Oncostatin M and IL-6 Induce u-PA and VEGF in Prostate Cancer Cells and Correlate *In Vivo*

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Abstract. *Background/Aim: Oncostatin M (OSM) and interleukin-6 (IL-6) are growth factors for prostate cancer (PC). Vascular endothelial growth factor (VEGF) and urokinase-type plasminogen-activator (u-PA) have been implicated in tumour progression. A possible interaction between IL-6, OSM, u-PA and VEGF in PC was investigated. Materials and Methods: Primary prostate epithelial cells (PPEC) and DU-145 PC cells were treated with IL-6 or OSM and the effects on u-PA and VEGF expression were studied. Plasma levels of IL-6, OSM, u-PA and VEGF were determined in patients with or without PC. Results: In DU-145 cells, OSM and IL-6 up-regulated u-PA and VEGF significantly. Higher levels of IL-6 and OSM in metastasising PC than in non-metastasising PC and benign prostatic hyperplasia (BPH) and correlations between IL-6, OSM, u-PA and VEGF were found. Conclusion: OSM and IL-6 increase u-PA and VEGF in DU-145 cells but not in PPEC and possibly, by promoting matrix degradation and angiogenesis, could play a role in the pathogenesis of prostate cancer.*

Prostate cancer (PC) is the most common cancer diagnosed in men and the second leading cause of cancer death among men in the USA (1). PC initially occurs as an androgen-dependent tumour. Thus, androgen-deprivation is a commonly used therapeutic strategy for the treatment of PC. While the initial response rate is excellent, the cancer eventually recurs in the androgen-depleted state. The underlying mechanisms of androgen-independent proliferation remain unknown. Among many putative PC growth factors is the pleiotropic cytokine and glycoprotein (gp) 130 ligand interleukin-6 (IL-6), which has been previously shown to act as a paracrine and autocrine growth factor for PC *via* activation of the androgen receptor on PC cells through the janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways (2, 3). Furthermore, elevated serum levels of IL-6 have been found in patients with androgen-independent disease, and both IL-6 protein and receptors (IL-6R) have been identified in PC specimens, while the absence of IL-6 significantly impaired proliferation of prostatic carcinoma cell line *in vitro* (4, 5). IL-6 is part of the "IL-6 superfamily", that also includes leukaemia inhibitory factor (LIF), IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and oncostatin M (OSM). Among these cytokines OSM has also been shown to induce the proliferation of human PC cells *in vitro* and blocking of signalling through gp130 inhibited IL-6 and OSM induced growth in such cells (4, 6, 7).

In various human tissues different members of the IL-6 family have been shown to modulate extracellular matrix degradation and angiogenesis, two events crucial for cancer progression and metastasis (8, 9).

Urokinase-type plasminogen activator (u-PA), a serine protease of the plasminogen/ PA system, has been implicated in promoting metastasis of many types of cancers (10).

It is assumed that the role of the plasminogen/PA system in PC is primarily by activating plasminogen into plasmin, which in turn would degrade matrix components directly and indirectly by generating active matrix metalloproteinases from their respective inactive precursors (11, 12). Consistent

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Table I. *Primers used for RT-PCR.*

Target	Annealing temperature	fwd-Primer	rev-Primer
VEGF	55˚C	5'-gga cat ctt cca gga gta-3'	5'-tgc aac gcg agt ctg tgt-3'
u-PA	65˚C	5'-aca cgc ttg ctc acc aca-3'	5'-ccc agc tca caa ttc cag tc-3'
GAPDH	60˚C	5'-aca gtc cat gcc atc act gcc-3'	5'-gcc tgc ttc acc acc ttc ttg-3'

Primers were designed using the Primer3 Software (http://frodo.wi.mit.edu/).

with this view u-PA and its receptor u-PAR have been found in malignant prostate tissue (13, 14). Furthermore u-PA plasma levels are significantly elevated in patients with PC and correlate with an aggressive phenotype and poor prognosis of human PC (14-18).

Together with matrix degradation, angiogenesis is a prerequisite for solid tumour growth and metastasis (19, 20). In PC microvessel density reflecting angiogenesis is associated with the development of metastasis and overall patient survival (21). Elevated plasma levels of the potent angiogenic mediator vascular endothelial growth factor (VEGF) have been found in patients with metastasising and hormone-refractory PC and VEGF and its receptors have been detected in malignant prostate tissue (22-29). While the role of VEGF as a highly potent angiogenic factor is well documented, direct effects of VEGF on PC cells by stimulating the motility of these cells and by inducing mitogenesis of PC cells *in vitro* have been suggested (28, 30).

The aim of the present study was to investigate whether IL-6 and OSM affect the expression of u-PA and VEGF in primary prostate epithelial cells (PPEC) and the PC cell line DU-145. In order to inspire discussion of the relevance of the *in vitro* data in a clinical setting, plasma levels of IL-6, OSM, VEGF and u-PA were also determined in patients with metastasising PC, non-metastasising PC, and benign prostate hyperplasia (BPH).

Materials and Methods

In vitro experiments.

Cell lines. PPEC were obtained from Cambrex Corporation, East Rutherford, NJ, USA. The DU-145 cells were purchased from DSMZ, Braunschweig, Germany.

Treatment of PPEC and DU-145. The PPEC (15.000 cells/cm^2) were incubated in Prostate Epithelial Cell Basal Medium (PrEBM; Cambrex Corporation) and the DU-145 cells (15.000 cells/cm^2) were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, St. Louis, MO, USA), containing 0.1% bovine serum albumin (BSA; Sigma) for 24 hours prior to treatment with the respective cytokine. Thereafter the medium was replaced with fresh PrEBM or RPMI 1640, respectively, containing 0.1% BSA and recombinant human (rh) IL-6, rh LIF or rh OSM, all obtained from R&D Systems, Minneapolis, MN, USA, respectively, was added at the concentrations indicated. After incubation for 24 hours, the culture supernatants were collected following removal of cell debris by centrifugation and stored at –70˚C until used. The total cell number of the respective cultures after trypsinisation was counted with a haemocytometer.

Determination of u-PA and VEGF antigen. The levels of u-PA and VEGF in the culture medium were determined by specific enzyme linked immunosorbent assays (ELISAs; Technoclone, Vienna, Austria for u-PA, R&D Systems for VEGF-A) according to manufacturers' instructions.

Real-time-PCR. Stimulation with rh IL-6 (100 ng/ml) , rh LIF (10^4 U/ml) or rh OSM (100 ng/ml) of the PPEC and DU-145 cells was performed as described above for 4 or 8 hours. mRNA was isolated using QuickPrepTM Micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK), according to the manufacturers' instructions. Real-time-PCR was performed using LightCycler-RNA Master SYBR Green I (Roche) according to the manufacturers' instructions. The primers, as shown in Table I, were designed using the Primer3 Software (http://frodo.wi.mit.edu/). The amplification conditions consisted of an initial incubation at 61˚C for 20 minutes, followed by incubation at 95˚C for 30 seconds, 50 cycles of 95˚C for 1 second, 60˚C for 10 seconds and 72˚C for 10 seconds, a melting step from 45˚C to 95˚C increasing 0.1˚C per second and a final cooling to 40˚C. The data were analysed using LightCycler Software Version 3.5 (Roche).

Clinical data.

Patients. A total of 47 patients were included in this cross-sectional study. Seven of these patients presented at their first visit with metastasising PC (Tx Nx M1), 20 with non-metastasising PC (≥T2 Nx M0) and 20 with BPH. Patients with BPH who underwent surgery were chosen as the control group to be certain of the histopathological diagnosis and to have the possibility of an age-matched control group. The clinical characteristics are summarized in Table II. The patients were selected from outpatients' clinics for urology at their first presentation to the specialist. Patients with hormonal therapy or former reproductive or endocrine disease were excluded. The study was carried out in compliance with the Helsinki Declaration and was approved by the Regional Ethics committee. All the subjects gave their written informed consent to participate.

Determination of plasma levels of IL-6, OSM, VEGF, and u-PA. The plasma levels of u-PA, VEGF, IL-6 and OSM of the patients were determined by ELISAs (R&D Systems for IL-6, OSM, and VEGF-A; Technoclone for u-PA) according to the manufacturers' instructions.

Table II. *Patient characteristics*.

	Benign hyperplasia (n=20)	Non-metastasising PC (n=20)	Metastasising PC (n=7)
Age (years; mean±SE)	66.84±2.06	67.39±1.61	76.31±1.61
PSA (ng/ml; mean±SE)	2.34±0.4	7.47±0.73	274.96±111.18
Smokers %	34	29	33
Diabetes %	15	18	16

PC: prostate carcinoma; PSA: prostate specific antigen.

Table III. *Effects of gp130 ligands on u-PA and VEGF mRNA*.

		u-PA	VEGF
OSM	4 hours	88±5	251±20*
	8 hours	265±17*	249±14*
IL-6	4 hours	81±7	100±13
	8 hours	141±9*	163±14*

DU-145 cells were incubated without (control) or with IL-6 (100 ng/ml) or OSM (100 ng/ml) and mRNA was analyzed by Real-time-PCR. u-PA and VEGF mRNA levels were normalized according to the respective GAPDH mRNA levels and are given as percent of control±SD. Control was set as 100 percent. *$p<0.05$; (n=3).

Statistical analysis. Data are represented as median and interquartile range or as mean and standard deviation (SD). After determination of the distribution pattern, statistical differences between groups were determined by analysis of variance (ANOVA) for plasma levels of u-PA and VEGF, and by ANOVA after log-transformation of IL-6 and OSM. *Post hoc* pairwise comparisons were conducted according to Bonferroni. Pearson's correlation was used to correlate log-transformed plasma levels of IL-6 and OSM with prostate specific antigen (PSA), u-PA and VEGF, respectively. Multivariate analysis was not performed due to the low number of subjects included in this study. All the *p*-values were two-tailed, and values lower than 0.05 were taken as indicators for statistical significance. All the calculations were performed using a computer programme (SPSS for Windows 16.0, SPSS Inc, Chicago, IL, USA).

Results

Effects of IL-6 and OSM on production of u-PA and VEGF in the PC cell line DU-145. As can be seen from Figure 1, the gp130 ligands IL-6 (100 ng/ml) and OSM (100 ng/ml), but not LIF (10^4 U/ml), increased the production of u-PA and VEGF in the human PC cell line DU-145 up to 2-fold and 4.5-fold, respectively. In contrast neither u-PA nor VEGF production was affected by OSM or IL-6 in the human PPEC. OSM dose dependently increased u-PA and VEGF in the DU-145 cells (Figure 2). The maximum effects were seen with 100 ng/ml rh OSM and 100 ng/ml rh IL-6.

Effects of gp130 ligands on u-PA and VEGF mRNA. Analysis by quantitative Real-Time-PCR (Table III) demonstrated an appreciable increase of u-PA and VEGF specific mRNA after treatment with IL-6 and OSM.

Plasma levels of IL-6, OSM, VEGF and u-PA in patients with metastasising PC, non-metastasising PC or benign prostatic hyperplasia. As can be seen from Table IV, the patients suffering from metastasising PC had significantly higher plasma levels of IL-6, OSM, VEGF and u-PA than the patients with non-metastasising PC or BPH.

The plasma levels of PSA in all 47 patients showed a significant correlation with the plasma levels of VEGF (R=0.611, $p<0.00001$) and u-PA (R=0.393, $p=0.007$) as well with levels of IL-6 (R=0.566, $p<0.000001$), whereas the plasma levels of OSM showed no association with the PSA levels (R=0.228, $p=0.127$).

Correlation of plasma levels of gp130 ligands with VEGF and u-PA. The plasma levels of IL-6 showed a subtle, yet significant correlation with the plasma levels of VEGF (R=0.585, $p<0.0001$) and with the plasma levels of u-PA (R=0.333, $p=0.022$). The plasma levels of the gp130 ligand OSM significantly correlated with the u-PA plasma levels (R=0.323, $p=0.027$) whereas the VEGF plasma levels showed no association with the OSM levels (R=-0.024, $p=0.873$).

Discussion

In addition to their growth promoting effect OSM and IL-6 might support tumour progression in PC indirectly by modulating the expression of particular biomolecules involved in tumour growth and metastasis in PC cells. In the present study IL-6 and OSM significantly increased the expression of u-PA and VEGF in PC DU-145 cells. IL-6 has been shown by us and others to induce the expression of u-PA and VEGF in various malignant and non-malignant cells (31-34). It should be emphasized, however, that these mediators did not affect the production of u-PA and VEGF in the PPEC. A third member of the IL-6 family tested, LIF, had no effect on these parameters in both cells types.

IL-6 and OSM could contribute indirectly to tumour progression in PC by supporting two critical events in tumour progression, extracellular matrix degradation and angiogenesis, through the up-regulation of the expression of u-PA and VEGF, respectively, in PC cells.

In an attempt to evaluate the significance of these *in vitro* results the u-PA and VEGF plasma levels were found to be significantly higher in patients with metastasising PC as compared to the respective levels measured in the plasma of patients with non-metastasising PC or BPH. This was in

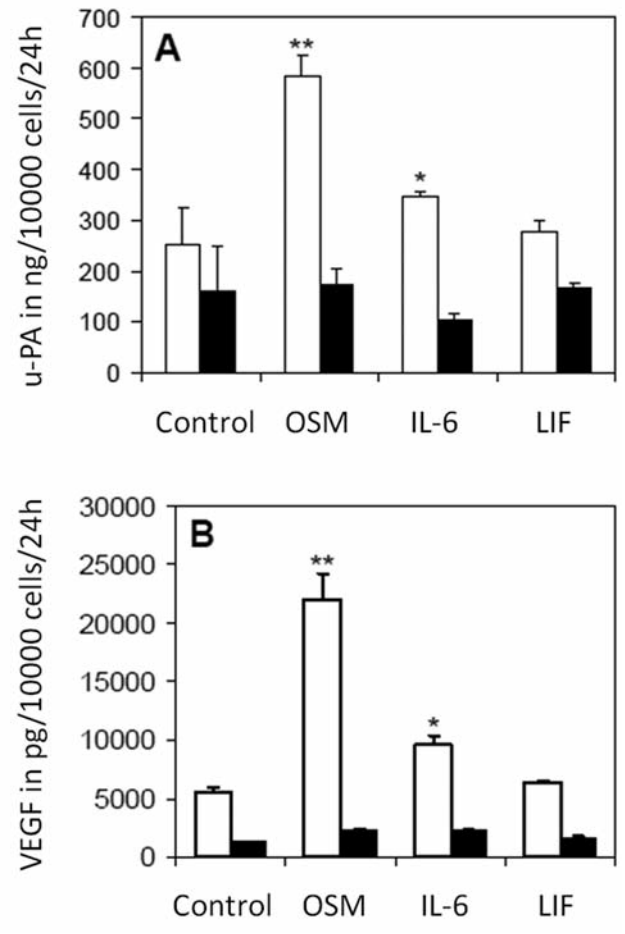

Figure 1. *Effects of OSM and IL-6 on u-PA (panel A) and VEGF (panel B) production in the human PC cell line DU-145 (open bars) and human primary prostate epithelial cells (PPEC) (black bars). The cells were incubated for 24 hours in the absence (control) or presence of rh IL-6 (100 ng/ml), rh OSM (100 ng/ml) or rh LIF (104 U/ml), and u-PA and VEGF were determined by ELISA. Mean values±S.D. of three independent determinations. Experiments were performed three times for each cell type, with similar results. A representative experiment is shown. **p<0.005, *p<0.05.*

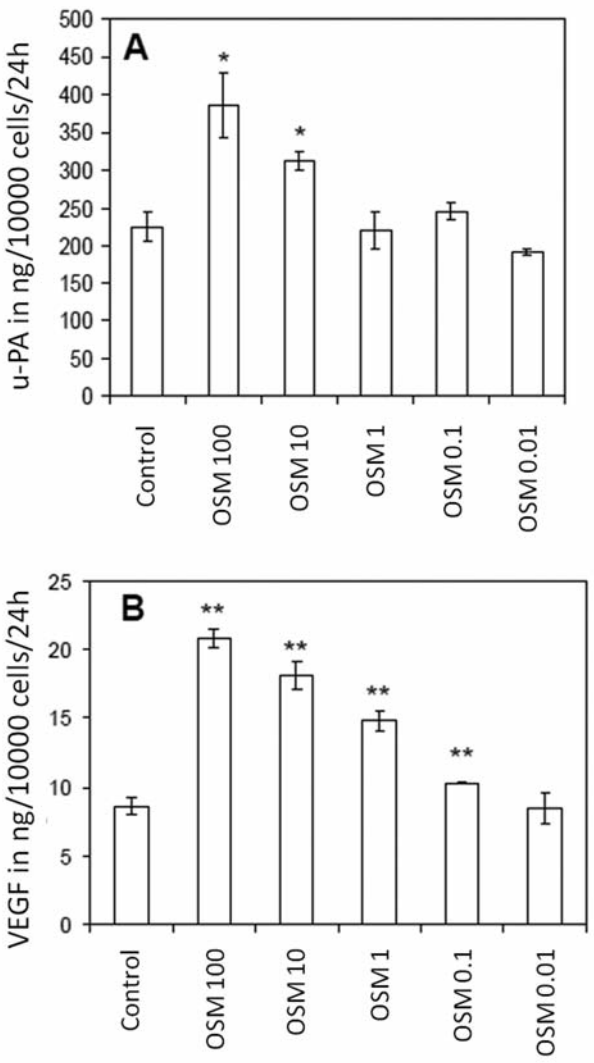

Figure 2. *Effect of OSM on u-PA (panel A) and VEGF (panel B) production in the human PC cell line DU-145. DU-145 cells were incubated for 24 hours in the absence (control) or presence of rh OSM. u-PA and VEGF were determined by ELISA. Mean values±S.D. of three independent determinations. Experiments were performed three times for each cell type, with similar results. A representative experiment is shown. **p<0.005, *p<0.05.*

agreement with earlier work (18, 22, 25, 29). However it should be mentioned that VEGF plasma levels in non-metastasising PC were lower than in BPH. An explanation for this finding remains elusive. Furthermore, also consistent with published data, significantly higher IL-6 plasma levels were measured in the patients with metastasising disease as compared to non-metastasising disease and BPH. In addition, to the best of our knowledge, we are the first to report also significantly increased OSM levels in the plasma of patients suffering from metastasising PC as compared to the respective plasma levels in patients with non-metastasising disease or BPH.

IL-6 and OSM have been shown to have a direct proliferative effect on PC cells. Our observation that the plasma levels of these mediators correlated with the plasma levels of u-PA and VEGF in all the patients supported our hypothesis that IL-6 and OSM might contribute to tumour progression in PC *via* modulation of extracellular matrix degradation and angiogenesis. It should be noted that plasma levels of OSM and IL-6 in these patients were in the picogram range whereas concentrations of these cytokines used in the *in vitro* experiments to stimulate u-PA and VEGF production in PC cells were in the nanogram range. One could speculate, however, that plasma levels might not reflect

Table IV. *Plasma levels of IL-6, OSM, VEGF, and u-PA.*

	Benign hyperplasia (n=20)	Non-metastasising PC (n=20)	Metastasising PC (n=7)
IL-6 (pg/ml)	0.81 (0.38-1.79)	1.32 (0.90-2.73)	5.81 (2.77-47.46)***
OSM (pg/ml)	<0.01 (<0.01-0.83)	<0.01 (<0.01-0.52)	2.73 (<0.01-19.87)**
VEGF (pg/ml)	20.26 (6.83-48.18)	11.08 (4.19-36.15)	51.47 (14.55-170.80)*
u-PA (ng/ml)	0.44 (0.34-0.56)	0.45 (0.38-0.60)	1.03 (0.57-1.15)***

Median values and interquartile range (in brackets). *p<0.016, **p<0.009, ***p<0.0001 compared to BPH and non-metastasising PC; p-values were determined by ANOVA.

local cytokine concentrations at the site of inflammation, and that therefore at the tumour site the concentration of IL-6 and OSM might be significantly higher as compared to levels measured in the circulation. Albeit showing such significant correlations between IL-6 and u-PA or VEGF and OSM and u-PA , respectively, the small number of patients included in the present study did not formally allow us to draw conclusions purely from the clinical findings. However, in combination with our *in vitro* data they strengthen the hypothesis that IL-6 and OSM might play a role in the pathophysiology of PC by affecting the expression of the serine protease u-PA and the angiogenic factor VEGF.

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