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Enhancement of Antiproliferative Effects of Interleukin-1β and Tumor Necrosis Factor-α on Human Prostate Cancer LNCaP Cells by Coculture with Normal Fibroblasts through Secreted Interleukin-6

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The cell-cell interactions between tumor cells and stromal cells are considered to be important in the regulation of tumor development at primary and metastatic secondary sites. We studied the effects of various cytokines on the cell-cell interactions between androgen-dependent LNCaP or androgen-independent PC-3 human prostate cancer cell lines and normal fibroblasts using a coculture system. Among the tested combinations of cytokines and fibroblasts, strong modulations of cytokine actions were seen in coculture with human normal fibroblasts WI-38. While interleukin (IL)-1β or tumor necrosis factor-α (TNF-α) partially suppressed LNCaP cell growth in monoculture, each cytokine completely inhibited it in the case of coculture with WI-38 cells. On the other hand, they did not inhibit PC-3 cell growth significantly, regardless of monoculture or coculture. Conditioned medium prepared from WI-38 cells pretreated with IL-1β or TNF-α also strongly inhibited LNCaP cell growth. In the conditioned medium, marked IL-6 secretion was induced from WI-38 cells by IL-1β or TNF-α. Furthermore, neutralizing antibodies to IL-6 or IL-6 receptor abrogated the antiproliferative effects of IL-1β- and TNF-α-pretreated WI-38 conditioned medium. These results demonstrate that the antiproliferative effects of IL-1β and TNF-α on prostate cancer cells are enhanced by coculture with normal fibroblasts through some diffusible factor(s), such as IL-6, from the stimulated fibroblasts.

Key words: Prostate cancer — Fibroblast — IL-1β — TNF-α — IL-6

Tissues of various organs consist of mainly epithelial and stromal layers. They interact with each other through diffusible signals and direct contact. Proliferation and differentiation of the epithelial cells are regulated by their interactions.[1, 2] Thus, the stromal cells play an important role to support and maintain the homeostasis and functions of the tissues. In this respect, many diseases including cancer are considered to result from imbalance of the cell-cell interactions. During tumor development, there are cell-cell interactions between tumor cells and stromal cells of the same tissue origin. Moreover, metastatic tumor cells may also interact with stromal cells of different tissue origin. In fact, infiltration or inclusions of macrophages and stromal cells are often seen in tumor tissues.[3, 4] Although their role in tumor regulation at primary and metastatic secondary sites is still unknown, they may influence tumor growth via cell-cell interactions.[5, 6] Thus, modulation of the cell-cell interactions between tumor cells and stromal cells is an attractive target for cancer chemotherapy. Therefore, we focused on fibroblasts among stromal cells and developed a coculture system of tumor cells and fibroblasts to study the cell-cell interactions.

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Prostate cancer is most common in Western countries, but the number of patients is also increasing in Japan as the life style becomes increasingly westernized.[7] In the early stages, most prostate cancer cells are androgen-dependent and respond well to androgen withdrawal in chemotherapy.[7] However, even after a response, relapse often occurs and new tumor cell populations develop.[7] During cancer progression, the prostate cancer cells lose androgen dependency and acquire metastatic ability. The androgen-independent prostate cancer cells become resistant to antiandrogens and also other anticancer drugs and tend to metastasize to bone.[8] Furthermore, antiandrogen therapy at early stages of prostate cancer might help to select androgen-independent tumors. Thus, new types of chemotherapy are needed. In this study, we examined the cell-cell interactions between prostate cancer cells and normal fibroblasts using a coculture system. We found that the antiproliferative effect of inflammatory cytokines against prostate cancer cells was greatly enhanced by coculture with normal fibroblasts.

MATERIALS AND METHODS

Reagents Recombinant human interleukin (IL)-1β, IL-10, transforming growth factor-β1 (TGF-β), and neutralizing antibodies to human IL-6 and IL-6 receptor were pur-

chased from R & D Systems (Minneapolis, MN); recombinant human IL-4, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) were from Pepro Tech (London, England); recombinant human interferon-γ (IFN-γ) was from Endogen (Woburn, MA); 5α-dihydrotestosterone (DHT) was from Sigma (St. Louis, MO).

Cells Androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines and normal human diploid fibroblast WI-38 cells derived from embryonic lung tissue were obtained from Dainippon Seiyaku (Osaka). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin at 37°C with 5% CO_2.

Coculture experiments WI-38 cells were first inoculated in 96-well plates at 5000 cells/well (100 μl/well) and cultured confluently for 2 days. Then, 50 μl of LNCaP or PC-3 cells (5000 cells/well) was inoculated onto a monolayer of confluent WI-38 cells. Various cytokines were added to the culture and the cells were further cultured for 3 days. The growth was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described.[9] Because MTT was reduced by both cell types, the measured absorbance at 570 nm in coculture corresponds to total values. For monoculture, the cells were inoculated at 5000 cells/well (150 μl/well) and cultured for 3 days with various cytokines. For the assay of androgen response, FBS was stripped with 0.5% charcoal at 55°C for 30 min.

Preparation of conditioned medium WI-38 cells were inoculated in 96-well plates at 5000 cells/well (100 μl/well) and cultured for 2 days with or without 5 ng/ml of IL-1β or TNF-α. The cultured supernatant was collected and added to fresh 96-well plates. Then, 50 μl of LNCaP or PC-3 cells (5000 cells/well) was inoculated into each well of the plates containing the conditioned medium and culture was continued for 3 days with or without further addition of IL-1β or TNF-α. For assay of neutralizing antibodies, neutralizing antibodies to IL-6 or IL-6 receptor were added to the conditioned medium 1 h before the inoculation of prostate cancer cells.

IL-6 production WI-38 cells were inoculated in 96-well plates at 5000 cells/well (100 μl/well) and cultured for 2

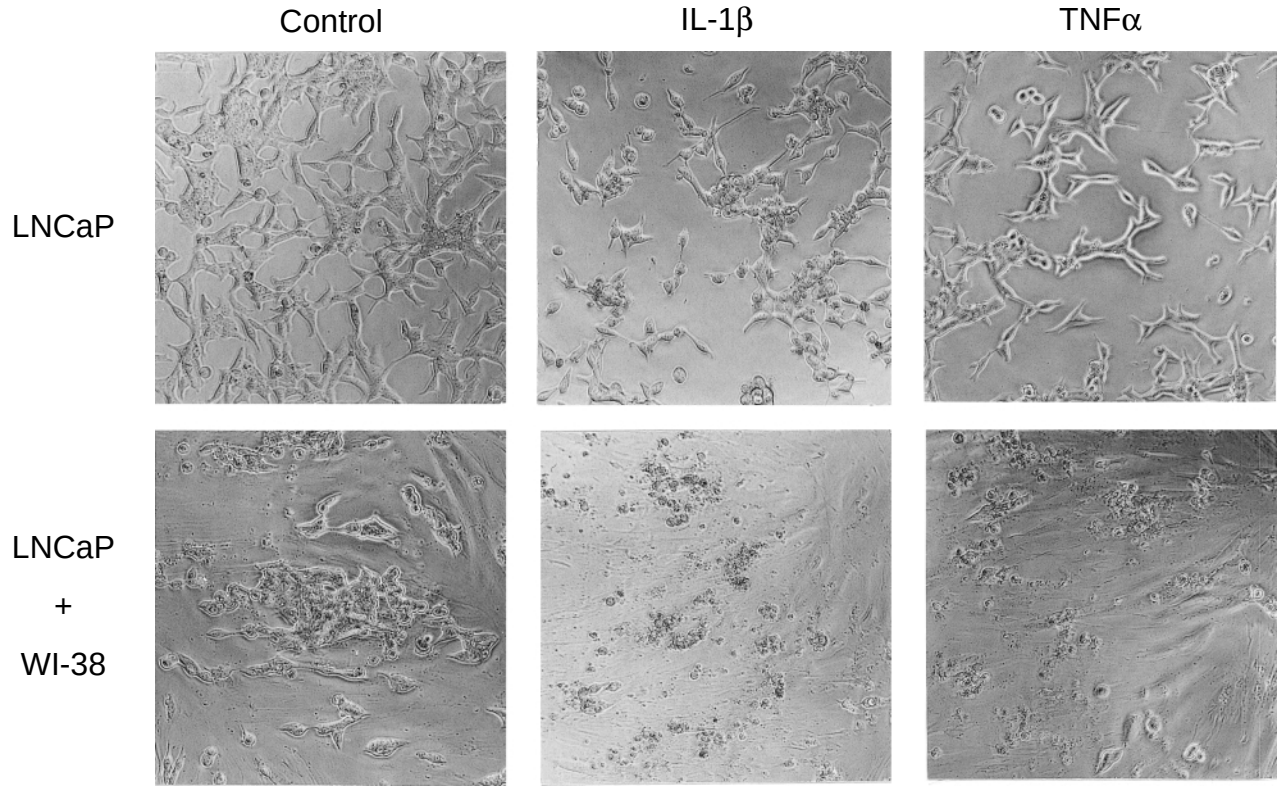

Fig. 1. Coculture of LNCaP cells and WI-38 cells. LNCaP cells were monocultured (above) or cocultured with WI-38 cells (below) for 3 days with or without 5 ng/ml of IL-1β or TNF-α.

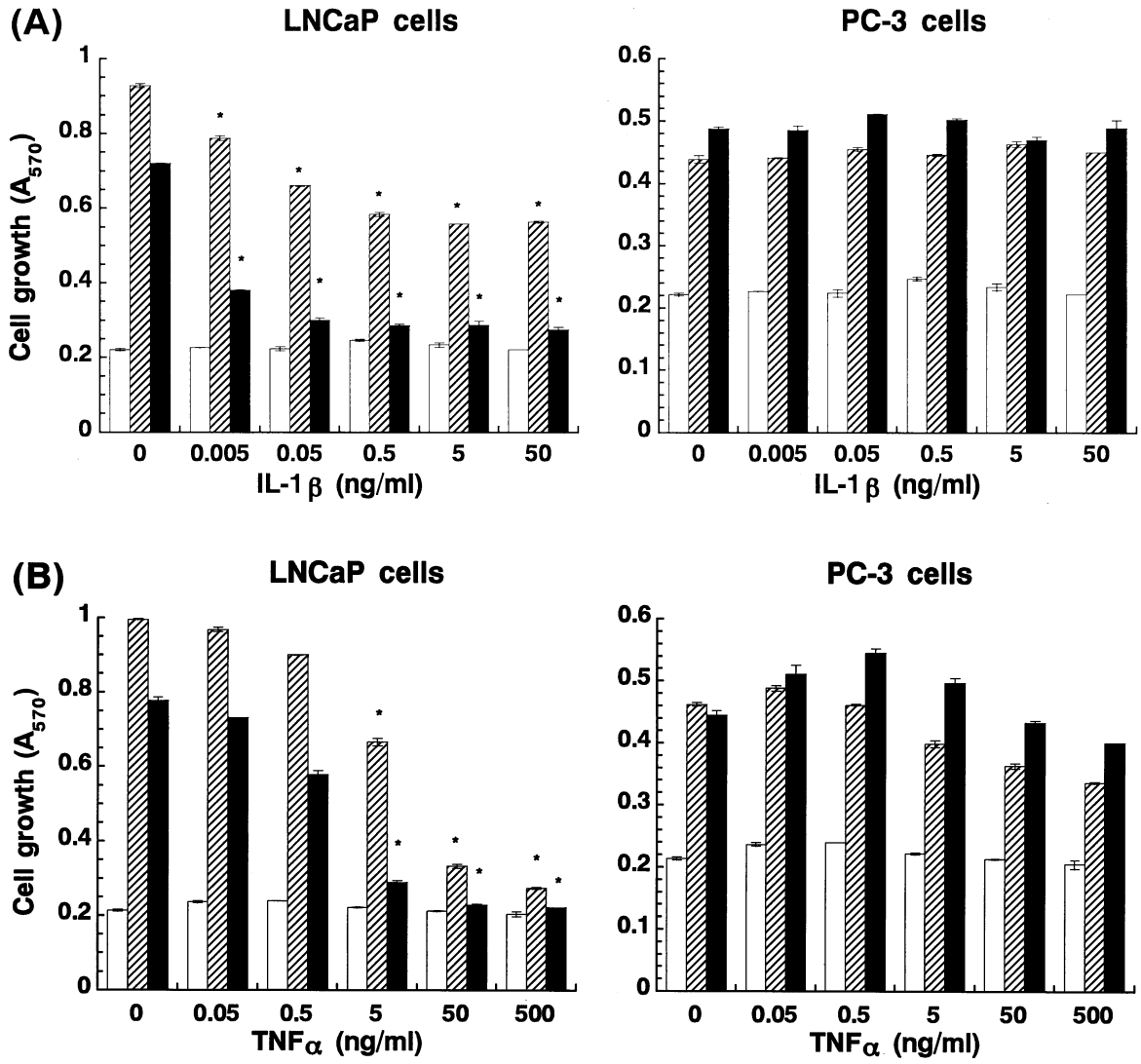

Fig. 2. Effects of IL-1β and TNF-α on coculture of prostate cancer cells and WI-38 cells. LNCaP (left), PC-3 (right), or WI-38 cells were cultured alone or cocultured with WI-38 cells with the indicated concentrations of IL-1β (A) and TNF-α (B) for 3 days. Cell growth of WI-38 cells alone (□), LNCaP or PC-3 cells alone (▨), and LNCaP or PC-3 cells cocultured with WI-38 cells (■) was determined using MTT. Values of ■ included the contribution of WI-38 cells. The values are means±SD of 3 independent duplicate determinations. * $P<0.001$ versus none.

days with or without 5 ng/ml of IL-1β or TNF-α. The cultured supernatant was collected and IL-6 content was assayed using an ELISA (Endogen).

Statistical analysis Statistical analysis was performed by using Student's unpaired *t* test.

RESULTS

Effects of IL-1β and TNF-α on coculture of prostate cancer cells and normal fibroblasts To study cell-cell interactions between tumor cells and stromal cells, we developed a coculture system using androgen-dependent LNCaP or androgen-independent PC-3 human prostate cancer cells and human normal fibroblasts WI-38 derived from embryonic lung tissue. Although we tested other fibroblasts, such as primary cultures derived from rat prostate, the modulations of cytokine actions described below were less pronounced than they were with WI-38 cells. Therefore, to examine the effect of cytokines on coculture, we used WI-38 cells in this study. Coculture

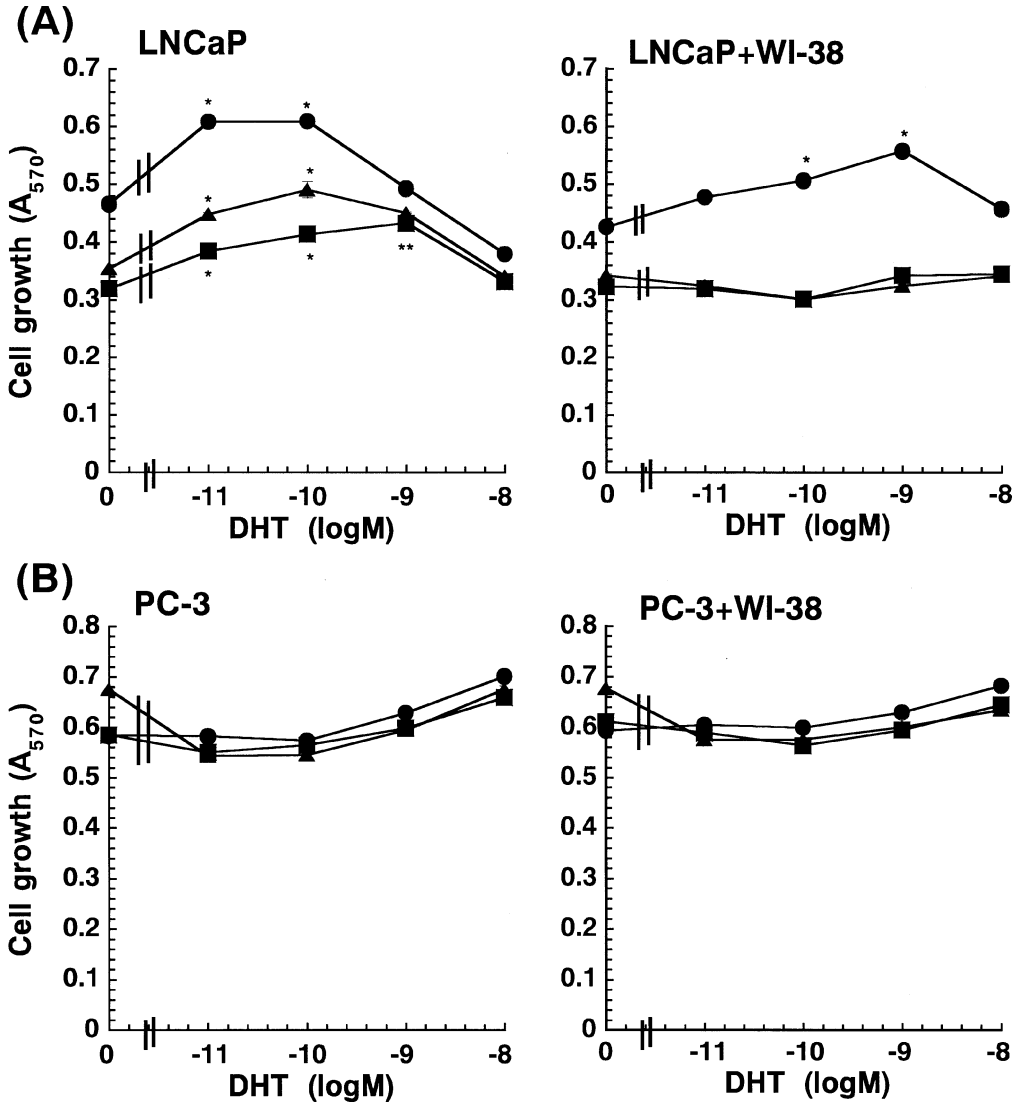

Fig. 3. Effects of IL-1β and TNF-α on DHT response in prostate cancer cells. LNCaP (A) or PC-3 (B) cells were cultured alone (left) or cocultured with WI-38 cells (right) with the indicated concentrations of DHT in the presence or absence (●) of 5 ng/ml of IL-1β (■) or TNF-α (▲) for 3 days. LNCaP cell growth was determined using MTT. Values (right) included the contribution of WI-38 cells. The values are means±SD of 3 independent duplicate determinations. * P<0.005 versus none, ** P<0.001 versus none.

experiments were performed in which LNCaP or PC-3 cells were inoculated onto a monolayer of precultured WI-38 cells. When LNCaP cells were cocultured with WI-38 cells, they aggregated and grew to form spheroids (Fig. 1). In contrast, PC-3 cells cocultured with WI-38 cells pushed aside the monolayer of WI-38 cells, showing the appearance of invading the fibroblast sheet (data not shown).

We first examined the effects of various cytokines, both inflammatory and antiinflammatory. Among IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, and TGF-β, we found that IL-1β or TNF-α strongly inhibited LNCaP cell growth in coculture with WI-38 cells. Even though IL-1β suppressed LNCaP cell growth in monoculture moderately (up to 40% inhibition) and did not affect WI-38 cells, it strongly inhibited the growth of LNCaP in coculture with WI-38 cells (Fig. 2A). At 5 ng/ml of IL-1β, LNCaP cells almost disappeared on a WI-38 monolayer sheet (Fig. 1). At 5 ng/ml, TNF-α also partially suppressed LNCaP cell growth in monoculture, while it strongly inhibited the

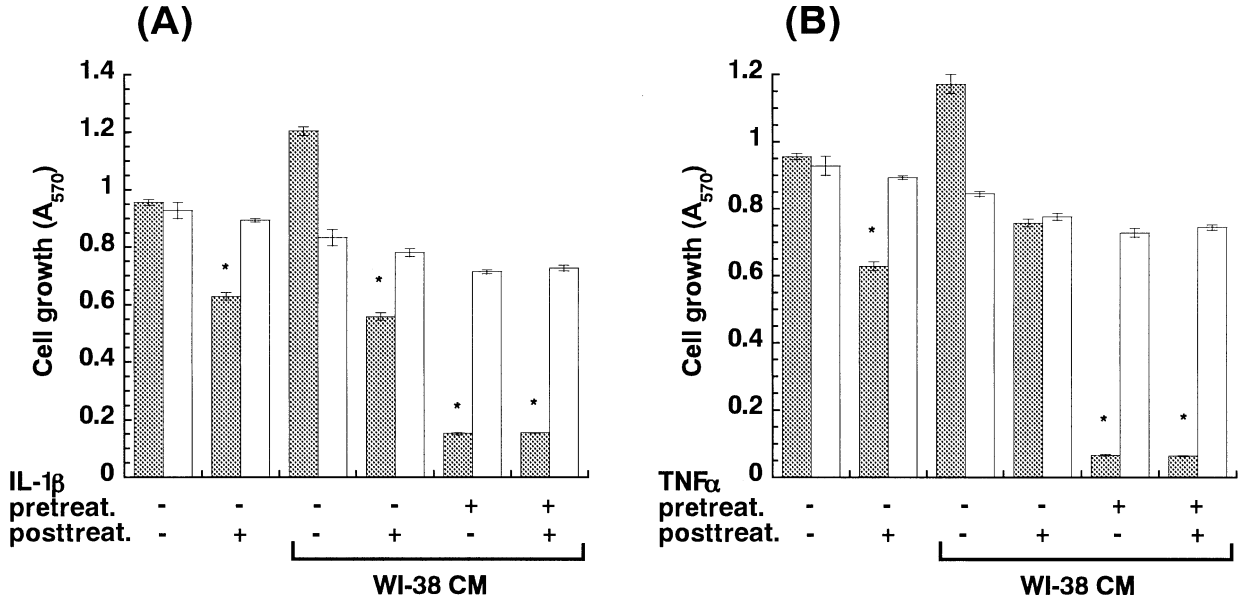

Fig. 4. Effect of cytokine-treated WI-38 conditioned medium on prostate cancer cell growth. Conditioned medium was prepared from WI-38 cells cultured for 2 days with or without 5 ng/ml of IL-1β (A) or TNF-α (B) (pretreat.). Then, LNCaP (▨) or PC-3 (□) cells were cultured in normal medium or the conditioned medium for 3 days with or without a further addition of 5 ng/ml of IL-1β (A) or TNF-α (B) (posttreat.). Cell growth was determined using MTT. The values are means±SD of 3 independent duplicate determinations. * $P<0.001$ versus none.

growth in coculture with WI-38 cells (Figs. 1 and 2B). These results showed that the antiproliferative effects of IL-1β and TNF-α against LNCaP cells were greatly enhanced by coculture with WI-38 cells. In contrast, high concentrations of TNF-α or IFN-γ moderately suppressed the growth of androgen-independent C-3 cells, but IL-1β, TNF-α, and other tested cytokines failed to show apparent inhibitory effect on the growth in coculture with WI-38 cells (Fig. 2 and data not shown).

Effects of IL-1β and TNF-α on androgen response in LNCaP cells Because IL-1β and TNF-α inhibited the growth of androgen-dependent LNCaP cells but not that of androgen-independent PC-3 cells, we examined the effects of IL-1β and TNF-α on androgen response in LNCaP cells. The experiments were performed in medium supplemented with serum that had been charcoal-stripped to remove residual hormones. LNCaP cells in monoculture responded to the proliferative effect of DHT at 10^{-11}–10^{-10} *M* (Fig. 3A). On the other hand, PC-3 cells showed almost no response to DHT (Fig. 3B). Although IL-1β and TNF-α suppressed LNCaP cell growth, the cells remained responsive to DHT (Fig. 3A). In contrast, LNCaP cells in coculture with WI-38 cells responded to DHT, but IL-1β and TNF-α completely inhibited the responsiveness to DHT (Fig. 3A).

Effect of cytokine-treated WI-38 conditioned medium on LNCaP cell growth To investigate the hypothesis that some diffusible factor(s) would be secreted from WI-38 cells under the influence of IL-1β or TNF-α, we prepared conditioned medium from WI-38 cells pretreated with the cytokines and cultured LNCaP cells in the conditioned medium. Conditioned medium prepared from untreated WI-38 cells did not affect LNCaP cell growth and the addition of IL-1β to the culture suppressed the growth a little more strongly as compared with the culture without conditioned medium (Fig. 4A). However, when LNCaP cells were cultured in conditioned medium prepared from WI-38 pretreated with IL-1β, the growth was completely inhibited with or without further addition of IL-1β to the culture (Fig. 4A). As well as IL-1β, conditioned medium prepared from TNF-α-pretreated WI-38 cells also completely inhibited LNCaP cell growth (Fig. 4B). On the other hand, these antiproliferative effects of the conditioned medium did not appear in PC-3 cells (Fig. 4). These results indicated that IL-1β or TNF-α stimulated WI-38 cells to secrete some diffusible factor(s) for growth inhibition of LNCaP cells.

Synergistic effect of IL-6 on IL-1β and TNF-α actions against LNCaP cells Because antiproliferative effects on LNCaP cells were only seen with IL-1β and TNF-α among the tested cytokines, we searched for some diffusible factor(s) secreted from IL-1β- or TNF-α-treated WI-38 cells by examining the synergistic effects of various cytokines on IL-1β and TNF-α actions against LNCaP

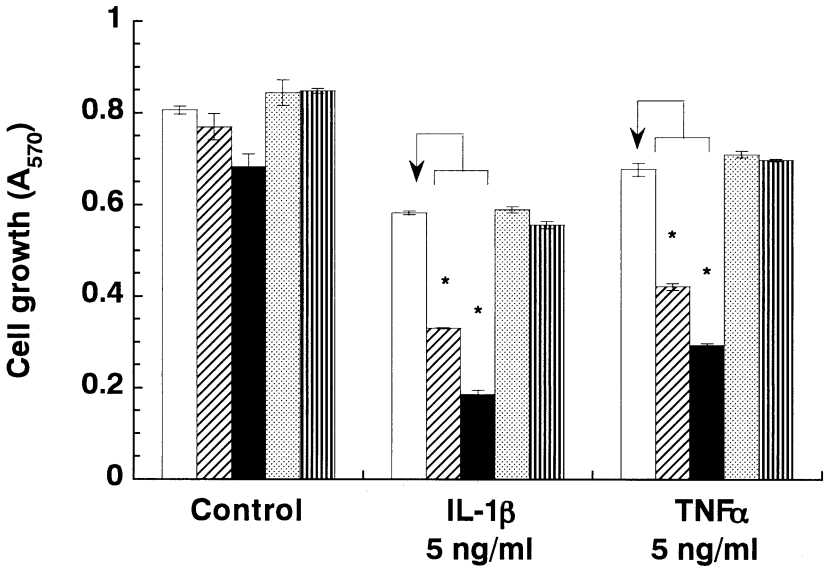

Fig. 5. Synergistic effect of IL-6 on IL-1β and TNF-α actions against LNCaP cells. LNCaP cells were cultured alone for 3 days with 0 (□), 5 (▨), and 50 (■) ng/ml of IL-6 or 50 ng/ml of IL-4 (▩) or IL-8 (▥) in the presence or absence of 5 ng/ml of IL-1β or TNF-α. Cell growth was determined using MTT. The values are means±SD of 3 independent duplicate determinations. * $P<0.001$.

Table I. Effect of IL-1β and TNF-α on IL-6 Production by WI-38 Cells

Treatment	IL-6 (ng/ml)
None	0.82±0.046
IL-1β 5 ng/ml	43.1±2.46*
TNF-α 5 ng/ml	4.70±0.814**

WI-38 cells were cultured for 2 days with or without IL-1β or TNF-α. Cell-free supernatants were assayed by ELISA for IL-6 content. The values are means±SD of 3 independent duplicate determinations. * $P<0.001$ versus none, ** $P<0.02$ versus none.

cells. Among IL-4, IL-6, IL-8, IL-10, IFN-γ, and TGF-β, only IL-6 was found to exert a synergistic effect. Although IL-6 alone did not affect the LNCaP cell growth up to 50 and 5 ng/ml of IL-1β or TNF-α alone also had no significant effect, IL-6 inhibited the growth in the presence of 5 ng/ml of IL-1β or TNF-α (Fig. 5). IL-4, IL-8 and other tested cytokines did not exert any synergistic effect even at 50 ng/ml (Fig. 5 and data not shown). Thus, IL-6 was found to act on LNCaP cells synergistically with IL-1β or TNF-α.

Increased production of IL-6 from WI-38 cells in the presence of IL-1β and TNF-α We examined whether IL-6 production by WI-38 cells is really stimulated by IL-1β and TNF-α using an ELISA. The same conditioned medium was used for growth inhibition assay (Fig. 4) and ELISA assay. In the absence of cytokines, WI-38 cells secreted IL-6 into the conditioned medium at 0.8 ng/ml (Table I). IL-1β and TNF-α increased the IL-6 secretion about 50 and 6 times, respectively (Table I). This result, therefore, showed that IL-1β and TNF-α stimulated WI-38 cells to produce significant amounts of IL-6.

Effect of IL-6 depletion on the action of cytokine-treated WI-38 conditioned medium To confirm the possibility that IL-6 secreted from WI-38 cells acts synergistically with IL-1β or TNF-α, we next examined the effect of IL-6 depletion on the action of cytokine-treated WI-38 conditioned medium using neutralizing antibodies to IL-6 or IL-6 receptor. Whereas neutralizing antibodies to IL-6 or IL-6 receptor did not affect the growth of LNCaP cells cultured in untreated WI-38 conditioned medium, these antibodies completely abrogated the antiproliferative effect of TNF-α-treated WI-38 conditioned medium (Fig. 6). Even though these antibodies failed to suppress the action of IL-1β-treated WI-38 conditioned medium, they inhibited it when the conditioned medium was diluted 100 times (Fig. 6). Thus, these results showed that IL-6 secreted from WI-38 cells in the presence of IL-1β or TNF-α inhibited LNCaP cell growth synergistically with IL-1β or TNF-α.

DISCUSSION

In coculture with fibroblasts, tumor cell growth is expected to be up-regulated or down-regulated through diffusible signals and direct contact. It was shown that

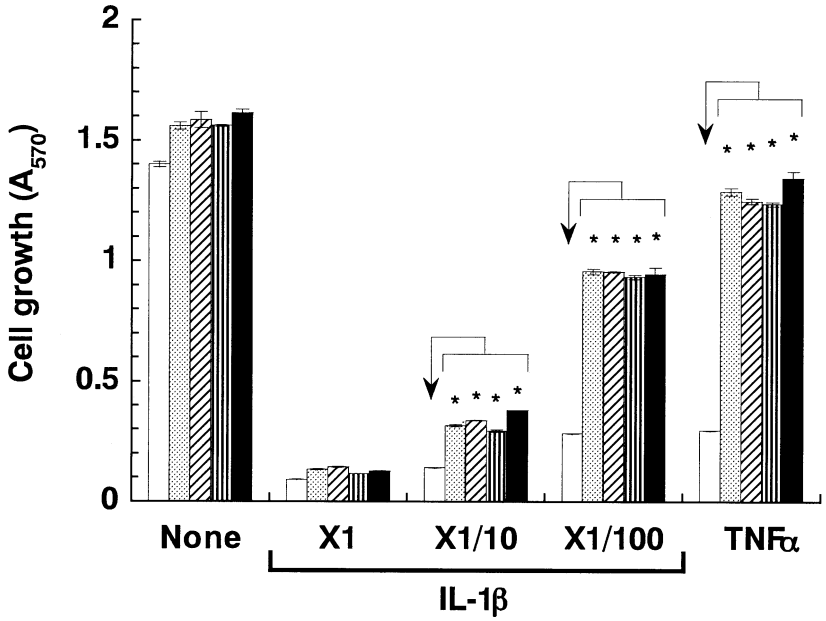

Fig. 6. Effect of IL-6 depletion on the action of cytokine-treated WI-38 conditioned medium. Conditioned medium was prepared from WI-38 cells cultured for 2 days with none or 5 ng/ml of IL-1β or TNF-α. LNCaP cells were cultured in the conditioned medium for 3 days with none (□), 5 (▩) or 10 (▨) μg/ml of anti-IL-6 antibody, or 25 (▥) or 50 (■) μg/ml of anti-IL-6 receptor antibody. The neutralizing antibodies to IL-6 and IL-6 receptor were added to the culture 1 h before the inoculation of LNCaP cells. The IL-1β-treated conditioned medium was diluted 1 (×1), 10 (×1/10), and 100 (×1/100) times with normal medium. Cell growth was determined using MTT. The values are means±SD of 3 independent duplicate determinations. ∗ $P<0.001$ versus none.

normal fibroblasts modulate breast tumor growth by co-culture experiments in which the cells were separated by a microporous membrane.[10] In this study, however, to model direct contact between tumor cells and fibroblasts, we performed a coculture experiment in which tumor cells were inoculated onto a monolayer culture of fibroblasts. Unexpectedly, neither LNCaP cell nor PC-3 cell growth was changed drastically by coculture with WI-38 fibroblasts. However, we found that the antiproliferative effects of inflammatory cytokines, IL-1β and TNF-α, on LNCaP cells were greatly enhanced by coculture with WI-38 cells. To study the cell-cell interactions between tumor and stromal cells in primary tumor development, some studies have been done using normal fibroblasts from the tissue of origin of the tumor.[10, 11] Although we tested other fibroblasts, including mouse and rat embryonic ones, and primary cultures derived from rat prostate, lung, and bone marrow, modulations of cytokine actions were less pronounced than with WI-38 cells. To examine the mechanism of enhancement of cytokine actions by coculture with fibroblasts, we used WI-38 cells in further experiments.

Although IL-1β and TNF-α have already been reported to suppress LNCaP cell growth, the growth-inhibitory effects are only partial.[12–14] On the other hand, in the coculture with WI-38 cells they inhibited LNCaP cell growth completely (Figs. 1 and 2). Using conditioned medium prepared from WI-38 cells, we showed that significant inhibition was conferred by some diffusible factor(s) secreted from WI-38 cells upon cytokine stimulation (Fig. 4). However, in these combinations of the cells and the cytokines, the influence of direct contact between tumor cells and fibroblasts was not negligible, because LNCaP cells almost disappeared on the coculture with WI-38 cells (Fig. 1). Further studies suggested that IL-1β and TNF-α stimulated WI-38 cells to secrete IL-6 and acted synergistically against LNCaP cells. It was reported that IL-1α inhibited the growth of human melanoma cells by increasing autocrine production of antiproliferative IL-6.[15] Autocrine production of IL-6 from LNCaP cells regardless of addition of IL-1β or TNF-α was below the limit of detection, i.e., less than pg/ml order, and far less than that from WI-38 cells (data not shown and Table I). Furthermore, IL-1β or TNF-α alone did not exert a strong growth-inhibitory effect in the absence of added IL-6. Thus, it was not the case that autocrine IL-6 exerted the antiproliferative effects against LNCaP cells. As IL-6 production from WI-38 cells in the presence of IL-1β was high, neutralizing antibodies to IL-6 or IL-6 receptor depleted it only when the conditioned medium was

diluted (Table I and Fig. 6). Because high concentrations of IL-6, even 5 μg/ml, partially suppressed LNCaP cell growth (data not shown) and the IL-1β content in the diluted conditioned medium was only a trace, secretion of some other factor(s) from WI-38 cells in the presence of IL-1β can not be excluded. However, our results suggested that some kinds of cancer cells such as LNCaP cells could be almost eliminated by early inflammatory defense in the presence of fibroblasts. Because IL-1 and TNF synergistically stimulate IL-6 production from fibroblasts,[16] these cytokines are considered to exert a strong antiproliferative effect on cancer cells at inflammatory sites *in vivo*. It has been reported that TNF-α induces apoptosis in LNCaP cells.[17] Furthermore, IL-6 up-regulates TNF receptor and potentiates the antiproliferative effect of TNF on human lymphoma cells.[18] The mechanism by which IL-6 enhances IL-1β and TNF-α actions against LNCaP cells is still being studied, but it seems possible that IL-6 sensitizes LNCaP cells to apoptosis induction through increasing the levels of cytokine receptors.

By contrast, growth of androgen-independent PC-3 cells was not significantly affected by the tested cytokines in our experiments. PC-3 cells were reported to secrete IL-6 as an autocrine growth factor to protect themselves from death.[14, 19] Moreover, it was also reported that neutralizing antibodies to IL-6 suppressed PC-3 cell growth.[19] However, in our experiments, neutralizing antibodies to IL-6 and IL-6 receptor had no effect on growth (data not shown). This discrepancy might be due to differences of antibodies and culture conditions. In contrast to the case of LNCaP cells, IL-1β and TNF-α did not exert growth-inhibitory activity on PC-3 cells. Concerning the antiandrogen effects of IL-1β and TNF-α, we showed that LNCaP cells in monoculture responded to the proliferative effect of DHT even in the presence of IL-1β or TNF-α, but they did not respond when in coculture with WI-38 cells (Fig. 3). Whether some diffusible factor(s) such as IL-6 secreted from the stimulated WI-38 cells or whether strong growth inhibition in coculture resulted in loss of responsiveness to DHT was not discriminated. On the other hand, concerning the relationship between androgen dependency and the responsiveness to inflammatory cytokines, our results suggested that loss of androgen dependency conferred resistance to inflammatory cytokines, and *vice versa*. Androgen-independent prostate cancer cells are often more metastatic and malignant than androgen-dependent cells.[8] Thus, we speculate that early carcinogenesis in the prostate is prevented by inflammation, but chronic mild inflammation might create inflammation-resistant prostate cancer cells, which then become androgen-independent. Although the mechanism of androgen independence in prostate cancer cells has been studied,[20–23] the relationship between androgen dependency and the responsiveness to IL-1β and TNF-α should be further investigated.

We think that this is the first report demonstrating the synergistic actions of IL-6 and IL-1β or TNF-α against LNCaP cells. Furthermore, our results suggest that growth of some kinds of tumor cells could be modulated by the addition of a stimulator, such as an inflammatory cytokine, indirectly though the mediation of fibroblasts. Coculture of tumor cells and fibroblasts should be a good system for discovering new types of anticancer drugs.

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