, IL. Aquasol liquid scintillation fluid from IDN, Boston, MA, and BCA reagent from Pierce Chemicals, Rockford, IL.

Cells and Cell-Culture Conditions

The MCF-7 cell line was obtained from Dr. J. Fogh of the Memorial Sloan-Kettering Cancer Center, NY; T-47D and HS578T cell lines were obtained from the ATCC. Both MCF-7 and T-47D are ER-positive human breast cancer cell lines. HS578T is an ER-negative cell line. MCF-7 cells were maintained in MEM. T-47D cells were maintained in RPMI 1640, and HS578T cells were grown in DMEM. The Daudi cell line, a Burkitt lymphoma cell line, was obtained from ATCC and grown in RPMI. Cells were grown in air:CO2 atmosphere (95:5 v/v) at 37°C. Adherent cells were grown in T-75 flasks and were harvested by an initial wash with calcium and magnesium-free Hanks’ balanced salt solution followed by a 2-min incubation with a 0.25% trypsin solution containing 1 mM EDTA.

IFN Receptor Assay

IFN-β interferon receptor measurement was performed by a method developed in our laboratory (20). rIFN-β interferon was radiolabeled by the chloro-
mine T procedure (21) to a specific activity of 80–150 μCi/μg, stabilized for storage in a 1.2% HSA solution and stored at -70°C. Binding of $[^{125}]$IFN-βser to MCF-7, HS578T and Daudi cells was assessed in a suspended cell assay. The adherent breast cancer cells were grown to subconfluence in 225-cm$^2$ flasks, gently trypsinized in a 0.5% (w/v) trypsin and 0.2% (w/v) EDTA solution for 2–3 min and resuspended in their respective growth media. Daudi cells, a suspended lymphoblastoid cell line, were grown in a 3-liter spinner flask.

Cells were collected by centrifugation, washed twice with cold RPMI and resuspended in RPMI containing 0.1% HSA (w/v) to give 6.0–10 × 10$^6$ cells/ml. Aliquots (0.5 ml) of the cell suspensions were added to 15–85-mm borosilicate glass tubes containing $[^{125}]$IFN-βser (1–2 × 10$^{-5}$–0.25 × 10$^{-5}$ ng/ml) and 3.5 ml of the incubation media. Parallel incubations containing 250 ng/ml of unlabeled IFN-βser were used to assess nonspecific binding. Nonspecific binding to Daudi, HS578T and MCF-7 was 15, 43, and 46%, respectively. For Scatchard analysis of binding parameters, increasing concentrations of unlabeled IFN-βser were added (0.12–250 ng/ml) to generate ligand titration curves.

Assays were incubated 18–24 h at 4°C and harvested by centrifugation (1000 × g, 5 min). Cells were washed once in 4 ml of ice cold phosphate buffered saline. The contents of each tube was resuspended in 0.5 ml and transferred to a new glass tube for counting. Radioactivity was determined in a gamma counter (Searle) with a 65% counting efficiency. All assay points were done in duplicate, three replicate experiments performed and all values corrected for nonspecific binding. Scatchard plots were analyzed using the Ligand program (Munson and Rodbard, Elsevier Scientific Software, Cambridge, UK).

### 2′,5′-Oligoadenylate Synthetase Assay

The activity of the 2′,5′-oligoadenylate synthetase enzyme in incorporating $^3$H-ATP into 2′,5′-oligoadenylate cores was measured (22). Cell lysates were incubated with poly(rI.rC)agarose, allowing the 2′,5′-synthetase enzyme to adhere, incubated for 20 h with 1.35 μM ATP buffer solution and digested with bacterial alkaline phosphatase. Aliquots (5 μl) were pipetted onto DEAE-cellulose paper, washed, eluted with 0.3 m KCl and counted. Enzyme activity, defined as picomoles ATP incorporated per 10$^5$ cells per hour, was calculated from the percent conversion of $[^{3}H]$ATP into oligoadenylate cores.

### Estrogen Receptor Assay

ER content was determined by a whole cell binding assay (23). Cells were plated in 24-well (16 mm) plates at 5 × 10$^4$ cells/well. For the initial assays, cells were allowed to reach semiconfluence (approximately 80%). The medium was replaced with 1 ml of medium containing 10% CSS. After 24 h, this medium was removed and replaced with fresh medium (CSS) with or without IFN-βser or IFN-γ for 24 or 48 h. For the estimations at low plating density, cells were allowed to adhere overnight and then incubated for 48 h in medium (CSS) with or without IFN-βser, IFN-γ, or IFN-α, or 5 days with low dose IFN-βser. CSS was used to decrease the chance of estrogen in the serum causing ER down-regulation. In some experiments (Table 3), phenol red, a weak estrogen, was also removed.

At the end of incubation, the medium was removed and replaced with 0.5 ml of medium containing a single saturating dose (6 nM) of $[^{3}H]$estradiol (99 Ci/mmol) alone or together with a 100-fold excess of estradiol to estimate total binding and nonspecific binding. For experiments where determinations of affinity and receptor content according to the method of Scatchard were performed, eight points were used over a range of $[^{3}H]$estradiol concentrations (0.02–18 nM). After 45-min incubation at 37°C, medium was removed and the cells washed four times with cold phosphate buffered saline with 0.1% bovine serum albumin. One ml of ethanol was then added to each well and left for 10 min. Ethanol was then transferred to a scintillation vial containing 10 ml of Aquasol and counted by liquid scintillation. Duplicate untreated wells were washed three times and then 1 ml of phosphate buffered saline diluted 1:10 with water was added to extract protein. These samples were stored at -20°C and subsequently thawed, sonicated, and analyzed for protein using the BCA assay (Pierce Chemicals, Rockford, IL). Scatchard analysis was performed using the Ligand program.

### Antiproliferative Assay

Cells were initially plated at a concentration of 5 × 10$^4$ cell/well in 2 ml of media in 6-well (35-mm) plates. Following overnight incubation, fresh media was added containing either IFN-βser, TAM, a combination of both drugs at the indicated concentrations or media alone. Cells were then incubated for 7 days. Media was changed every 2–3 days with fresh media containing the same concentration of drug prepared at the onset of the experiment. After 7 days, adherent cells were dissociated for counting by adding 2 ml per well of saline (0.15 M NaCl) containing 0.15% (w/v) protease. After incubation for 2 h at 37°C, single-cell suspensions were obtained by vigorous pipetting and the contents of each well was transferred to a counting vial. Cell number was determined using a Coulter counter (Coulter Instrument Corporation, Hialeah, FL).

For the sequence experiments, cells were plated as above, but IFN alone was added for the first 5 days. The IFN-containing media was aspirated and replaced with media containing TAM and incubated for an additional 5 days. This was compared to incubation with both drugs for 5 days. Potentiation was estimated by multiplying the percent of cells remaining compared to control of each individual agent (24). Supra-additivity was defined as

$$
\% AB = \frac{\% A \times \% B}{1} < 1,
$$

additivity was defined as

$$
\% AB = \frac{\% A \times \% B}{1} = 1,
$$

and subadditivity was defined as

$$
\% AB = \frac{\% A \times \% B}{1} > 1,
$$

where $A$ and $B$ are the effects of each individual agent and $AB$ is the effect of the combination.

### Statistics

One-way analysis of variance (one-way ANOVA) was used to test the effects of interferon type and dose level on estrogen receptors. The stationary and log phase data were analyzed separately. Power calculations were based on $t$ tests between control and each interferon type where the standard deviations were estimated from a one-way ANOVA on interferon type only. Pooling dose levels was justified since the mean squared error for the ANOVA did not increase.

### RESULTS

IFN Receptors and 2′,5′A Synthetase Induction in Breast Cells. Specific binding of $[^{125}]$IFN-βser by untreated MCF-7 and HS578T cells was determined by a single-point assay of suspended cells. HS578T, the ER-negative cell line, bound approximately 2.7× more IFN-βser per 10$^6$ cells than MCF-7, the ER-positive cell line (0.996 ± 0.56 fmol/10$^6$ cells versus 0.373 ± 0.06 fmol/10$^6$ cells). Similar results were obtained whether the binding was done to adherent cells in plates (data not shown) or to suspended cells following brief trypsinization. As a reference, the amount of binding is shown for an equivalent number of Daudi cells, a lymphoblastoid cell line highly sensitive to the synthesis of IFN-induced proteins and the antiproliferative effect of IFN-βser. Daudi cells bound 3- to 9-fold more IFN-βser than HS578T and MCF-7 cells, respectively (2.90 ± 0.56 fmol/10$^6$ cells). The LIGAND program was used to analyze titration binding data for estimation of receptor molecular weight and number. Using a nonspecific binding correction estimated by the program, linear Scatchard plots were obtained for both breast cell lines. Both cell lines exhibited...
high affinity binding of IFN-βser with a molecular weight in the pm range (57 ± 15 for HS578T and 124 ± 43 for MCF-7). The difference in total binding between the two cell lines was primarily a result of the different number of receptor sites per cell (3523 ± 672 and 1014 ± 532 respectively) rather than differing receptor affinity.

IFN-βser increased 2′5′ oligoadenylate synthetase activity 12 fold and 374 fold in MCF-7 and HS578T respectively (Table 1). There was maximal induction of 2′5′A synthetase at low doses of IFN-βser, unlike the antiproliferative effect of IFN which was dose responsive over the same range, from 0.1 to 10 ng/ml (see below). No difference in 2′5′A synthetase induction was seen between cells assayed when growing at low or high density (data not shown). Tamoxifen had no effect on 2′5′A synthetase.

Effect of IFN on ER. The effect of IFN on ER levels was examined, initially in single-dose assays. Semiconfluent MCF-7 cells were incubated for 48 h with IFN-βser or IFN-γ (0.1–100 ng/ml, 10–1000 IU/ml). No significant changes in ER binding occurred in response to any dose level of IFN (Table 2). Similar results were seen after 24 h of IFN incubation (data not shown). Scatchard analysis of a single ER binding experiment at each IFN dose level confirmed that no changes were seen in receptor affinity.

The effect of 48 h of treatment with IFN on ER levels at low plating densities (10) was also examined using both single dose and multiple dose ligand assays. No significant change in ER binding was seen (Table 2). Scatchard analysis revealed no changes in affinity or receptor concentration. When IFN-α6 was used, results were identical to those with IFN-βser (Table 2). MCF-7 cells plated at low density were also incubated for 5 days with low dose IFN-βser 0.1 ng/ml, again without significant increase in ER as determined by Scatchard analysis in the presence or absence of phenol red (Table 3). A representative Scatchard plot of [3H]estradiol binding to MCF-7 cells plated at low density and treated with a low dose of rIFN-βser (0.1 ng/ml) for 48 h demonstrates the lack of differences in both receptor number and affinity (Fig. 1). The intrassay variation in the measurement of IFN effects on ER was 10%. However between experiments there was a larger variation in the absolute values of ER as well as the percentage differences with treatment as noted by others (13). Given this variation, a difference of less than 30–50% in ER receptor number could not be statistically detected.

Antiproliferative Effects. The effect of combining IFN-βser 0.5 ng/ml (100 IU/ml) and TAM (10⁻⁸ - 5 x 10⁻⁶ M) was first compared to either drug alone. As previously reported (8, 10, 11), the effect was additive rather than supra-additive. At each concentration of TAM, the antiproliferative effect of the combination was greater than either drug alone (Fig. 2A) but not more than would be predicted by an additive model. Similar results were obtained when TAM was combined with either 0.05 ng/ml or 2.5 ng/ml of rIFN-βser (10 IU/ml and 500 IU/ml: data not shown). Simultaneous administration of rIFN-βser and TAM was also tested on the T-47D cell line (Fig. 2B). Both compounds had an antiproliferative effect individually; the combination was additive. The effect of IFN-γ and TAM was tested in MCF-7 cells. Additivity was also observed over a range of TAM combinations with 5 ng/ml (100 IU/ml) of IFN-γ (Fig. 2C) or with 50 ng/ml (1000 IU/ml) of IFN-γ (data not shown).

To test whether pretreatment of MCF-7 cells with IFN-βser at a low dose of 0.05 ng/ml (10 IU/ml) would increase the antiproliferative effect of subsequent TAM administration, cells were treated with IFN-βser or media alone for 5 days followed by 5 days of varying concentrations of TAM (10⁻⁸-10⁻⁶ M) or 5 days of the combination of IFN and TAM (Fig. 3A). Again the simultaneous combination of drugs resulted in an additive antiproliferative effect. In contrast, sequential treatment with IFN-βser alone followed by TAM showed a subadditive effect...
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Fig. 2. A, effect of IFN-βser (0.5 ng = 100 IU), tamoxifen, and the combination are compared to the predicted outcome as a percentage of the control MCF-7 cells (for the calculation see "Materials and Methods"). Results are shown as the mean and standard error of four experiments. A, tamoxifen; •, IFN-βser; □, combination. B, as per A in T-47D cells, results are a mean of three experiments. A, tamoxifen; •, IFN-βser, D, combination. C, as per A, IFN-γ (10 ng = 100 IU) in MCF-7 cells. Results are a mean of three experiments. A, tamoxifen; •, IFN-γ; □, combination.

DISCUSSION

In view of previous work suggesting synergy with other antiproliferative agents such as chemotherapy and radiation, we examined the combination of IFN with TAM to see if this would enhance the antiproliferative effects of IFN. The antiproliferative effect of the combination was additive in two ER-positive cell lines (MCF-7 and T47D). Since extending exposure from 7 to 10 days resulted in synergy with the TAM analogue, toremifene (25), MCF-7 cells were exposed to 11 days of the combination of IFN-βser and TAM which also did not result in any increase in effect over additivity (data not shown).

A report of an increase in ER levels by IFN-α2b (9) led us to examine whether IFN-βser or IFN-γ could modulate ER. Potentiation of receptor expression by IFNs might lead to a synergistic antiproliferative effect with antiestrogens. Neither IFN-β nor IFN-γ caused increases in ER expression in semi-confluent MCF-7 cells at low or high doses. Similarly, low dose IFN-βser in cells plated at low density failed to demonstrate significant increases in ER content for either 48-h or 5-day exposure. Our two experiments with IFN-α6 in MCF-7 also failed to show any difference in ER expression, in contrast to the findings of the two previous reports that showed increased levels of ER (9, 10). Thus we feel that the different IFNs used in those experiments (IFN-α2b and fibroblast-derived IFN-β, respectively) may not explain the differences in our findings.

Lack of biologically significant upregulation of ER by IFN was supported by the antiproliferative results which showed that the use of low-dose IFN-βser prior to TAM did not result in a synergistic antiproliferative effect. Furthermore, we demonstrated a dose-response effect of IFN pretreatment on the sequential treatment across all three TAM concentrations. However when comparing the effect of sequence versus combination at individual TAM concentrations, the combination was significantly better than the sequence at TAM 10^{-8} M (P < 0.0001).

When MCF-7 cells were treated sequentially with IFN-βser (0.05, 0.5, and 5 ng/ml) for 5 days followed by 5 days of TAM (10^{-8} -10^{-6} M) the antiproliferative effect of the sequence increased with increasing doses of both agents (Fig. 3B).

The antiproliferative effects of IFN-βser and IFN-γ as single agents were also compared in an ER negative cell line HS578T. Growth inhibition by both IFNs in HS578T (Fig. 4) was equivalent to that observed in the two ER-positive cell lines. The antiproliferative effect was proportional to duration of exposure to IFN.
antiproliferative effect of TAM. If ER modulation was occurring as a unique effect of exposure to low-dose IFN, one would have expected the reverse, namely that preincubation with low-dose IFN would result in TAM having a greater antiproliferative effect than incubation with higher IFN doses. The two previous reports used different cell lines, ZR-751 (9) and CG-5 (10), to demonstrate the effect of low-dose IFN on ER upregulation. However, neither report presented evidence for a synergistic antiproliferative effect as a result of pretreatment with low-dose IFN.

A number of reports have demonstrated the sensitivity of human breast carcinoma cell lines in vitro and human tumor xenografts in nude mice to both type I and II IFNs (11, 12, 15–18). Despite the absence of ER modulation, the combination may merit further exploration for therapy of breast cancer. The antiproliferative effect of IFN, unlike TAM, was not related to modulation of ER. Consistent with its receptor expression, IFN was effective in inducing 2-5A synthetase and antiproliferative effects in both ER-negative and ER-positive cells. Thus, although IFN and TAM were additive in their antiproliferative effect against ER-positive cell lines, their different mechanisms of action may result in synergy against the typical hormonally heterogeneous breast cancer.

REFERENCES


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