Interleukin 1β mediates the modulatory effects of monocytes on LNCaP human prostate cancer cells

Z Culig1, A Hobisch1, M Herold2, A Hittmair3, M Thurnher1, IE Eder1, MV Cronauer1, C Rieser1, R Ramoner1, G Bartsch1, H Klocker1 and G Konwalinka2

1Departments of Urology. 2Internal Medicine and 3Pathology. University of Innsbruck. Anichstraße 35. A-6020 Innsbruck. Austria

Summary Proliferative and secretory responses in androgen-sensitive prostate cancer LNCaP cells are regulated by steroid and peptide hormones and by differentiation-promoting substances. In the present study, we evaluated whether peripheral blood monocytes that exhibit anti-tumour activity in haematopoietic and solid tumours influence growth and secretion in the LNCaP cell line. For this purpose, LNCaP cells were incubated with monocyte-conditioned medium (MCM), and proliferation as well as expression of androgen receptor (AR) and secretion of prostate-specific antigen (PSA) were assessed. Conditioned medium from monocytes reduced proliferation in a dose-dependent manner. Incubation with 40% MCM caused a 50% reduction in cell proliferation. AR protein decreased by 70% and PSA levels in supernatants from LNCaP cells were reduced by approximately 80% following treatment with MCM. We focused on the contribution of two major products of activated monocytes, prostaglandin E2 and interleukin 1β (IL-1β), to the MCM modulatory action. LNCaP cells treated with prostaglandin E2 showed neither a reduction in proliferation nor a down-regulation of AR and PSA levels. The effects of MCM on cellular proliferation, AR protein and PSA secretion were abolished by pretreatment of MCM with a neutralizing anti-IL-1β antibody. In addition, recombinant IL-1β was able to replace MCM for the inhibition of proliferation and down-regulation of AR and PSA proteins. LNCaP cells were shown to express the IL-1β receptor type I, which transduces IL-1β signal. Our findings reveal that monocyte-derived IL-1β inhibits the proliferation of androgen-responsive prostate tumour cells and reduces AR and PSA levels.

Keywords: prostate cancer cell; monocyte; proliferation; androgen receptor; prostate-specific antigen; interleukin 1β

The LNCaP cell line, which was derived from a lymph node metastasis of a patient who did not respond to endocrine therapy, is frequently used as a model for studying human prostate cancer (Horoszewicz et al. 1983). It is known that steroid and peptide hormones as well as differentiation-promoting substances regulate proliferative and secretory responses in LNCaP cells. The cells respond to androgen stimulation by accelerating division and producing prostate-specific antigen (PSA). The patterns of androgenic regulation of growth and secretory function in the LNCaP cell line are different (Lee et al. 1995). The maximal growth rate is achieved at 0.1 nm of dihydrotestosterone (DHT), whereas higher doses of this androgen induce growth arrest. It was proposed that the inhibition of cellular proliferation by high androgen doses is mediated by transforming growth factor β (TGF-β) (Kim et al. 1996). In contrast to the proliferative response, the percentage of PSA-positive cells and the levels of PSA protein in LNCaP supernatants increase at concentrations of 1 nm dihydrotestosterone and beyond (Lee et al. 1995). In addition to androgens, polypeptide growth factors such as epidermal growth factor (EGF), TGF-α, insulin-like growth factors and basic fibroblast growth factor (bFGF) are mitogenic for LNCaP cells (Wilding et al. 1989; MacDonald and Habib. 1992; Nakamoto et al. 1992; Ritchie et al. 1997). Positive effects of triiodothyronine and inhibitory effects of vitamin D, retinoic acid, luteinizing hormone-releasing hormone, phenylacetate and activin on growth of LNCaP cells have also been described (Limonta et al. 1992; Skowronski et al. 1993; Young et al. 1994; Esquet et al. 1995; Dalkin et al. 1996; Walls et al. 1996). Secretion of PSA was found to be enhanced after treatment with triiodothyronine, vitamin D and phenylacetate (Skowronski et al. 1993; Esquet et al. 1995; Walls et al. 1996), whereas divergent results on PSA regulation by retinoic acid were reported (Fong et al. 1993; Young et al. 1994). LNCaP cells express a mutant androgen receptor (AR), which binds oestrogenic and progesterogenic steroids and non-steroidal anti-androgens hydroxylutamide and nilutamide with higher affinity than the wild-type AR. These substances induce reporter gene activity in the presence of LNCaP AR more efficiently than in the presence of the wild-type AR (Veldscholte et al. 1990).

Studies on interactions between leucocytes that produce various cytokines and differentiation factors and prostate tumour cells may improve understanding of prostate cancer biology. In one of the initial studies in this field Hsieh et al. (1995) have shown that phytohaemagglutinin (PHA)-stimulated lymphocytes produce substance(s) that reduce cell proliferation, increase expression of cytoskeleton proteins and down-regulate AR and PSA in LNCaP cells. These effects were observed after treatment with various concentrations of T-lymphocyte-conditioned media (TCM). However, the T-cell-derived factor(s) that mediate these effects have so far not been identified. Interactions between other cells of the immune system and various tumours have been described (Wang et al. 1996; Iversen et al. 1997). For example, monocytes were found to suppress cell viability and colony formation in human leukaemias and lung tumours. In this study, we address the issue of whether peripheral blood monocytes influence growth and secretion in androgen-responsive prostate epithelial cells. We
show that monocyte-derived interleukin 1β (IL-1β) is a mediator of the modulatory effects on LNCaP cells.

**MATERIALS AND METHODS**

**Materials**

MCDB-131 and RPMI-1640 media were purchased from Sigma (Deisenhofen, Germany) and HyClone (Logan, UT, USA) respectively. Fetal calf serum (FCS) and antibiotics (penicillin/streptomycin) were from Biological Industries (Kibbutz Beth Haemek, Israel). Phosphate-buffered saline (PBS) was from PAA Laboratories (Linz, Austria). Cell culture vessels were from Costar (Cambridge, MA, USA), Sarstedt (Nümbrecht, Germany) and Falcon (Lincoln, NE, USA).

Phytohaemagglutinin-M (PHA-M) was purchased from Difco Laboratories (Detroit, MA, USA). The murine monoclonal anticytokertin 8 and 18 antibody CAM 5.2 was purchased from Becton Dickinson (San Jose, CA, USA) and a polyclonal rabbit anti-IL-1β antibody from Genzyme (Cambridge, MA, USA). The mouse monoclonal antibody MEM18 (IgG1 anti-CD14) was from An Der Grub, Vienna, Austria. Biotinylated monoclonal antibody 6B5 (IgG2a anti-IL-1 receptor type I) was purchased from Pharmingen (San Diego, CA, USA). Both radiolabelled (specific activity 83.2 Ci mmol⁻¹) and unlabelled synthetic androgen methyltrienolone (R 1881) were purchased from New England Nuclear (Breinchenhain, Germany). IL-1β was provided by Sigma. Prostaglandin E₃ was from Boehringer Ingelheim (Heidelberg, Germany). The scintillation liquid Optiphase was from Pharmacia (Uppsala, Sweden). The commercial methyl-thiobetrazole (MTT) assay Eₐₐ was purchased from Biomedica (Vienna, Austria). An immunoenzymometric assay for the quantitative measurement of human IL-1β (minimum detectable concentration 2 pg ml⁻¹) was provided by Medgenix Diagnostics (Fleurus, Belgium). The PSA IMX enzyme immunoassay (sensitivity 0.1 ng ml⁻¹) was from Abbott Laboratories (Abbott Park, IL, USA). FACS Calibur apparatus and CellQuest software were from Becton-Dickinson.

**Culture of peripheral blood monocytes**

Peripheral blood was collected from three healthy volunteers on several occasions. Monocytes were obtained from peripheral blood mononuclear cells after Ficoll separation by standard adherence (90 min, 37°C) and cultured in RPMI-1640 medium that was supplemented with 10% charcoal-stripped FCS (CS FCS). The purity of monocytes as determined by staining for naphthol acetate esterase and by measuring CD14 expression was > 90%. For preparation of monocyte-conditioned medium (MCM), 2.5 x 10⁶ monocytes ml⁻¹ were cultured with and without PHA. After 48 h, the supernatants were cleared by centrifugation and frozen at −20°C until use.

**Proliferation assays in LNCaP cells**

The LNCaP cell line was purchased at passage 21 from the American Type Culture Collection (Bethesda, MD, USA). LNCaP cells were seeded into 24-well plates at 2 x 10⁴ cells per well in MCDB-131 medium supplemented with 10% FCS. Medium was changed 24 h later and the final concentration of CS FCS was 3%. Culture medium was supplemented with MCM (10–60%). Prostaglandin E₃ (1–10 μM) or IL-1β (0.1–10 ng ml⁻¹). Control experiments were carried out in the absence of any supplement in medium or in the presence of PHA-M. In neutralization experiments, MCM was preincubated with the antibody against IL-1β overnight. It has previously been determined that 1 μg of this antibody is capable of neutralizing approximately 1000 units of natural or recombinant IL-1β. Cell proliferation was determined after 72 h incubation by means of the MTT assay. This assay is based on the ability of living cells to reduce slightly coloured tetrazolium salts to intensely coloured formazan derivatives. The assay was performed as described previously (Cronauer et al. 1996). In selected experiments the MTT assay results were compared with those obtained with a cell counter and were found to be identical.

**Immunohistochemical analyses**

Following treatment with MCM or medium without supplements, LNCaP cells were trypsinized, cyospun, resuspended in PBS, fixed in 1% paraformaldehyde, permeabilized by adding 0.1% Triton X-100 and stained for cytokertin expression. Cytokeratin immunohistochemistry was performed according to a streptavidin–biotin–peroxidase protocol. The immunohistochemical procedure was described previously (Hobisch et al. 1995).

**Androgen receptor-binding assay**

LNCaP cells were cultured in the absence (untreated control) or presence of respective supplements in 5% CS FCS for 72 h. Then they were scraped off, washed once, resuspended in medium and incubated with [³H]methyltrienolone, at concentrations of 0.3–5 nm, for 90 min at room temperature. Non-specific binding was measured in the presence of a 200-fold molar excess of unlabelled methyltrienolone. The pellets were recovered after incubation by centrifugation (3800 g, 3 min) and washed twice with 500 μl of ice-cold medium. The cell pellets were then lysed in 1 ml of scintillation liquid and the radioactivity was determined in a β-counter. Cellular protein was determined according to the method described by Bradford (1976). Bₐₐ and Kₐ were calculated by Scatchard analysis.

**Determination of prostate-specific antigen in supernatants from LNCaP cells**

The cells were grown on 24-well plates in the presence of 5% CS FCS with or without supplements. The medium was removed after 72 h and the PSA level was measured by an enzyme immunoassay. PSA values were corrected for cell number according to the results of the proliferation assay.

**Determination of IL-1β concentration in monocyte conditioned media**

IL-1β in MCM was determined by a solid-phase enzyme amplified sensitivity immunoassay. The assay is based on an oligoclonal system in which several monoclonal antibodies directed against distinct epitopes of IL-1β are used. The use of several distinct monoclonal antibodies avoids assay hyperspecificity. The assay was performed on a microtitre plate. Samples containing IL-1β react with capture antibodies coated on a plastic well and with monoclonal antibodies labelled with horseradish peroxidase. After
an incubation period of 2 h at room temperature the microtitre plate was washed three times to remove unbound enzyme-labelled antibodies. The revelation solution (tetramethylbenzidine-hydrogen peroxide) was added and incubated for 15 min at room temperature. The reaction was stopped with sulphuric acid and the microtitre plate was read at 450 nm. The concentrations of IL-1β in MCM ranged between 1.6 and 2.4 ng ml⁻¹.

**Measurement of surface antigen expression by flow cytometry**

To determine surface antigen expression, peripheral blood monocytes were labelled with primary mouse monoclonal anti-CD14 antibody MEM18 followed by fluorescein isothiocyanate (FITC) -conjugated goat anti-mouse Ig. LNCaP cells were labelled with biotinylated primary mouse monoclonal anti-IL-1 receptor antibody 6B5 followed by FITC-conjugated streptavidin. Washes were in PBS containing 0.2% bovine serum albumin (BSA). After the last wash, the cells were stored in PBS containing 0.2% BSA and 2% formaldehyde. The samples were analysed on a FACS Calibur. Data were analysed and presented using CellQuest software.

**RESULTS**

**Monocyte-conditioned medium-treated LNCaP cells show changes in cell shape**

To study the influence of monocytes on prostate cancer cells, LNCaP cells were exposed to medium conditioned by peripheral blood monocytes homogeneously positive for CD14 (Figure 1A). Distinct morphological changes in LNCaP cells were observed after treatment with monocyte-conditioned medium (MCM). The cells became elongated and showed dendrite-like processes, which were connected to each other (Figure 2). These morphological changes were notable even with 10% MCM in LNCaP culture medium. Expression of cytoskeletal proteins was evaluated by semiquantitative immunohistochemistry. A monoclonal antibody directed against luminal cytokeratins 8 and 18 was used for this purpose. The expression and staining intensity of these cytokeratins were previously reported to increase in the more differentiated prostatic luminal epithelium (Peehl et al. 1993). In the case of LNCaP cells treated with MCM, we observed neither an increase in the percentage of cytokeratin-immunopositive cells nor a change in staining intensity.
Monocyte-conditioned medium causes a dose-dependent reduction in cellular proliferation

LNCaP cells were cultured in the absence or presence of MCM for 3 days. After this period, cellular growth was assessed by means of the MTT assay. Conditioned medium from PHA-M-activated monocytes reduced cellular proliferation in a dose-dependent manner (Figure 3). MCM at 40% reduced proliferation by 50%. Higher concentrations of MCM in culture media did not result in any further reduction in cell number. PHA-M itself did not display any effect on the proliferation of LNCaP cells. Conditioned medium from monocytes that were not treated with PHA-M induced a similar decrease in cell proliferation (data not shown). Thus, it seems that the presence of PHA in monocyte cultures is not required for inhibition of growth of LNCaP cells. The proliferative effect of 0.01 nm of R1881 was abolished by 40% MCM (Figure 3).

Androgen receptor levels and PSA secretion decrease after treatment with MCM

Specific binding of radioactively labelled methyltrienolone in MCM-treated LNCaP cells as well as in controls was determined. MCM caused a dose-dependent reduction in androgen binding. Figure 4 shows a decrease in specific binding of [3H]R1881 in cells treated with 40% MCM. The maximum decrease in the AR level was approximately 70% in the presence of 40% MCM. There was no change in AR binding affinity following MCM treatment. Secretion of the AR-regulated PSA protein was measured in supernatants from LNCaP cells after 72 h of incubation with MCM. A decrease in PSA was observed in the presence of 20–40% of MCM in culture media. PSA protein was reduced by 80% with 40% MCM. MCM also antagonized stimulatory effects of the synthetic androgen R1881 on PSA secretion into the supernatant (Figure 5).

IL-1β is responsible for the modulatory effects of MCM

We focused on the contribution of individual factors to the MCM modulatory effects. Initially, we investigated the role of prostaglandin E2, which is a major product of activated monocytes (Venkataprasad et al., 1996). LNCaP cells treated with prostaglandin E2 showed neither a reduction in proliferation nor a down-regulation of AR and PSA levels. Thus, prostaglandin E2 can be excluded as a mediator of the inhibitory effects of monocytes on androgen-responsive prostate cancer cells. Next, we examined the role of the proinflammatory cytokine IL-1β, which exhibits anti-tumour activity in several models (Kilian et al., 1991; Braunschweiger et al., 1996), in the interaction between the immune system and prostate cancer cells. In order to determine whether IL-1β mediates MCM effects on LNCaP cells, MCM was preincubated with a neutralizing polyclonal anti-IL-1β antibody. The effectiveness of this antibody was previously demonstrated in other assay systems (Salem et al., 1990; Lisak and Bealmeir, 1991). MCM pretreated with the antibody did not reduce LNCaP cell proliferation (Figure 6A). The MCM effect on AR protein was completely abolished after preincubation of MCM with the polyclonal anti-IL-1β antibody (Figure 6B), and the PSA levels were almost completely restored (Figure 6C). Moreover, addition of recombinant IL-1β to cultures of LNCaP cells exerted effects that
DISCUSSION

The major finding of the present study is that peripheral blood monocytes are capable of modulating cellular events in androgen-responsive LNCaP cells by secreting IL-1β. This ability of monocytes is similar to that previously reported for T lymphocytes in the same cell line (Hsieh et al. 1995). Both cell types secrete substances that cause a reduction in cellular proliferation, diminish levels of AR protein and down-regulate PSA in LNCaP cells. In our experiments, MCM was effective regardless of the presence of PHA in monocyte cultures. In contrast, unstimulated lymphocytes provoked only a minimal growth inhibition of LNCaP cells (Hsieh et al. 1995).

LNCaP cells became elongated and exhibited dendrite-like processes after contact with MCM and TCM. Similar changes in cell shape were described after treatment of LNCaP cells with analogues of cAMP (Bang et al. 1994). There was, however, one difference between MCM- and TCM-treated LNCaP cells. The expression of cytokeratins 8 and 18 and their staining intensity increased twofold after incubation with TCM as determined by a total intensity score procedure (Hsieh et al. 1995). In the case of MCM we did not see any differences in the expression of these cytoskeleton proteins by semiquantitative immunohistochemistry. Contrasting results on expression of cytokeratins may be due to use of different antibodies and immunohistochemical techniques. It was suggested by others that TCM- and cAMP analogue-treated LNCaP cells undergo neuroendocrine differentiation (Bang et al. 1994; Hsieh et al. 1995). TCM treatment provoked an increase in total intensity score of two neuroendocrine markers, neuron-specific enolase and serotonin. However, the concept of neuroendocrine differentiation of this cell line was questioned recently (Noordzij et al. 1996). In that publication and in our previous experimental studies neuroendocrine cells within the LNCaP cell line were not identified (unpublished data). Thus, a differentiation process induced by leukocytes in androgen-responsive prostate cancer cells is not well characterized to date.

The MCM-induced growth-inhibitory effect was associated with decreased AR expression. Association between AR expression and growth regulation of prostate cancer cells can be studied in AR-positive LNCaP cells and in AR cDNA-transfected PC-3 cells. It was demonstrated that the expression of AR in PC-3 cells leads to a decrease in proliferation (Yuan et al. 1993). We infer that more reliable information on AR involvement in growth regulation may be obtained in its natural cellular environment, i.e. in LNCaP cells. Growth inhibition and AR reduction were previously observed in LNCaP cells incubated with retinoic acid and TCM (Young et al. 1994; Hsieh et al. 1995). Regarding AR expression, conditioned media from T lymphocytes and monocytes, and retinoic acid differ from anti-androgens cyproterone acetate, hydroxyflutamide and bicalutamide, which are commonly used in endocrine therapy for prostate cancer. These compounds bind to the AR with low affinity and do not permit acquisition of a transcriptionally active form of the receptor (Kempainen et al. 1992; Kallio et al. 1994). However, they did not down-regulate AR protein itself. AR is expressed in relapsed prostate tumours and in their metastases, which were obtained before onset of therapy and during tumour progression (van der Kwast et al. 1991; Hobsich et al. 1995, 1996). Several recent publications support the view that reduction in androgen concentration is not sufficient to prevent transmission of extracellular signals via the AR (Veldscholte et al. 1990; Culig et al. 1993, 1994; Kokontis et al. 1994; Nazareth and Weigel, 1996). Prostate cancer cells may adapt to an environment with very low androgen concentration by increasing their AR expression and transcriptional activity (Kokontis et al. 1994). Furthermore, mutant ARs discovered in prostate cancer frequently exhibit a gain in function: they are efficiently activated by other steroids and non-steroidal AR antagonists (Veldscholte et al. 1990; Culig et al. 1993). Finally, AR activity is up-regulated by several non-steroidal substances such as polypeptide growth factors and second messengers (Culig et al. 1994; Nazareth and Weigel, 1996). Therefore, strategies aimed at reducing AR levels, such as administration of secretory products from the immune cells, may be beneficial in metastatic prostate cancer. AR down-regulation was followed by reduction of PSA protein in LNCaP supernatants. Thus, the effect of MCM is different to that of phynylacetate and vitamin D. substances that have been reported to inhibit proliferation of LNCaP cells and increase PSA secretion (Walls et al. 1996; Zhao et al. 1997).

The mediator role of IL-1β was revealed in experiments in which the neutralizing anti-IL-1β antibody abolished the effects of MCM. Furthermore, IL-1β itself exhibited a dose-dependent inhibition on LNCaP proliferation. AR protein level and PSA secretion. In contrast to our findings with MCM, a compound that is responsible
for the growth-modulatory effects of TCM on androgen-responsive prostate cancer cells has not been identified. Antibodies against TNF-α, TGF-β, FGF, EGF and IL-2, -4, -5, -6 or -8 were tested, but none of them was capable of neutralizing TCM effects (Hsieh et al. 1995). Based on semipurification of growth-modulatory activity, it was proposed that a protein in the molecular weight range of 13–24 kDa is responsible for TCM action. It is not unlikely that growth-modulatory effects of TCM and MCM could be, at least in part, attributed to the same cytokine.

Monocytes are known as the major source of secreted IL-1 (Dinarello, 1988). Human IL-1β is a 17-kDa protein that is secreted during an inflammatory process. Two functionally almost equivalent forms of IL-1, IL-1α and IL-1β, which display about 27% homology at the protein level, exist. Both forms of IL-1 bind to the
same cell-surface receptor and therefore show similar biological activities. IL-1 was found to exhibit anti-tumour activity in various neoplasms either by augmentation of cellular immune response or by inhibition of proliferation (Braunschweiger et al. 1988; Kilian et al. 1991). In agreement with our results, IL-1 was previously found to provoke a dose-dependent growth inhibition in LNCaP cells (Hsieh and Chiao, 1995; Ritchie et al. 1997). IL-1 diminished DHT effect on the proportion of the replicating LNCaP cells (Hsieh and Chiao, 1995). However, our results with regard to AR binding differ from those of Hsieh and Chiao. These authors failed to show a negative effect on AR protein levels after IL-1x treatment. Ritchie et al. (1997) also treated LNCaP cells and androgen-independent PC-3 and DU-145 cells with increasing concentrations of IL-1b. Supplementation of prostate cell cultures with IL-1b caused a decreased proliferation of all three cell lines. However, the negative effect of IL-1b on proliferation was far more pronounced in LNCaP cells than in the two androgen-independent cell lines. Although determination of AR protein was not performed in this study, a marked effect in LNCaP cells suggests an interaction between IL-1b and AR pathways. IL-1b also decreased cell chemotaxis in the three prostate cancer cell lines (Ritchie et al. 1997). Interestingly, inhibition of growth of MCF-7 breast cancer cells by IL-1 is associated with down-regulation of oestrogen receptor (Danforth and Saggiya, 1991).

A different type of interaction between monocytes and LNCaP cells was recently reported (Klein et al. 1997). Expression of matrilysin, which is a member of the matrix metalloproteinase superfamily, in LNCaP cells is induced by conditioned medium from the monocytic cell line THP-1. In addition, recombinant IL-1 up-regulated matrilysin expression. Biological neutralization experiments revealed that the effect of THP-1 conditioned medium was abolished by an anti-IL-1b antibody, and it was concluded that IL-1b is a mediator of matrilysin induction. This up-regulation of matrilysin by IL-1b may be harmful in prostate cancer. It was shown that matrilysin-transfected prostate cancer cells have a high invasive potential in immunodeficient mice (Powell et al. 1993).

IL-1b activates several second-messenger systems in target tissues (Munoz et al. 1990; Roberts et al. 1992; Carman-Krzan and Wise, 1993; Cole et al. 1995; Sjöholm 1995). For example, IL-1b induces cAMP in human decidual cells. It stimulates secretion of nerve growth factor in astroglial cultures by activation of the phospholipase A2-lipoygenase pathway. In pancreatic β-cells ceramide may be involved in transducing the cytokine and cytosolic actions of IL-1b. Involvement of protein kinase C and nitric oxide pathways in IL-1b signalling was also described. It remains to be determined in future studies which of these signalling pathways may be operative in prostatic epithelial cells.

In summary, this study demonstrates that the monocyte secretory product IL-1b is a potentially important negative regulator in LNCaP cells. Therapeutic application of this pleiotropic cytokine in prostate cancer will probably be further explored.

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