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Recombinant interferon-α2b affects proliferation, steroid receptors and sensitivity to tamoxifen of cultured breast cancer cells (CG-5)

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Exposure of CG-5 human breast cancer cells to recombinant interferon (IFN)-α2b results in a significative inhibition of cell proliferation; this is observed when cells are cultured in their standard conditions and is not modified if serum concentration present in the culture medium is lowered. Estrogen receptors are increased in CG-5 cells following a 5 day treatment with concentrations of IFN-α2b ranging from 10 to 1000 IU/ml of culture medium. Progesterone receptors seem to be more influenced by a longer treatment with the drug (7 days). The K_d of both receptors is not modified by the exposure of cells to IFN-α2b. Finally, the antiproliferative effect of tamoxifen on CG-5 cells is amplified by the simultaneous addition to culture medium of IFN-α2b even at very low concentrations.

Key words: Antiestrogens, breast cancer, cell proliferation, interferon, steroid receptors.

Introduction

Much attention has been focused on the capability of interferon (IFN) of regulating steroid receptor expression and influencing the response to hormones and antihormones. A recent report showed that natural IFN-β affects cell proliferation of a highly estrogen-sensitive human breast cancer cell line (CG-5).[1]

Moreover, IFN-β enhances both estrogen receptors (ER) and progesterone receptors (PR), and promotes the antiproliferative activity of tamoxifen (TAM) and medroxyprogesterone acetate in CG-5 cells.[1–4] In agreement with these results, van den Berg *et al.*[5] demonstrated that recombinant IFN-α2 modifies ER content in ZR-75-1 cells and sensitizes them to the anti-growth action of TAM.

However, Goldstein *et al.*[6] failed to demonstrate any significant increase in ER content using IFN-β_{ser} and IFN-α in MCF-7 cells after 48 h or 5 days of exposure to the drugs, using different plating densities and different concentrations of IFNs.

With the aim of verifying if IFN-α2b behaves like IFN-β in CG-5 cells, we studied the effect of this type of IFN on cell growth, receptor content and sensitivity to the antiestrogen TAM.

Materials and methods

Cells and culture conditions

The human and mammary origin of CG-5 cell line has been previously described.[7] Cells were maintained routinely in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, UK) supplemented with 10% fetal calf serum (FCS, Flow Laboratories), 20 mM HEPES (Sigma, St Louis, MO), 200 IU/ml penicillin and 200 μg/ml streptomycin (Flow Laboratories), and grown in an air-CO_2 atmosphere (95:5) at 37°C.

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Materials

Recombinant IFN-α2b was kindly provided by Schering Plough, Italy. Its biological activity was 2×10^8 IU/mg protein. TAM was kindly donated by ICI-Pharma, Italia, Italy. [2,4,6,7-^{3}H(*N*)]Estradiol ([^{3}H]E_2, specific activity 93.3 Ci/mmol) was purchased by New England Nuclear (Boston, MA). Diethylstilbestrol (DES) was provided by Sigma. 16-α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]-pregn-4-ene-3,dione ([^{3}H]ORG 2058, specific activity 57.0 Ci/mmol) and unlabeled ORG 2058 were purchased by Amersham International (Buckinghamshire, UK).

Cell growth experiments

The ability of IFN-α2b to inhibit the growth of CG-5 cells was determined by plating out the cells at an initial density of 50 000 cells/ml in 60 mm plastic Petri dishes in DMEM supplemented with 10% FCS, HEPES and antibiotics. After 24 h, the medium was changed with DMEM supplemented with 10 or 5% FCS or 5% charcoal-treated FCS (CH-FCS), HEPES, antibiotics and various concentrations of IFN-α2b ranging from 5 to 1000 IU/ml of culture medium.

In experiments concerning the effect of IFN-α2b associated with TAM, CG-5 cells, plated 24 h before in the medium previously described, were exposed to various concentrations of IFN-α2b and 10^{-7} M TAM, in medium supplemented with 5% CH-FCS, HEPES and antibiotics.

Triplicate cultures were set up in all experiments for each drug concentration and medium was renewed after 3 days. Cells were counted in a hemocytometer after 3 and 6 days of exposure to the drugs. Control dishes, without drugs, were run in parallel.

Steroid hormone receptor assay

CG-5 cells were plated at an initial density of 50 000 cells/ml in 35 mm plastic Petri dishes in DMEM supplemented with 10% FCS, which was renewed after 24 h with the addition of 10, 100 or 1000 IU/ml of IFN-α2b. Cell dishes were set up in triplicate and control dishes were cultured in parallel. Medium was changed every 3 days.

Steroid receptor determination was performed by a whole cell assay after 5 and 7 days of IFN-α2b treatment. At each time, cells were washed 3 times with medium without FCS and left for 1 h in the same medium at 37°C in a humidified air with 5% CO_2. Then medium was renewed with DMEM containing 5 nM of [^{3}H]E_2 in the presence or absence of a 100-fold excess of DES for the determination of ER, and 5 nM[^{3}H]ORG 2058 in the presence or absence of a 200-fold excess of unlabelled ORG 2058 for the determination of PR.

B_{max} and K_d were determined using a six-point Scatchard analysis for ER and an eight-point Scatchard analysis for PR. In this case the labeled ligand was used in a concentration range of 0.1–5 nM.

After 1 h incubation with the ligands, cells were washed twice with Hanks' balanced salt solution at room temperature prior to extracting radioactivity with 1 ml of 80% ethanol overnight.

Radioactivity was measured by liquid scintillation counting. ER and PR content was expressed as the number of sites/cell. Cell number was determined in a series of plates which underwent the same experimental procedure.

Data analysis

Analysis of variance with multiple comparison (Fisher's test) was used to test the effect of IFN-α2b alone or combined with TAM on cell growth. To evaluate the effect of the combination of two drugs we used the following definitions:[8]

$SF_{A+B} > (SF_A) \times (SF_B)$ antagonist effect
$SF_{A+B} = (SF_A) \times (SF_B)$ additive effect
$SF_{A+B} < (SF_A) \times (SF_B)$ synergistic effect

where SF is the cellular surviving fraction; A and B indicate the agent used alone, while A + B is the combination of two drugs.

Statistical differences in receptor levels, which showed a large variability both in control and IFN-treated cells, were calculated by means of Wilcoxon's signed rank test.

Results

Effect of IFN-α2b on CG-5 cell proliferation

Figure 1 shows the effect of 3 and 6 days of exposure to IFN-α2b on proliferation of CG-5 cells grown in their standard conditions (presence of 10% FCS). After 3 days of treatment the maximum inhibition was observed at concentrations of 500–1000 IU/ml

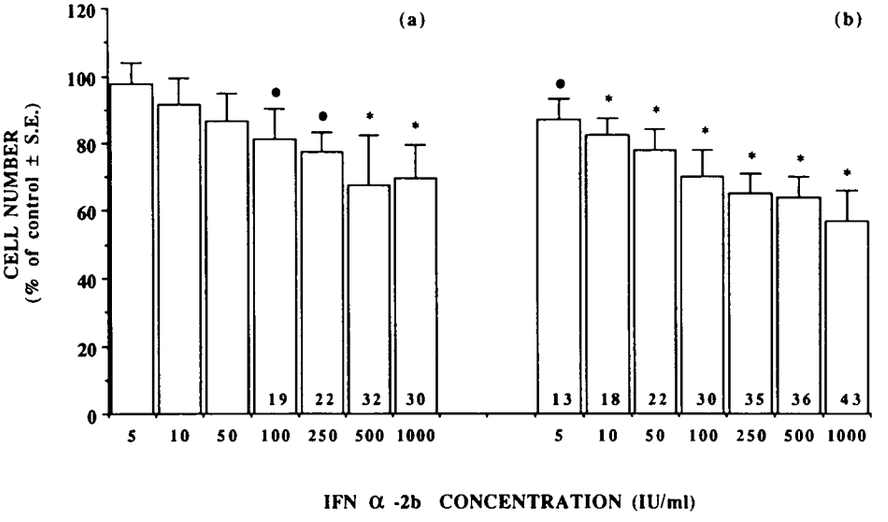

Figure 1. Effect of various concentrations of IFN-α2b on the growth of CG-5 cells cultured in medium supplemented with 10% FCS after (a) 3 days and (b) 6 days of treatment. The absolute number of cells/dish in control cultures was 970 000 after 3 days and 3 454 000 after 6 days. Columns represent the average of three different experiments. Bars represent standard error. The number at the column bottom is the percentage of growth inhibition with respect to control. •$p < 0.05$, *$p < 0.01$ (Fisher's test versus control).

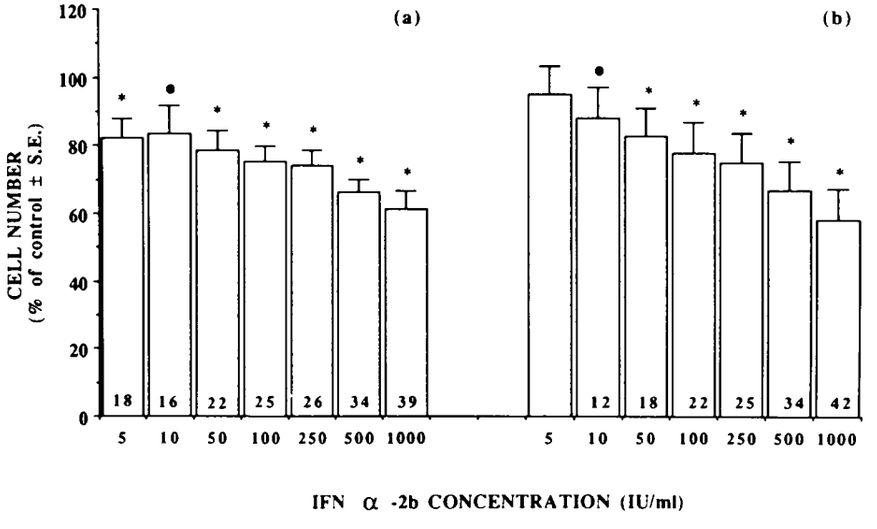

Figure 2. Effect of various concentrations of IFN-α2b on the growth of CG-5 cells cultured in medium supplemented with 5% FCS after (a) 3 days and (b) 6 days of treatment. The absolute number of cells/dish in control cultures was 870 000 after 3 days and 3 132 000 after 6 days. Columns represent the average of three different experiments. Bars represent standard error. The number at the column bottom is the percentage of growth inhibition with respect to control. •$p < 0.05$, *$p < 0.01$ (Fisher's test versus control).

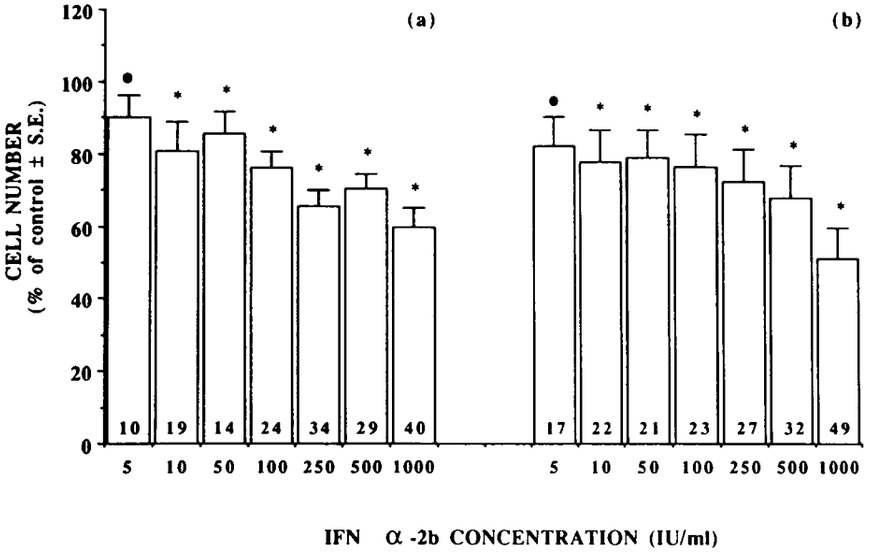

Figure 3. Effect of various concentrations of IFN-α2b on the growth of CG-5 cells cultured in medium supplemented with 5% CH-FCS after (a) 3 days and (b) 6 days of treatment. The absolute number of cells/dish in control cultures was 507 000 after 3 days and 1 799 000 after 6 days. Columns represent the average of three different experiments. Bars represent standard error. The number at the column bottom is the percentage of growth inhibition with respect to control. •$p < 0.05$, *$p < 0.01$ (Fisher's test versus control).

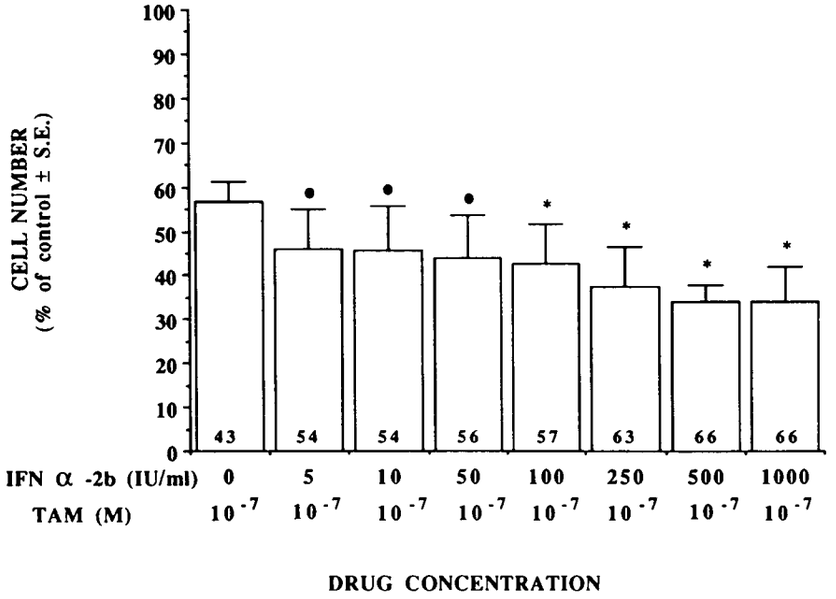

Figure 4. Effect of various concentrations of IFN-α2b and 10^{-7} M TAM on the growth of CG-5 cells cultured in medium supplemented with 5% CH-FCS after 6 days of treatment. Columns represent the average of three different experiments. Bars represent standard error. The number at the column bottom is the percentage of growth inhibition with respect to control. •$p < 0.05$, *$p < 0.01$ (Fisher's test versus TAM-treated cells).

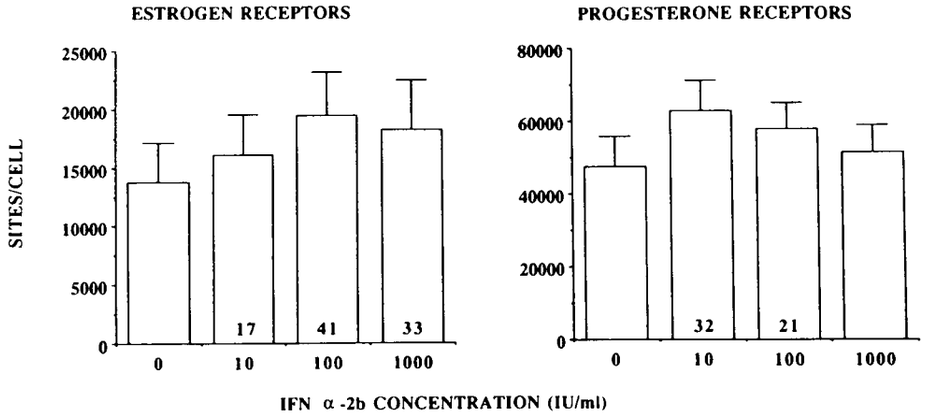

Figure 5. Effect of IFN-α2b on estrogen receptors (after 5 days of treatment) and progesterone receptors (after 7 days of treatment) in CG-5 cells. Columns represent the average of seven different experiments. Bars represent standard error. The number at the column bottom is the percentage of the augmentation induced by IFN-α2b treatment.

(about 30% with respect to control, $p < 0.01$); after 6 days the inhibition of cell proliferation reached 43% with respect to control at the highest concentration used ($p < 0.01$).

If more stringent culture conditions (inclusion of 5% FCS or 5% CH-FCS) are used, cells grow slowly, but no further enhancement of the inhibition of cell proliferation induced by IFN-α2b is observed (Figures 2 and 3).

Simultaneous exposure of CG-5 cells to IFN-α2b and 10^{-7} M TAM for 6 days leads to an increase of the antiproliferative effect compared with that

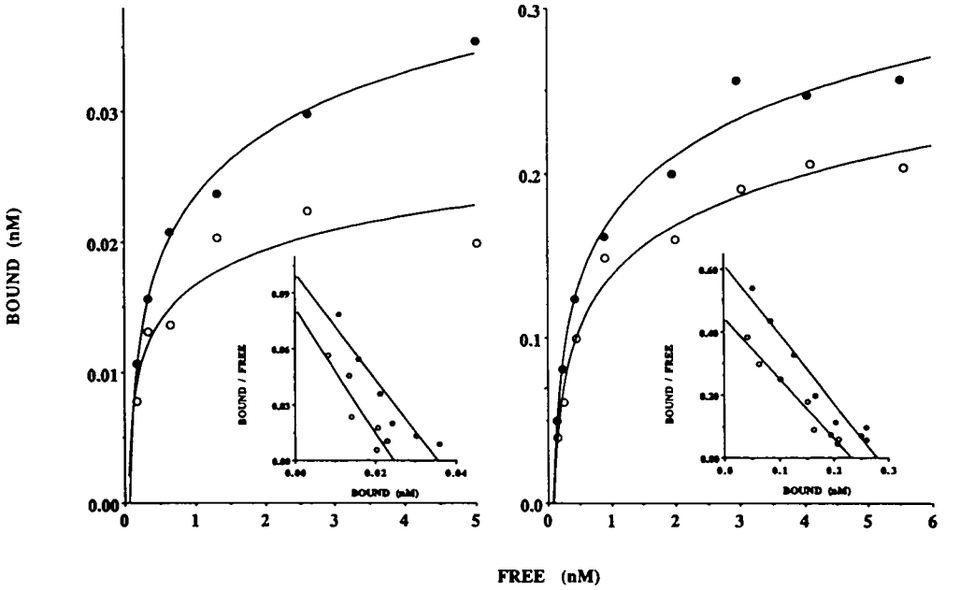

Figure 6. Left: specific binding of $[^3H]E_2$ in CG-5 cells untreated (○) or treated with 100 IU/ml IFN-α2b (●) for 5 days. The inset shows the Scatchard plot from the same data. Resultant parameters were 15 428 sites/cell and K_d 0.326 nM for control cells, and 22 365 sites/cell and K_d 0.386 nM for treated cells. Right: specific binding of [^{3}H]ORG 2058 in CG-5 cells untreated (○) or treated with 100 IU/ml IFN α-2b (●) for 7 days. The inset shows the Scatchard plot from the same data. Resultant parameters were 73 815 sites/cell and K_d 0.520 nM for control cells, and 91 438 sites/cell and K_d 0.560 nM for treated cells.

induced by TAM or IFN-α2b alone. The percentage inhibition with respect to control ranged from 54 to 66% at IFN-α2b concentrations from 5 to 1000 IU/ml ($p < 0.01$). The combination IFN-α2b plus TAM had an additive effect, even when very low concentrations of IFN were used (5 IU/ml). The difference with respect to TAM-treated cells was always statistically significant (Figure 4). When comparing the effect of the association versus individual IFN-α2b concentrations, the combination was significantly better than IFN-α2b alone (data not shown).

Effect of IFN-α2b on ER and PR level

Treatment of CG-5 cells for 5 days with various concentrations of IFN-α2b resulted in an enhancement of ER which was seen at all the doses tested ($p \leq 0.02$), as can be seen in Figure 5, which shows mean values from seven different experiments. The ER modification persists for 7 days, but the statistical significance is reached only at 10 IU/ml IFN-α2b (data not shown). As far as PR are concerned, after a 5 day treatment no statistically significant variation was seen (data not shown), while a detectable increase was observed after 7 days of exposure to the drug at 10 ($p \leq 0.02$) and 100 IU/ml of IFN-α2b ($p \leq 0.05$), see Figure 5.

Figure 6 shows the specific binding curves and the Scatchard plot of binding data from a representative experiment concerning the effect of IFN-α2b on ER and PR, respectively. The Scatchard analyses show a parallelism between the straight lines of control and IFN-α2b treated cells, indicating that the effect of IFN consists in an enhancement of B_{max} without any significant modification of the receptor affinity.

Discussion and conclusions

Both our previous findings and reports by other authors led us to study in CG-5 cells the effect of IFN-α2b on cell proliferation, steroid receptor levels and antiproliferative action of TAM.

IFN-α2b produces a remarkable inhibition of CG-5 cell growth in standard culture conditions. The inhibition is not enhanced if the FCS concentration in the medium is decreased (5% FCS or 5% CH-FCS), as happens with IFN-β.[2] This suggests that IFN-α2b is able to reduce cell growth, irrespective of possible antagonizing factors present in the serum.[9] Otherwise, stringent culture conditions do not make CG-5 cells more sensitive to IFN-α2b action.

Our data show that IFN-α2b and TAM have an additive effect, in agreement with reports by other authors.[5,10]

The expression of steroid hormone receptors is regulated by IFN-α2b as well as by IFN-β in CG-5 cells. These findings are in agreement with data reported by van den Berg *et al.*[5] in the ZR-75-1 cell line and Bezwoda and Meyer[11] in MCF-7 cells.

In our opinion, the role played by ER increase in the potentiation of the antiestrogen effect merits further investigation. In fact, the action on ER is not the unique event produced by IFN treatment which can influence the antiestrogen action. The IFN capability of regulating other steroid receptors[12,13] or the promotion of the synthesis of 'negative' growth factors, such as transforming growth factor-β,[14] cannot be excluded. Moreover, preliminary experiments performed in our laboratory indicated that IFN-β decreases insulin and epidermal growth factor binding to the CG-5 cell membrane (F Iacopino *et al.*, manuscript in preparation),[15] in agreement with reports by other authors.[16,17]

The relevance of receptor increase in the observed action of IFN and TAM is supported by the findings of Bezwoda and Meyer.[11] In fact, these authors reported that the synergistic effect of IFN-α2b and TAM in MCF-7 cells reaches a maximum when the maximum IFN-induced increase of ER is evident. Since clinical studies showed a greater effectiveness of TAM in breast cancer patients with high ER levels, the combination of IFN and antiestrogens seems full of promise.

Acknowledgments

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