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Androgen receptors and hormone sensitivity of a human prostatic cancer cell line (PC-3) are modulated by natural beta-interferon

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Abstract Androgen receptors are expressed at a low level in the cell line PC-3, which does not respond to either androgens or antiandrogens. If these cells are exposed to natural beta-interferon (β-IFN) a reduction in cell growth and an increase in androgen receptors, evaluated by both biochemical and immunocytochemical techniques, occur. This increase seems not to be related to a selective block of PC-3 in any phase of the cell cycle. Pretreatment with β-IFN determines in PC-3 cells a partial responsiveness to the androgen dihydrotestosterone as reflected by the increase in cell number. Moreover, the antiandrogen hydroxyflutamide shows agonistic properties by increasing the cell number of PC-3 cells pre-exposed to β-IFN. When the antiandrogen is tested in combination with interferon, it produces a reduction in the β-IFN-induced inhibition of cell growth. It is not known whether these unexpected effects are due to the increase in androgen receptors or to other mechanisms.

Key words Androgen receptors · Hormone sensitivity · Hydroxyflutamide · Interferon · Prostatic cancer

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It is well known that interferons (IFNs), regarded primarily as antiviral agents, affect cell growth and differentiation [17]. Recently published studies have suggested that IFNs also influence the expression of steroid hormone receptors both in vitro and in vivo [15, 21, 25] and sensitize breast cancer cells to the antitumor action of antiestrogens [6, 11, 25], drugs that mainly act through the estrogen receptor.

In a previous paper we provided evidence that natural beta-interferon (β-IFN) inhibits cell growth of PC-3 cells, a human prostatic adenocarcinoma cell line [19]. In addition, at concentrations ranging from 100 to 1000 IU/ml of culture medium, it produces an enhancement of androgen receptor (AR) level, evaluated by a whole cell assay [20]. However, data on androgen binding to PC-3 cells are controversial, some authors reporting that they express AR [26] or a very low level of normal AR [4] while others have found that they contain only nuclear AR or lack AR [3, 10].

In the present study we approached this problem again by an investigation of the presence and characteristics of AR in this cell line. Moreover, we explored the interaction of β-IFN with AR, growth fraction and the capability of modulating the hormone sensitivity of these cells, which have been reported to be androgen-insensitive [10, 18].

Materials and methods

Materials

β-IFN, induced in human fibroblasts by poly(I) · poly(C), was kindly supplied by Serono (Rome, Italy). It was dissolved at the appropriate concentrations in culture medium. 5α-Dihydro[1,2,4,5,6,7-^{3}H]testosterone ([^{3}H]DHT) (specific activity 100 Ci/mmol) was purchased from Amersham (UK). 5α-Dihydrotestosterone (DHT) was provided by Sigma (St. Louis, Mo.). The antiandrogen hydroxyflutamide (FOH) was a gift from Dr. R. Neri (Schering Corporation, Brookfield, N.J.). These compounds were dissolved in ethanol. Mouse monoclonal anti-human AR antibody (F39.4.1) against the N-terminal domain was produced and characterized as described previously [16].

Cells

PC-3 cell line was a gift from Prof. F. Labrie (Laval University, Quebec, Canada). Cells were routinely cultured at 37°C under a humidified atmosphere in a 5% CO_2 incubator. They were maintained in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, UK), supplemented with 5% fetal calf serum (FCS, Flow Laboratories), 10 mM HEPES buffer (Sigma), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B (Flow Laboratories).

Cell growth experiments

In the experiments performed to assess the responsiveness to androgens and antiandrogens, PC-3 cells were plated at an initial density of 20000 cells/ml in 60-mm plastic Petri dishes, in standard culture conditions and allowed to attach. Twenty-four hours later the medium was renewed with DMEM containing 5% charcoal-treated FCS (FCS-CH), HEPES buffer, antibiotics and DHT or FOH concentrations ranging from 10^{-9} to 10^{-6} M. Culture medium was again changed after 3 days. Control plates, which received vehicle only, were run in parallel.

In experiments performed to assess whether pretreatment of PC-3 cells with β-IFN would modify the effect of subsequent androgen or antiandrogen administration, cells were plated in standard culture conditions at a density of 25000 cells/ml in 100-mm plastic Petri dishes and allowed to attach. Twenty-four hours later the medium was changed with DMEM containing β-IFN at the concentration of 1000 IU/ml. After 3 days of treatment, cells were trypsinized and plated in 60-mm plastic Petri dishes at a density of 20000 cells/ml in standard culture conditions. Twenty-four hours later the medium was renewed with DMEM containing 5% FCS-CH, HEPES buffer, antibiotics and either DHT or FOH concentrations ranging from 10^{-9} to 10^{-6} M. In control plates medium containing vehicle only was present.

In experiments in which β-IFN and FOH were combined, cells were plated at a density of 20000 cells/ml in 60-mm plastic Petri dishes and 24 hours later medium was renewed with DMEM containing 5% FCS-CH, HEPES buffer, antibiotics and concentrations of FOH ranging from 10^{-9} to 10^{-6} M associated with 100 or 1000 IU/ml of β-IFN.

In all the experiments the final ethanol concentration in the control and treated cell medium never exceeded 1%. Cell counts were performed with a hemocytometer after 6 days of treatment with the drugs. Cell viability was assessed by trypan blue dye exclusion.

Androgen receptor binding studies (whole cell assay)

PC-3 cells were plated out at an initial density of 20000 cells/ml in 60-mm plastic Petri dishes in standard culture conditions. Twenty-four hours later DMEM was changed and cells were incubated with DMEM supplemented with 1000 IU/ml β-IFN. This concentration of β-IFN was chosen because, in our previous experience, it produced the greatest enhancement of AR, evaluated using a single saturating dose (10 nM) of [^{3}H]DHT [20]. In control plates no β-IFN was added. After 3 days the medium was renewed with DMEM supplemented with 5% FCS-CH, HEPES buffer and antibiotics. Twenty-four hours later, monolayers were washed three times with DMEM and left in this medium for 1 h in the incubator. Then, medium was removed and cells incubated in triplicate with DMEM containing increasing concentrations of [^{3}H]DHT (0.1–10 nM), either in the presence or in the absence of a 100-fold excess of unlabeled DHT, for 1 h at 37°C. At the end of incubation, cells were washed three times with Hanks' balanced salt solution to remove free steroids and monolayers were incubated with 80% ethanol at room temperature overnight to extract radioactivity. Ethanol extracts were transferred to vials and radioactivity counted in a liquid scintillation counter. Parallel cultures were processed in the same manner but did not receive either labeled or unlabeled hormone and were used to determine cell number. This allowed AR to be expressed as sites per cell.

Immunocytochemical analysis

Immunocytochemical analysis was carried out on coverslips with PC-3 cells plated at a density of 20000 cells/ml in standard culture conditions and treated with β-IFN at concentration of 1000 IU/ml. At the end of β-IFN treatment, medium was removed, coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% buffered formaldehyde for 5 min. Slides were then rinsed with PBS and post-fixed in methanol 100% for 5 min at −20°C followed by acetone 100% for the same time and at the same temperature. Slides were preincubated for 10 min with 0.1% buffered bovine serum albumin (BSA) to block nonspecific binding and then covered with a 1:1000 dilution of monoclonal antibody F39.4.1 [16] at 4°C overnight. After washing, reactivity was visualized using an immunoperoxidase procedure. Peroxidase-conjugated rabbit antimouse IgG (Dako Laboratories, Denmark), diluted 1:50 in PBS, was added as the conjugate for 1 h at room temperature followed by 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide mixture as the substrate for 10 min. As a negative control coverslips with lymphatic tissue sections were used (not shown).

Cell cycle analysis

PC-3 cells were trypsinized and plated in 100-mm plastic Petri dishes at 20000 cells/ml in standard culture conditions. Twenty-four hours later, medium was renewed with fresh medium with or without 1000 IU/ml β-IFN. At 24, 48, 72 and 96 h of treatment, PC-3 cells were trypsinized and fixed in cold PBS-buffered acetone–methanol mixture for 30 min. Then the cells were centrifuged, resuspended in cold PBS, and stored at 4°C. For the analysis cells were again centrifuged and resuspended with 500 µl DNA staining solution. This was freshly prepared and contained 50 µg/ml propidium iodide (PI), 1–2 mg/ml (50–75 Ku/mg) RNase A and 0.1% Nonidet P40 in PBS. Cells were incubated for 30 min at room temperature followed by cooling on ice, and analyzed both immediately and after an overnight incubation at 4°C. Flow cytometric analysis was accomplished with a Becton-Dickinson FACScan equipped with a 15-mW 488-nm argon laser and suitable filter set for the red emission of PI. Fluorescence signal was collected with linear amplification, the flow rate was maintained at less than 300 cells per second and the data were collected, stored and analyzed in a Consort 30 Data Management System (Hewlett-Packard series 300 computer). For each experimental condition 30000 events were acquired. The percentage of cells in the various phases of the cell cycle, i.e., G_0–G_1, S and G_2–M, was determined by computerized cell cycle analysis of the DNA histograms performed with a commercially available software package (Sum of Broadened Rectangles, Becton-Dickinson).

Statistical analysis

Data from all cell proliferation experiments were subjected to analysis of variance; multiple comparisons were made using Fisher's test only if the analysis of variance demonstrated a significant effect of the treatment.

Table 1 Effect of DHT or FOH on the growth of PC-3 cells with or without pre-exposure to 1000 IU/ml β-IFN

Compound	Concentration (M)	Cell no. without pre-exposure to β-IFN (mean % of control ± SE)	Cell no. with pre-exposure to β-IFN (mean % of control ± SE)
DHT	10^{-9}	103.9 ± 11.4	134.5 ± 11.1[b]
	10^{-8}	109.8 ± 11.4	123.0 ± 11.1[b]
	10^{-7}	110.1 ± 11.0	120.9 ± 11.8[b]
	10^{-6}	100.0 ± 13.0	112.7 ± 6.2[a]
FOH	10^{-9}	93.1 ± 7.8	127.1 ± 3.5[b]
	10^{-8}	97.1 ± 8.3	123.9 ± 3.2[b]
	10^{-7}	102.7 ± 6.1	125.6 ± 7.8[b]
	10^{-6}	109.8 ± 7.2	118.6 ± 5.9[b]

DHT, Dihydrotestosterone; *FOH,* hydroxyflutamide
Cell number was determined after 6 days of treatment with DHT or FOH. Values represent the mean of nine observations from three separate experiments
[a] $P < 0.05$ (Fisher's test vs control: see Materials and methods)
[b] $P < 0.01$ (Fisher's test vs control: see Materials and methods)

Table 2 Effect of FOH combined with β-IFN on the growth of PC-3 cells

Compounds	Concentration	Cell number (mean % of control ± SE)
β-IFN	100 IU/ml	73.6 ± 7.2[a]
FOH	10^{-9} M + β-IFN, 100 IU/ml	77.2 ± 12.8[a]
	10^{-8} M + β-IFN, 100 IU/ml	78.4 ± 6.4[a]
	10^{-7} M + β-IFN, 100 IU/ml	74.3 ± 7.4[a]
	10^{-6} M + β-IFN, 100 IU/ml	91.0 ± 8.7[b]
β-IFN	1000 IU/ml	44.0 ± 9.8[a]
FOH	10^{-9} M + β-IFN, 1000 IU/ml	54.1 ± 10.9[a]
	10^{-8} M + β-IFN, 1000 IU/ml	55.6 ± 14.2[a]
	10^{-7} M + β-IFN, 1000 IU/ml	57.0 ± 9.7[a]
	10^{-6} M + β-IFN, 1000 IU/ml	62.2 ± 10.6[a,b]

FOH, Hydroxyflutamide; *β-IFN,* beta-interferon
Cell number was determined after 6 days of treatment. Values represent the mean of nine observations from three separate experiments
[a] $P < 0.01$ (Fisher's test vs control: see Materials and methods)
[b] $P < 0.05$ (Fisher's test vs β-IFN treated cells: see Materials and Methods)

Results

Growth studies

The addition of DHT and FOH to PC-3 cells did not produce any statistically significant growth variation after 6 days of exposure to the drugs (Table 1). Conversely, sensitivity of PC-3 cells to both DHT and FOH appeared to be modified by β-IFN pretreatment. Pre-exposing PC-3 cells to 1000 IU/ml β-IFN induced a statistically significant increase in cell number at all DHT and FOH concentrations evaluated after 6 days of treatment (Table 1). No statistically significant difference in the effect produced by the different concentrations of DHT or FOH was seen.

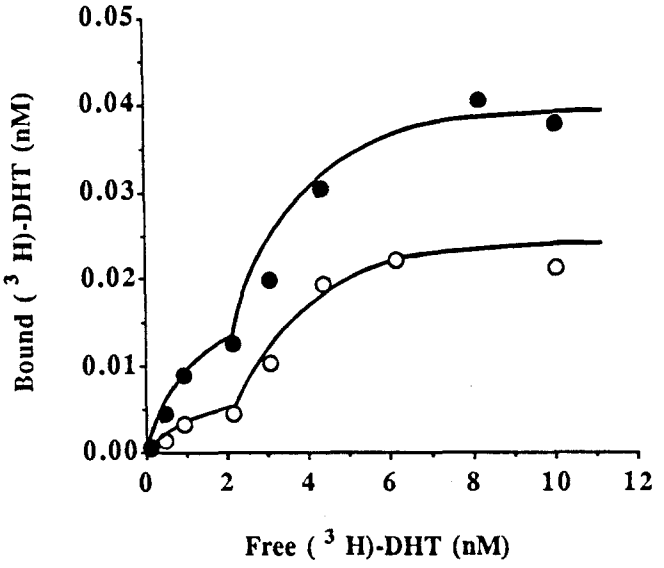


Fig. 1 Saturation analysis of dihydrotestosterone (DHT) binding to PC-3 cells in monolayers. Results from a representative experiment are expressed as specific [^{3}H]DHT binding for control and beta-interferon (β-IFN)-treated cells; ○ control; ● β-IFN, 1000 IU/ml

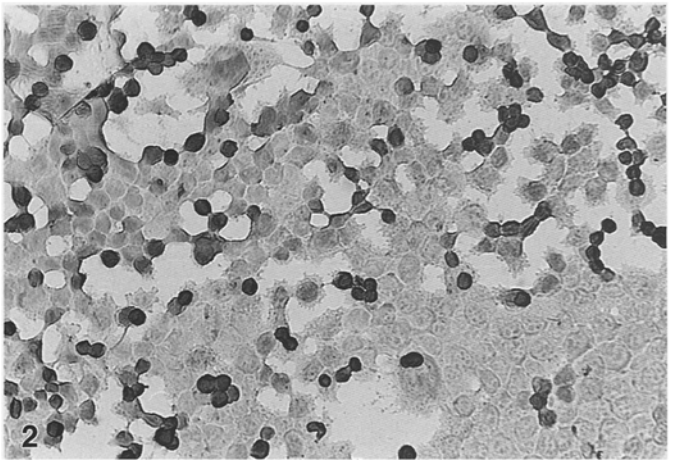
IFN alone reduced cell growth by approximately 30% at 100 IU/ml and 60% at 1000 IU/ml. The combination of 1000 IU/ml β-IFN with different concentrations of FOH revealed that 10^{-6} M FOH partially counteracted the antiproliferative effect of β-IFN while the association of IFN with the other concentrations of FOH did not result in any variation of the antiproliferative effect observed with IFN alone. This was seen also when 10^{-6} M FOH was combined with 100 IU/ml β-IFN (Table 2).

In all experiments performed, viability testing showed that >95% of adherent cells remained viable. A few nonviable floating cells were noted in the supernatants.

Androgen receptor studies

Figure 1 illustrates the specific binding of [^{3}H]DHT in untreated and β-IFN-treated PC-3 cells. The saturation curves show a biphasic trend for both treated and untreated cells with an apparent plateau observed between 2 and 3 nM for the first binding component. These data yielded a nonlinear Scatchard plot (not shown) which did not allow accurate estimation of either the association constant or the number of binding sites. Hill analysis [8] of the data yielded a Hill coefficient of 1.2 for control cells and 1.3 for treated cells, suggesting the presence of multiple binding components with positive cooperativity. A reasonable estimate for B_{max} of type I binding component can be obtained from the saturation curves, specific binding being higher in PC-3 cells exposed to β-IFN. K_D was estimated from the Hill plot analysis. The binding characteristics of type I AR are summarized in Table 3.

At the concentrations used the saturation of a second class of binding sites was apparently between 8 and 10 nM.



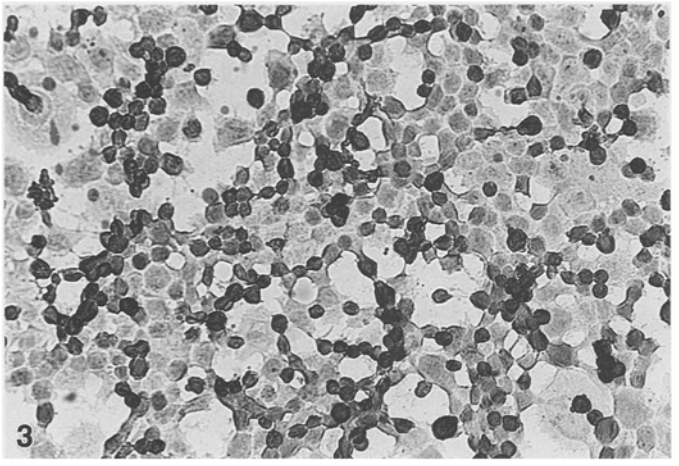


Fig. 2 Immunocytochemical staining of PC-3 cultured for 3 days in the absence of β-IFN. Original magnification ×100

Fig. 3 Immunocytochemical staining of PC-3 cells exposed for 3 days to 1000 IU/ml β-IFN. Original magnification ×100

Table 3 Type I androgen binding sites in PC-3 cells

Parameters	Treatment	
	Control	β-IFN, 1000 IU/ml
Sites per cell	2124.8 ± 516.7	6523.1 ± 880.0
Saturation (nM)	2.12	2.12
K_D (nM)	0.57 ± 0.027	0.61 ± 0.195

Data refer to the mean ± SE of three different experiments

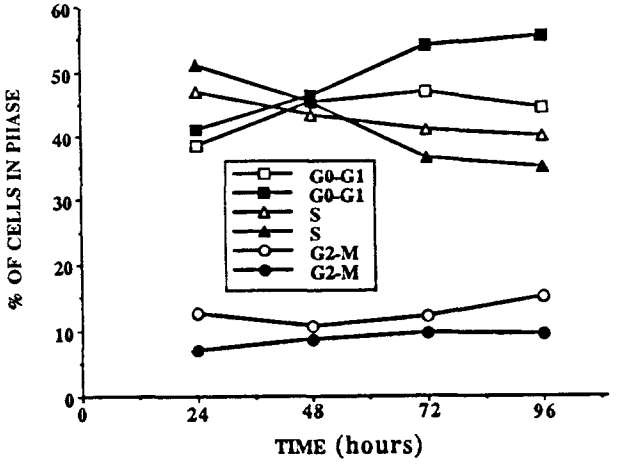


Fig. 4 Computerized mathematical analysis (Sum of Broadened Rectangles model) of the histograms of PI-3 cells DNA distribution obtained by flow cytometry. Values are from three different experiments. *Open symbols,* control cells; *filled symbols,* cells treated with 1000 IU/ml β-IFN. Cells with the same DNA content (i.e. G_0 and G_1, or G_2 and M) cannot be discriminated by the single-parameter assay used here, and are therefore counted as single populations

Immunocytochemical studies

The presence of AR in PC-3 cells was confirmed by results obtained using the newly developed highly specific anti-AR monoclonal antibody F39.4.1. Figure 2 shows the staining pattern of untreated PC-3 cells, which exhibited a specific nuclear staining with a wide range of intensity. β-IFN at 1000 IU/ml seemed to induce a higher number of nuclei to become positive with an enhanced staining intensity (Fig. 3). Viability testing performed after the end of β-IFN treatment showed that >90% of adherent cells were viable.

Cell cycle studies

Exposure of PC-3 cells to 1000 IU/ml β-IFN for up to 96 h altered the cell cycle distribution slightly. As depicted in Fig. 4, there was a detectable increase of cells in the G_0–G_1 phase of the cycle, with a concomitant, although less marked, reduction of cells in the S and G_2–M phases. These effects first became evident after 72 h of treatment.

Discussion

The present study confirms our previous report on the presence of AR in the cell line PC-3 [20]. In our experiments AR were evaluated using whole cell binding to avoid the bias of fractionation and homogenization procedures. Moreover DHT was used as ligand in cultures that had been maintained in medium supplemented with unstripped FCS. Other published studies regarding AR in PC-3 cells have been conflicting. Some authors found no detectable androgen binding in PC-3 cells [10], while others found only nuclear receptors, drawing the conclusion that these cells have an altered receptor system [3]. More recently, Culig et al. [4], using the polymerase chain reaction, concluded that a low level of normal AR is present in PC-3 cells. On the other hand, it has been suggested that in human prostatic cancer the presence of

nuclear receptors correlates better with androgen sensitivity than does the presence of cytosolic receptor [5, 7]. The high level of heterogeneity and genetic instability of PC-3 cells [2, 10], which in different laboratories may present different characteristics, could explain the controversial data. Moreover, it is important to note that the demonstration of steroid hormone receptors in monolayer cell cultures is critically dependent on the cell density, the conditions provided prior to analysis and the ligand used in the assay. AR level in PC-3 cells is, however, lower than that of cells known to be androgen-responsive, such as LNCaP. AR in LNCaP are found to be expressed at a high level using different tracers (i.e., DHT or R1881) and different culture conditions [23, 27].

Two classes of androgen binding sites have been identified in PC-3 cells. The K_D of type I AR we found in PC-3 cells is similar to that reported in literature for other cell lines [27]. The presence of a double class of AR has been reported by other authors in both normal and neoplastic prostatic cells [13, 24]. The role of type II AR is still debated. They could represent a storage compartment for maintaining elevated levels of intracellular androgens or be involved in the response to other ligands as hypothesized for the type II estrogen receptor [12]. Treatment of PC-3 cells with β-IFN produces an increase in type I AR that leaves the K_D unchanged. Moreover, it also seems to influence the type II binding component.

The presence of AR in PC-3 cells was confirmed using an anti-AR monoclonal antibody and the receptors appeared to be preferentially located in the cell nucleus. A high degree of heterogeneity was observed, with strongly positive, weakly positive and non-stained nuclei. Such heterogeneity is consistent with the reported heterogeneity of the PC-3 cell line [2, 10]. β-IFN treatment seems to produce an increase in strongly positive cells with respect to total cells.

As some steroid hormone receptors are preferentially synthesized during specific phases of the cell cycle [9, 22], it was important to ascertain whether β-IFN selectively blocked the normal progression of PC-3 cells through the different phases of the cell cycle. We observed that β-IFN did not exert a selective activity on the cell cycle, although it induced a detectable reduction in the percentage of cells in the proliferative compartments, i.e. S and G_2–M. Such a decrease in the growth fraction is consistent with the reduced number of cells recovered from β-IFN-treated cultures [19]. Thus, it is concluded that the enhanced AR content observed upon β-IFN treatment does not merely reflect a concomitant alteration of cell cycle distribution, but rather a specific activity of β-IFN on the physiological regulation of AR.

In keeping with a previous report [1, 18], PC-3 cells were unresponsive both to androgen as reported by other authors [10] and antiandrogen. This androgen and antiandrogen insensitivity could be due to the relatively low AR level. Moreover, PC-3 cells are composed of several subpopulations of which only a minority seems to express AR strongly, as demonstrated by the immunocytochemical assay. Pretreating cells with β-IFN allowed the response to androgen and antiandrogen in terms of an increase in cellular growth. β-IFN-induced modulation of AR could be involved in this phenomenon. The proliferative effect of FOH has been already described by other authors in LNCaP cells, in which an altered AR is present [14, 26, 27]. This apparently paradoxical phenomenon can be explained by the binding of the antihormone in the absence of the specific hormone. Under such circumstances, the action of the latter cannot be antagonized but a slight agonistic effect is likely to occur, following the same chain of reactions which characterize activation of a hormone-receptor system.

The interference observed between β-IFN and FOH in association experiments is well explained by the opposite action on cell growth exerted by the two drugs. The stimulation of cell growth produced by FOH counteracts, at least in part, the antiproliferative effect of β-IFN. This effect seems to be dose-dependent, because the lower dose of β-IFN (100 IU/ml) was less effective in antagonizing FOH stimulation than the higher. Although it is possible to hypothesize that an augmentation of AR is involved in the phenomenon, we are not able, at present, to quantify the relevance of the receptor increase to the sensitivity of PC-3 cells to androgens and antiandrogens.

The results of the present study confirm that β-IFN enhances AR in PC-3 cells and indicate that the two classes of binding sites are modified by β-IFN treatment. This is of great interest, because not only the intracellular accumulation of the hormone, which is likely to be dependent on type II AR, but also the interaction of the hormone with its effective mediator, which is supposed to be a type I receptor, is affected. In addition, our data suggest that β-IFN may restore hormone sensitivity in androgen or antiandrogen insensitive cells, but further studies are needed, in view of possible clinical applications.

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