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ORIGINAL PAPER

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Effect of tumor necrosis factor-α and interferon-γ on the growth of human prostate cancer cell lines

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Abstract Human recombinant tumor necrosis factor-α (rTNF-α, 10^{-12}–10^{-8} M) inhibited the proliferation of androgen-dependent LNCaP cells by 32–56%. In contrast, proliferation of androgen-independent PC-3 and JCA-1 cells was only slightly inhibited, or not inhibited at all, respectively. Human recombinant interferon-γ (rIFN-γ, 500 U/ml) decreased proliferation of PC-3 and JCA-1 cells by 35% and 53%, respectively, but had no effect on LNCaP cells. Interestingly, the combination of rIFN-γ and TNF-α had greater antiproliferative effects on JCA-1 cells than treatment with either cytokine alone. However, the antiproliferative effects of this combination were similar to those observed for PC-3 or LNCaP cells treated with rIFN-γ or TNF-α alone, respectively. These data suggest that some forms of androgen-independent prostate cancer may benefit from a combination therapy of IFN-γ and TNF-α, while the use of IFN-γ alone may be more efficacious in others.

Key words Tumor necrosis factor-α · Interferon-γ · Prostate cancer

The reduction of androgens by hormonal therapy provides an effective first-line treatment for 75–80% of patients with metastatic advanced prostate cancer (PCA) [10]. Unfortunately, the majority of patients ultimately relapse, and hormonal therapy and/or cytotoxic chemotherapy fail to provide effective responses against these relapsed, hormone-resistant PCAs. Therefore, new therapeutic approaches, possibly including the application of biological response modifiers, will be required to treat PCA more effectively.

Tumor necrosis factor-α (TNF-α) is a cytokine that mediates a wide range of biological activities *in vivo* and *in vitro* [21]. For example, TNF-α may be either cytostatic or cytotoxic on a variety of tumor cell lines [19]. Recently, several studies have reported conflicting data regarding the effect of TNF-α on PCA cell lines [3, 7, 15, 23]. Thus, the susceptibility of PCA cell lines to TNF-α remains unclear.

Interferons (IFNs) are a family of glycoproteins exhibiting antitumor, antiviral, antimitogenic and immunomodulatory activities. Treatment of human cancer cells with rTNF-α and interferons often results in an effect greater than that seen with either agent alone. For instance, a synergistic cytostatic effect of rTNF-α and rIFN-γ has been observed for bladder carcinoma, colon carcinoma, and melanoma [19, 20, 22]. Little is known regarding the effects of IFN-γ against PCA cells.

The present study demonstrates that TNF-α had antiproliferative effects on the androgen-dependent human PCA cell line LNCaP, but induced only slight or no growth inhibition for androgen-independent PC-3 and JCA-1 cells, respectively. In contrast, IFN-γ had an antiproliferative effect on the androgen-independent, but not androgen-dependent, cell lines. Moreover, the IFN-γ-mediated antiproliferative effect against JCA-1, but not PC-3, cells was enhanced in the presence of TNF-α. These data suggest that IFN-γ may be useful in the treatment of androgen-insensitive PCA, and this effect may be enhanced by the combination of IFN-γ and TNF-α.

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Materials and methods

Cell lines and tissue culture

The human PCA cell line PC-3 [5] was obtained from the American Type Culture Collection (Rockville, Md., USA). The human PCA cell line JCA-1 was established as previously described [8]. The human PCA cell line LNCaP [4] was a gift from Dr. Warren D. W. Heston (Memorial Sloan-Kettering Cancer Center, New York, N.Y., USA). PCA cell lines were routinely propagated in monolayer culture in a humidified incubator at 37 °C in a 5% CO_2/95% air atmosphere. Cells were grown in RPMI 1640 medium supplemented with 5% or 10% heat-inactivated fetal calf serum (FCS, Hyclone), 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Adherent PCA cells were removed for passage and experiments by trypsinization with trypsin ethylenediaminetetra-acetic acid (EDTA) (Gibco).

Cytokines and general reagents

The following were purchased: rTNF-α (specific activity 2×10^7 units/mg) and rIFN-γ (2.5×10^7 units/mg) from Genzyme (Cambridge, Mass., USA); charcoal-stripped fetal bovine serum (CSS) from Sigma (St. Louis, Mo., USA); and [^{3}H]thymidine ([^{3}H]TdR) from New England Nuclear (Boston, Mass., USA). 5α-Dihydrotestosterone (DHT) was a gift from Dr. Bernard I. Weinstein (New York Medical College, Valhalla, N.Y., USA).

[^{3}H]Thymidine incorporation assay

The PCA cells (5000–10 000/well) were seeded for 24 h in triplicate wells of flat-bottomed 96-well microtiter plates (Corning). Cytokines were added to the cells and incubated at 37 °C/5% CO_2 for 2–5 days. The plates were pulsed with 1 µCi [^{3}H]TdR/well, incubated for 6 h at 37 °C, trypsinized, and harvested onto strips of fiberglass filter paper with the use of a semiautomatic cell harvester (Skatron). The radioactivity of each sample was measured in a liquid scintillation counter. The effects of rTNF-α (10^{-12}–10^{-8} M), rIFN-γ (5–500 U/ml; 10^{-11}–10^{-9} M), and combinations of these cytokines were assessed at different time points (24, 48, 72, and 120 h).

Statistics

The unpaired Student's *t*-test was used for comparison of each group.

Results

Characterization of cell lines

The responsiveness to androgens for growth was verified in each of the cell lines. Proliferation of the LNCaP cell line was increased in the presence of DHT (10^{-8} M), when using medium containing 5% charcoal stripped fetal bovine serum (CSS medium) (Fig. 1). In addition, proliferation was greatly increased when cells were cultured in androgen-containing FCS (5%) compared with proliferation in androgen-depleted CSS (Fig. 1). These data indicate that LNCaP cells are androgen responsive. In contrast, both the JCA-1 and PC-3 cell lines proliferated in the presence or absence of DHT, and thus exhibited androgen-independent growth, as previously described (Fig. 1).

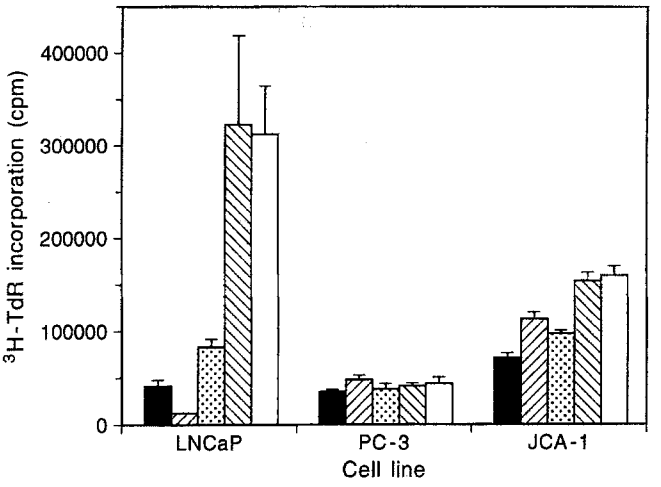


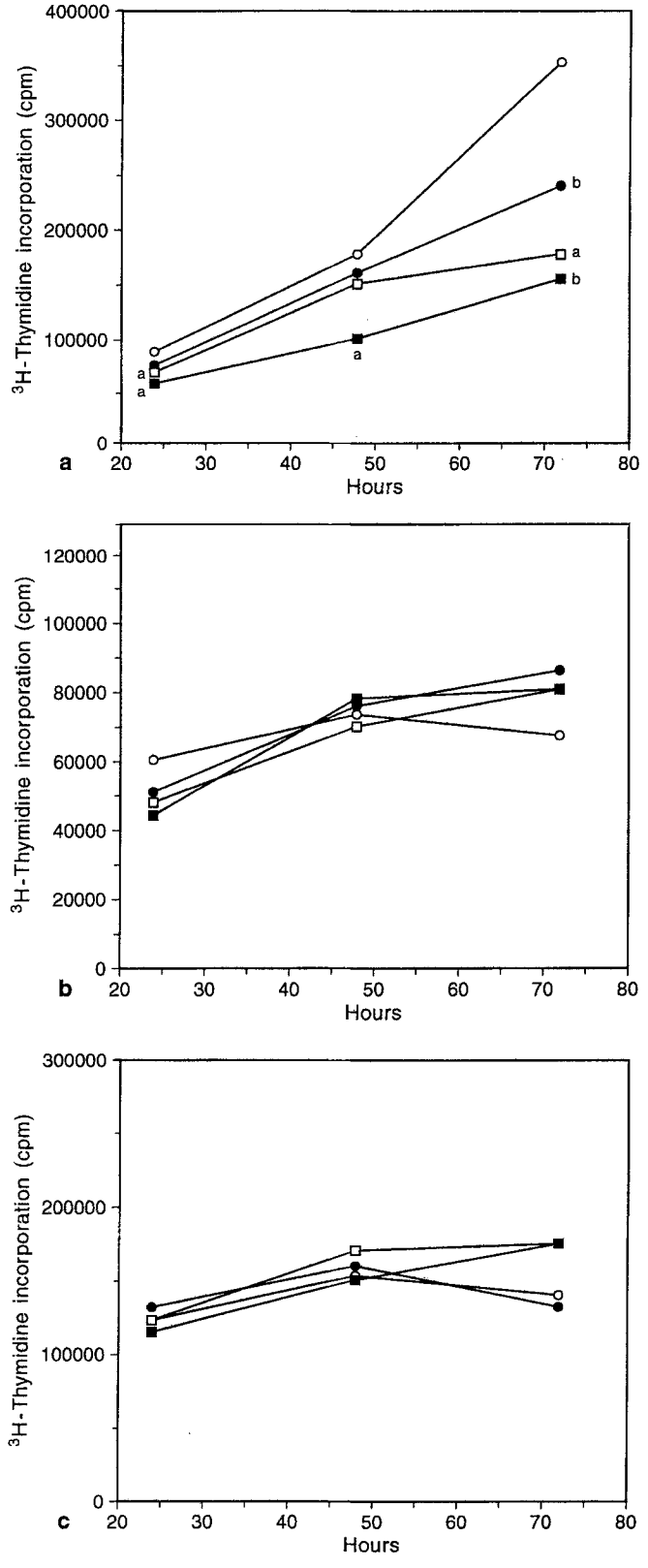
Fig. 1 Sensitivity of PCA cells to DHT. Cells in RPMI 1640 medium with 5% charcoal-stripped serum (CSS) were plated in 96-well plates, as described in "Materials and methods". Cells were allowed to attach for 3 days, replenished with fresh CSS medium, CSS and DHT (10^{-8} M), 5% FCS, or 5% FCS and DHT (10^{-8} M) for another 3 days. Cells were pulsed with [^{3}H]TdR for 6 h, harvested, and radioactivity was measured. Values represent mean ± SD of six wells for each treatment. All experiments were performed twice. ■ CSS day 0; ▨ CSS only; ▩ CSS + DHT; ▧ 5% FCS; □ 5% FCS + DHT

Effect of rTNF-α on PCA cell lines

The effects of rTNF-α on each cell line were examined at various time points. Dose- and time-dependent inhibitory effects of rTNF-α were observed for LNCaP cells; the greatest inhibitory effect was observed after a 72-h incubation period with 10^{-8} M TNF-α (Fig. 2a). In contrast, rTNF-α did not inhibit the proliferation of PC-3 or JCA-1 cells at any of the doses or times tested (Figs. 2b, c). These results were confirmed by parallel cell-counting experiments (data not shown). These data suggest that androgen-independent PCA cells may be resistant to the cytotoxic effects of TNF-α, since this cytokine only inhibited the proliferation of an androgen-dependent PCA cell line, LNCaP, but had no effect on the growth of two androgen-independent cell lines, PC-3 and JCA-1.

The effect of androgens on the susceptibility of rTNF-α (10^{-8}) was examined to determine if the growth-inducing properties of androgens could negate the antiproliferative effect of TNF-α. In the absence of DHT, rTNF-α (10^{-8} M) inhibited LNCaP proliferation by 46% compared with the control (Fig. 3). Similarly, rTNF-α inhibited LNCaP proliferation by 44% in the presence of DHT (10^{-8} M). As expected, rTNF-α

had little effect on PC-3 or JCA-1 proliferation in the absence or presence of DHT (Fig. 3). Thus, the antiproliferative effect of rTNF-α on LNCaP cells does not appear to be influenced by the presence of DHT.



Effect of rIFN-γ on PCA cells

The effects of IFN-γ on PCA cell lines *in vitro* are not clear and, thus, were examined in the present study. A dose- and time-dependent antiproliferative effect of rIFN-γ was observed for PC-3 and JCA-1 cells (Fig. 4a, b); however, no inhibitory effect was observed for LNCaP cells (Fig. 4c). rIFN-γ (500 U/ml) induced significant decreases of PC-3 proliferation after 48- to 120-h incubations. In contrast, the onset of the rIFN-γ-mediated antiproliferative effect was evident by 20 h in JCA-1 cells, since rIFN-γ (50 and 500 U/ml) induced significant decreases of JCA-1 proliferation at each of the time points tested (Fig. 4b). Maximal antiproliferative effects in response to rIFN-γ (500 U/ml) were seen at 120 h for PC-3 (35%) and 72 h for JCA-1 (53%), respectively.

Combined effects of rTNF-α and rIFN-γ on PCA cells

The combined effects of rTNF-α and rIFN-γ were investigated to evaluate whether these cytokines acted synergistically on PCA cell lines. rTNF-α (10^{-8} M) caused a 40% reduction in androgen-dependent LNCaP proliferation versus control (no addition, Fig. 5). In contrast, rIFN-γ (500 U/ml) failed to inhibit proliferation of LNCaP cells, and did not affect the TNFα-mediated antiproliferative effect against LNCaP cells. However, a 32% decrease of proliferation was observed for androgen-independent JCA-1 cells after addition of rIFN-γ, while rTNF-α had no effect at 24 h. Further, when rTNF-α and rIFN-γ were combined, the antiproliferative effect was increased to 59%, suggesting that these two cytokines had a synergistic effect on JCA-1 cells. On androgen-independent PC-3 cells, rTNF-α or rIFN-γ alone inhibited proliferation by 25% and 47%, respectively, and rTNF-α/rIFN-γ (500 U/ml) inhibited proliferation by 54%. These data indicate that the rTNF-α/rIFN-γ combination had no significant synergistic effect against PC-3 cells.

Fig. 2a–c Effect of rTNF on PCA cells. **a** LNCaP; **b** PC-3; **c** JCA-1. Cells in RPMI 1640 medium containing 5% FCS were plated in 96-well plates, and were allowed to adhere for 24 h before addition of rTNF-α (10^{-12}–10^{-8} M) for various times. Cells were pulsed with [^{3}H]TdR for 6 h, harvested and radioactivity was measured. Values represent mean of triplicate determinants. Standard deviations (not shown) were less than 20% of mean values. All experiments were performed at least twice. *a*, $P < 0.025$, *b*, $P < 0.005$ (vs. no addition at each time point). —○— no addition; —●— TNF 10^{-12} M; —□— TNF 10^{-10} M; —■— TNF 10^{-8} M

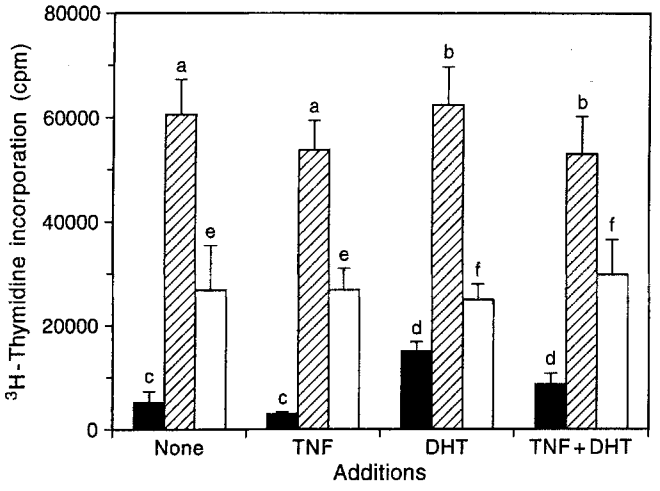


Fig. 3 Androgen effect on TNF susceptibility. Cells in RPMI 1640 medium with 5% charcoal-stripped serum were plated in 96-well plates, as described in "Materials and methods". Cells were allowed to attach for 3 days, replenished with fresh CSS medium, and treated with rTNF-α (10^{-8} M), DHT (10^{-8} M), rTNF-α and DHT, or untreated control for another 3 days. Cells were pulsed with [^{3}H]TdR for 6 h, harvested and radioactivity was measured. Values represent mean ± SD of six wells for each treatment. All experiments were performed twice. *a, b*: $P < 0.05$; *c*: $P < 0.005$; *d*: $P < 0.0005$; *e, f*: NS. ■ LNCaP; ▨ PC-3; □ JCA-1

Discussion

The biggest problem in the management of patients with metastatic PCA is the poor prognosis as a result of tumors that have become androgen insensitive. Thus, possible means to inhibit the proliferation of these tumors should be proposed since current therapies are not always effective. This study demonstrated that rIFN-γ inhibited the proliferation of androgen insensitive PCA cell lines, and indicated that the combination of rTNF-α and rIFN-γ acts in a synergistic manner to inhibit the proliferation of some androgen-insensitive PCAs.

rTNF-α inhibited proliferation of the androgen-dependent cell line LNCaP but had no effect on the proliferation of androgen-independent JCA-1 and PC-3 cells. The effect of rTNF-α was not influenced by the presence of exogenous DHT and was unrelated to TNF-α production, since these PCA cells neither transcribed the gene for TNF-α, nor produced biologically

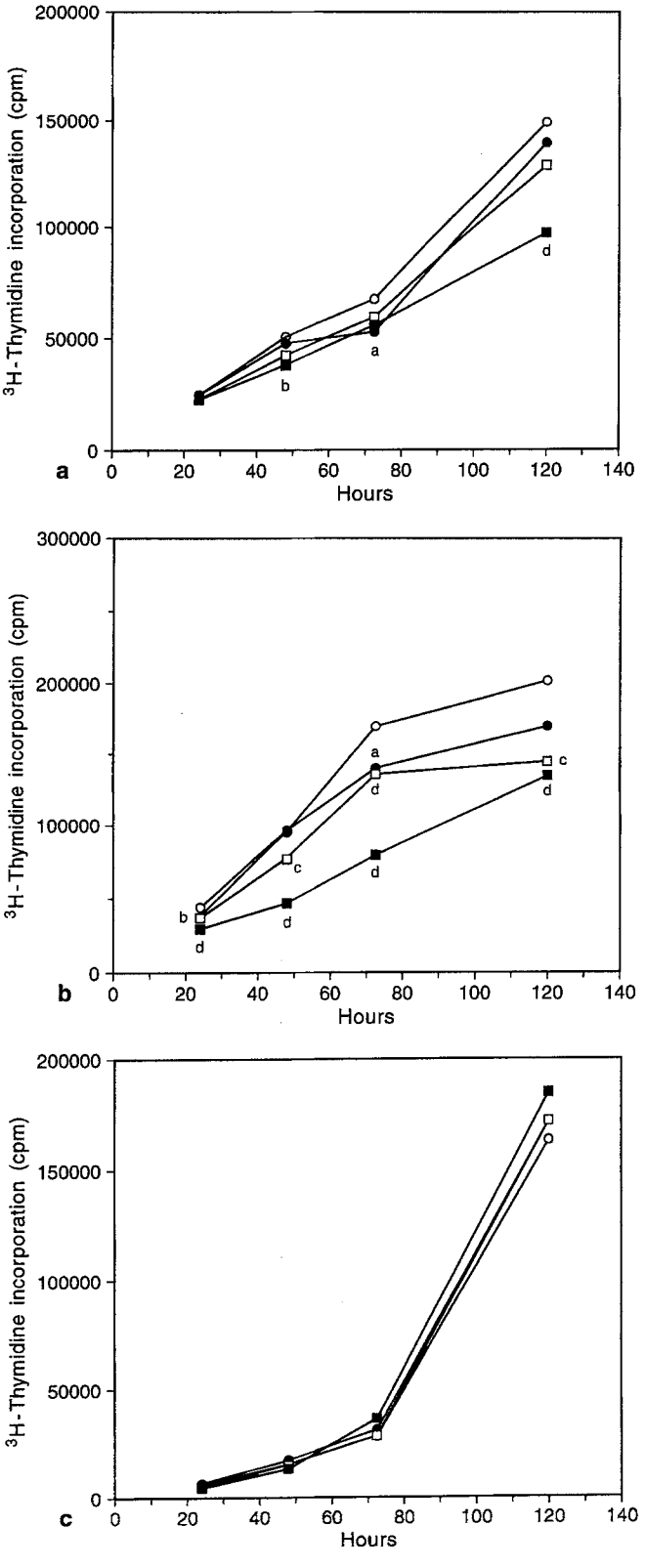


Fig. 4a–c Effect of rIFN-γ on PCA cells. **a** PC-3; **b** JCA-1; **c** LNCaP. Cells in RPMI 1640 medium with 5% FCS were plated in 96-well plates, as described in "Materials and methods". Cells were allowed to attach for 24 h, and treated with rIFN-γ (5–500 U/ml) for various times. Cells were pulsed with [^{3}H]TdR for 6 h, harvested at each time point, and radioactivity was then measured. Values represent mean of six wells for each treatment. Standard deviations (not shown) were less than 20% of mean values. All experiments were performed 3 times. *a*: $P < 0.05$; *b*: $P < 0.025$; *c*: $P < 0.005$; *d*: $P < 0.0005$ (vs. no addition at each time point). —○— no addition; —●— IFN 5 U/ml; —□— IFN 50 U/ml; —■— IFN 50 U/ml

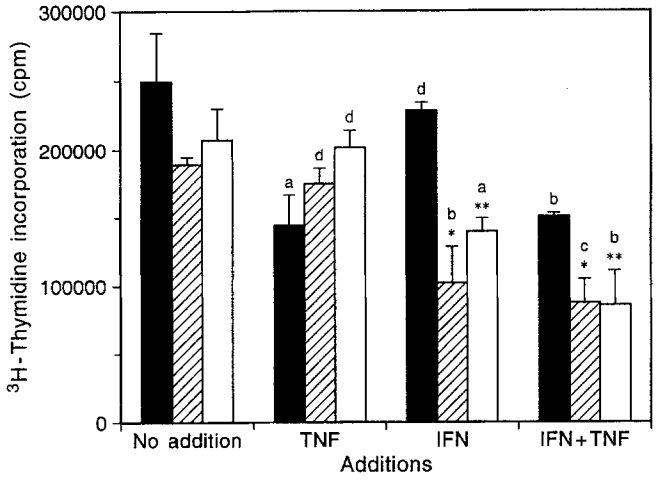


Fig. 5 Effect of rTNF-α on PCA cells with or without rIFN-γ preincubation. Cells in RPMI 1640 medium with 5% FCS were plated in 96-well plates, as described in "Materials and methods". Cells for "TNF-α" or "IFN" were allowed to attach for 48 h, and treated with 10^{-8} M of rTNF or 500 U/ml of rIFN-γ for 48 h after medium change. Cells for "IFN + TNF-α" were allowed to attach for 24 h, preincubated with 500 U/ml of rIFN-γ for 24 h, and treated with 10^{-8} M rTNF-α for another 48 h after medium change. Cells for "no addition" were allowed to attach for 48 h, and were incubated for another 48 h with only new medium. Cells were labeled with [^{3}H]TdR for 6 h, harvested and radioactivity was then measured. Values represent mean ± SD of triplicates. All experiments were performed 3 times. *a*: $P < 0.01$ vs. no addition; *b*: $P < 0.005$ vs. no addition; *c*: $P < 0.0005$ vs. no addition; *d*: NS vs. no addition. $^*P < 0.025$ vs. each other; $^+$NS vs. each other. ■ LNCaP; ▨ PC-3; □ JCA-1

active TNF-α (data not shown). Zhao et al. [23] reported that inhibition of LNCaP proliferation by rTNF-α was substantially higher in media containing androgen-depleted serum than in media supplemented with synthetic androgen. This suggested that the presence of androgen might antagonize the antiproliferative effect mediated by rTNF-α. In contrast, the present study demonstrates that the effects of rTNF-α for both androgen-sensitive LNCaP and androgen-insensitive PC-3 and JCA-1 were unchanged in the absence or presence of androgen (DHT). Thus, we suggest that TNF-α susceptibility amongst these cell lines is independent from the responsiveness to androgen, and that each cell line has its own characteristic response against rTNF-α.

The basis for the sensitivity or resistance of transformed cells to TNF-α remains unknown, as does the mechanism of action for other effects of TNF-α. Spriggs et al. [17] showed that rTNF-α treatment induced both TNF-α mRNA accumulation and resistance to TNF-α cytotoxicity in TNF-α-sensitive human breast carcinoma cells. Other investigators have also suggested that a mechanism of TNF-α resistance in tumor cells is related to the endogenous production of TNF-α mRNA and protein [9, 13]. Therefore, we examined whether PCA cells produced TNF-α by using TNF-α-sensitive WEHI-164 cells and Northern blot analysis (data not shown). However, no significant TNF-α mRNA accumulation or production was observed for any of the PCA cell lines. The differential effects of TNF-α also do not appear to be related to expression of TNF-α receptors, but may be linked to production of IL-6 (Y. Nakajima, A. DelliPizzi, C. Mallouh, N.R. Ferreri, unpublished work).

rIFN-γ did not affect proliferation of LNCaP cells, but inhibited proliferation of both PC-3 and JCA-1 cells. Further, the combination of rTNF-α and rIFN-γ inhibited proliferation of androgen-independent, TNF-α-insensitive JCA-1 cells in a synergistic manner. In contrast, the combination of these cytokines was not synergistic for androgen-dependent LNCaP cells, or another androgen-independent cell line, PC-3. Thus, the synergistic effects of rTNF-α and rIFN-γ cannot be attributed solely to insensitivity to androgens, since rTNF-α and rIFN-γ were not synergistic for androgen-insensitive PC-3 cells. Preincubation with rIFN-γ, or simultaneous treatment of rIFN-γ and rTNF-α, enhanced cytotoxicity or the antiproliferative effects of rTNF-α in some tumor cell lines *in vitro* [11, 12, 18, 19, 22], and results in the present study indicate that synergistic effects may also be achieved in some PCA cells.

The mechanism of IFN-γ-mediated effects is unclear since many genes are induced by this cytokine. Addition of rTNF-α, after a 24-h preincubation with rIFN-γ, potentiated the antiproliferative effect of rIFN-γ in JCA-1 cells. However, lesser effects were observed when either a 4- or 8-h pretreatment was done (data not shown). Thus, the delayed effects mediated by rIFN-γ may reflect the induction of a gene that encodes a protein whose effects require time to be expressed. The combination of rTNF-α and rIFN-γ may be the best way to maximize the effects of both cytokines, since this combination induced a greater inhibition of JCA-1 proliferation than did either cytokine alone. These data suggest that rIFN-γ may be more effective than rTNF-α against androgen-insensitive PCA, and that appropriate combination of these cytokines may offer advantages over the use of rIFN-γ alone in some instances.

Clinical trials with rTNF-α have been conducted for urological tumors. In a phase I-II trial of intravesical immunotherapy with rTNF-α against superficial bladder tumors, 3 complete responses (23%) out of 13 patients were obtained [14]. Kramer et al. reported that intraprostatic injection of rTNF-α combined with systemic use of rIFN-α-2b reduced prostatic volume by 38.3% and serum PSA levels by 18–87% in ten patients with locally advanced-hormone resistant PCA [6]. Growth inhibition of human PCA cell lines has been observed after treatment with rIFN-β and rIFN-α-2b *in vitro*, and modest toxicity associated with limited efficacy has been reported in clinical trials of patients with advanced PCA [1, 2, 16]. Moreover, rTNF-α and rIFN-γ have been shown to have an antineoplastic

effects *in vivo* against the PC-3 and DU145 cell lines [20]. The results of the present study suggest that the combination of rTNF-α and rIFN-γ may have synergistic effects against some androgen-insensitive PCAs. Further investigation will be required to determine how to maximize the aforementioned effects of these cytokines. These results may serve as a rationale for clinical trials employing this combination in patients with hormone-resistant advanced PCA.

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