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An autocrine loop for vascular endothelial growth factor is established in prostate cancer cells generated after prolonged treatment with interleukin 6

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Abstract

Concentrations of interleukin 6 (IL-6) and its receptor are increased in human prostate cancer. Prostate cancer LNCaP-IL-6+ cells, established after prolonged treatment with IL-6, have been found to acquire a growth advantage. Vascular endothelial growth factor (VEGF) may accelerate the growth of various tumours by stimulation of VEGF receptor 2 (VEGFR-2). To understand better the regulation of proliferation of LNCaP-IL-6+ cells, the expression of VEGF and VEGFR-2 was here investigated in the LNCaP-IL-6+ subline. VEGF was measured in cellular supernatants by enzyme-linked immunoassay. The expression of VEGFR-2 was assessed by Western blot. LNCaP-IL-6+ and control LNCaP-IL-6- cells were treated with a neutralising antibody against VEGFR-2. VEGF concentrations were 20-fold higher in LNCaP-IL-6+ than in LNCaP-IL-6- cells. The stimulatory effect of IL-6 on VEGF production was abolished by an inhibitor of the signalling pathway for phosphoinositol 3 kinase in LNCaP-IL-6+ and LNCaP-IL-6- cells. Exogenous VEGF did not stimulate proliferation in either LNCaP-IL-6+ cells or controls. VEGFR-2 was detected only in LNCaP-IL-6+ cells, in which the neutralising antibody caused a partial inhibition of cell proliferation. It was concluded that a VEGF autocrine loop is established in prostate cancer cells generated after chronic treatment with IL-6. Because of the upregulation of IL-6 in patients with prostate cancer, these findings might be clinically relevant.

Keywords: Prostate cancer; Cytokines; Interleukin 6; Vascular endothelial growth factor; Autocrine signalling

1. Introduction

Although androgens are key regulators of the proliferation and survival of prostate cancer cells, the signalling of peptide hormones and cytokines is frequently deregulated in this malignant tumour. It is recognised that interleukin 6 (IL-6) acts as a multifunctional regulator of prostate cancer cell growth (for review see [1]). Binding of IL-6 to the gp80 subunit of the IL-6 receptor is followed by the initiation of signal transduction through the gp130 subunit, the phosphorylation of Janus kinases (JAK) and signal transducers, and the activation of transcription (signal transducer and activator of transcription; STAT) factors and their subsequent translocation to the nucleus, where they bind to response elements and promote the transcription of specific genes. In addition to the JAK/STAT pathway, the incubation of cells with IL-6 may lead to the activation of the signalling cascades of mitogen-activated protein kinase (MAPK) and phosphoinositol 3 kinase (PI3-K).

IL-6 reportedly has divergent effects on the growth of the androgen-responsive cell line LNCaP [2,3]. In our laboratory, we find that LNCaP cells are inhibited by IL-6 [4]. By using prolonged treatment with this cytokine

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we have generated a subline, LNCaP-IL-6+, that does not show the growth-inhibitory response and upregulates endogenous IL-6. This subline might be useful for experimental studies on prostate cancer because it mimics the conditions in patients who have elevated IL-6 [5,6]. LNCaP-IL-6+ cells grow more rapidly in nude mice than do their counterparts, LNCaP-IL-6-, which were established after serial passaging in the absence of IL-6 [7]. In LNCaP-IL-6+ cells, there is an upregulation of cyclin-dependent kinase 2 and reduced expression of the tumour suppressors pRb and p27. On considering a variety of IL-6 effects in the prostate, we reason that other mechanisms, such as IL-6-induced changes in the expression and function of growth factors, are also implicated in the growth regulation of LNCaP-IL-6+ cells.

Vascular endothelial growth factor (VEGF) has been identified as a growth factor not only for endothelial cells but also for various types of malignant cells [8]. In prostate cancer, there apparently exists a VEGF autocrine loop [9,10]. VEGF receptor 1 (VEGFR-1) (Flt-1) and VEGFR-2 (Flk-1/KDR) are expressed in several prostate cancer cell lines and in clinical specimens [10]. Growth-regulatory effects of VEGF on prostate cells are mediated by VEGFR-2, whereas VEGFR-1 is involved in cellular migration [9]. The plasma VEGF is elevated in patients with metastatic prostate cancer [11] and VEGF expression is increased in malignant human and rat cell lines compared to those of benign origin [10,12].

Because of its association with proliferation, survival and angiogenesis, the regulation of VEGF in prostate cancer is of interest [13]. In various cell lines, treatment with IL-6 for 6-48 h leads to the induction of VEGF mRNA [14]. We have now investigated changes in the expression of VEGF and VEGFR-2 during the IL-6-driven selection of prostate cancer cells and considered the implications of the presence of VEGF and VEGFR-2 for tumour biology.

2. Materials and methods

2.1. Cell lines

RPMI medium was purchased from HyClone (Logan, UT). Fetal calf serum (FCS) and penicillin/streptomycin were from Biological Industries (Kibutz Beth Haemek, Israel). High-passage (nos 60–73) LNCaP-IL-6+ and LNCaP-IL-6- cells were cultured as previously described [4]. PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD).

2.2. Chemicals

Human recombinant VEGF isoform 121 was purchased from PromoCell (Heidelberg, Germany). For Western blot analysis, a polyclonal anti-VEGFR-2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used. Neutralisation experiments were carried out with a goat polyclonal IgG affinity-purified antibody specific for human VEGFR-2 (R&D Systems, Minneapolis, MN). Enzyme-linked immunoassays (ELISA) for VEGF and human recombinant IL-6 were also purchased from R&D. Non-immune goat IgG was from Sigma (St. Louis, MO). The synthetic androgen methyltrienolone (R1881) was purchased from New England Nuclear (Dreieichenhain, Germany). The JAK-selective inhibitor AG 490 and the MAPK inhibitor PD 98059 were from VWR International (Darmstadt, Germany). The PI 3-K inhibitor LY 294002 was purchased from Calbiochem (San Diego, CA).

2.3. VEGF measurement

To measure VEGF in cell supernatants, LNCaP-IL-6+ and LNCaP-IL-6- cells were seeded on 6-well plates at a density of 80 000 cells/well. After 24 h, the cells were supplemented with medium containing 3% charcoal-stripped FCS (v/v) in the absence or presence of R1881 or IL-6. In experiments in which the involvement of specific signalling pathways in the upregulation of VEGF was investigated, AG 490, PD 98059 or LY 294002 were added to the culture medium 30 min before the IL-6. The supernatants were removed after a 72-h incubation and cell numbers were counted with a haemocytometer. The supernatants were frozen at −80 °C until ELISA; the assay followed the manufacturer's recommendations.

2.4. Proliferation studies

LNCaP-IL-6+ and LNCaP-IL-6- cells were cultured in serum-free medium in the absence or presence of VEGF 121, the neutralising anti-VEGFR-2 antibody or the non-immune control antibody for 48 or 72 h. The chosen concentrations of the control antibody were the same as those of the anti-VEGFR-2 antibody. In selected experiments, anti-VEGFR-2 was added simultaneously with exogenous VEGF. Afterwards, the cells were counted with a haemocytometer.

2.5. VEGFR-2 Western blot

LNCaP-IL-6+, LNCaP-IL-6- and PC-3 cells were cultured for 48 h, collected and lysed in a buffer containing 20 mM NaH_2PO_4, pH 7.5, 1 mM EDTA and 10% glycerol (v/v). Protein determination was according to Bradford [15]. Aliquots were prepared with NuPAGE sample buffer (Invitrogen, Leek, The Netherlands) and equal amounts of protein were used for polyacrylamide gel electrophoresis. The dilution of the anti-VEGFR-2 antibody was 1:100 and that of the

Table 1
Basal and androgen-induced vascular endothelial growth factor (VEGF) in the LNCaP sublines

R1881 (nM)	VEGF (pg/10^5 cells/72 h)[a]	
	LNCaP-IL-6-	LNCaP-IL-6+
0	36.8±0.2	743.6±83.9
0.01	34.5±0.8	771.2±29.5
0.1	97.7±21[b]	923.4±23.6[b]
1	155.9±47.2[b]	1016.3±198.5[b]

IL-6, interleukin 6.
[a] Values are mean±SEM from three independent experiments
[b] $P<0.05$, treated cells versus untreated control (Mann–Whitney *t*-test).

secondary antirabbit antibody (Santa Cruz) 1:2000. The Western blot has been described elsewhere [7]. To develop the blot the ECL plus substrate (Amersham, Amersham Place, UK) was used. To confirm the presence of equal protein loading, a Western blot for β-actin with a monoclonal antibody from Chemicon (Hofheim, Germany) was performed.

3. Results

3.1. VEGF secretion in LNCaP-IL-6+ cells

Untreated LNCaP-IL-6+ cells secreted 20-fold higher concentrations of VEGF into the supernatants than did their counterparts routinely passaged in the absence of IL-6 (Table 1). In previous studies, it was shown that androgens upregulate VEGF in prostate epithelial cells [16,17]. To examine whether this regulation also occurs in a cell line with markedly upregulated endogenous growth factor, VEGF was measured in the supernatants from LNCaP-IL-6+ and LNCaP-IL-6- cells treated with androgen. R1881, at concentrations of 0.1 and 1 nM, induced VEGF expression in both sublines. With 1 nM of R1881, there was a 4.3-fold increase in VEGF in the supernatants of control cells and a less than 2-fold increase in cells established after continuous exposure to IL-6.

In order to understand better the signal-transduction pathways involved in VEGF regulation by IL-6, we treated both LNCaP sublines with IL-6 for 72 h and measured VEGF. It was earlier shown that STAT3 is required for the induction of VEGF downstream of the gp 130 subunit in cardiac myocytes [18]. In primary cultures of prostate epithelial cells, both MAPK and PI3-K pathways are involved in VEGF stimulation by insulin-like growth factor I [19]. LNCaP sublines were pretreated with inhibitors of IL-6 signalling and the supernatants were used for the measurement of VEGF. In some of our experiments carried out after incubation in the absence or presence of exogenous IL-6, absolute amounts of VEGF were lower in both sublines than those measured in experiments in which androgen was supplemented. Such differences might occur because of the influence of various serum factors. However, in each experiment LNCaP-IL-6+ cells grew faster than their counterparts and VEGF was on average 15-fold higher in the LNCaP subline generated after continuous treatment with IL-6 than in the control subline (Table 2). Interestingly, treatment with IL-6 caused an increase in VEGF in both LNCaP-IL-6+ and LNCaP-IL-6- cells. AG 490, the inhibitor of the JAK/STAT pathway, abolished the effect of IL-6 only in control cells. Whereas PD 98059, the inhibitor of MAPK, did not diminish IL-6-induced VEGF secretion, LY 294002, the PI 3-K inhibitor, did lower the concentrations of the growth factor in both sublines. Taken together, our results suggest that the regulation of VEGF in the LNCaP-IL-6+ subline depends on a functional PI 3-K pathway.

Table 2
Regulation of vascular endothelial growth factor (VEGF) secretion in LNCaP-IL-6- and LNCaP-IL-6+ cells by interleukin 6 (IL-6)

Treatment	VEGF (relative to untreated LNCaP-IL-6- cells)[a]	
	LNCaP-IL-6−	LNCaP-IL-6+
0	100	1556.1±69.4
10 ng/ml IL-6	145.9±11[b]	2454±63.5[b]
25 ng/ml IL-6	214.1±7[c]	3142±51.8[c]
1 μM AG 490	96.7.1±1.5	1860.3±62.8
25 ng/ml IL-6+1 μM AG 490	94.3±2.6[d]	2915.1±140.6
50 μM PD 98059	125.5±9.5	1665.8±11
25 ng/ml IL-6+50 μM PD 98059	241.6±15.2	3289.4±209.8
20 μM LY 294002	94.9±2.4	2229.4±36.4
25 ng/ml IL-6+20 μM LY 294002	85.7±0.6[d]	1147.5±70.8[d]

[a] Values are mean±SEM from two to five independent experiments. VEGF concentrations measured in untreated LNCaP-IL-6- cells were set as 100 and all other values are expressed in relation to that value.
[b] $P<0.05$, treatment with IL-6 (10 ng/ml) versus untreated control.
[c] $P<0.01$, treatment with IL-6 (25 ng/ml) versus untreated control.
[d] $P<0.05$, treatment with a respective signal-transduction inhibitor and IL-6 versus treatment with IL-6 alone (25 ng/ml) (Mann–Whitney *t*-test–.

3.2. Effect of exogenous VEGF on the proliferation of LNCaP sublines

We then investigated the regulation of the growth of LNCaP-IL-6+ cells and their counterparts by exogenous VEGF. As in previous studies, untreated LNCaP-IL-6+ cells showed a growth advantage compared to controls (Fig. 1) [4,7]. VEGF concentrations between 10 and 100 ng/ml were used in the proliferation studies. The results obtained from four independent experiments with the highest VEGF concentration are shown in Fig. 1. Interestingly, exogenous VEGF did not cause a proliferative effect in LNCaP-IL-6+ or LNCaP-IL-6- cells.

3.3. Expression of VEGFR-2

To study VEGF-mediated cellular events in LNCaP-IL-6+ cells, we investigated the expression of VEGFR-2. Stimulation of the proliferation of prostate cancer cells by VEGF depends on the presence of VEGFR-2 [10]. To allow a better comparison with published data, we included PC-3 cells as a positive control. As expected, VEGFR-2 was detectable in PC-3 cells (Fig. 2). Importantly, the receptor was present in LNCaP-IL-6+, whereas its expression was not detectable in LNCaP-IL-6- cells.

3.4. Neutralisation of VEGF effects

The neutralising antibody against VEGFR-2, at concentrations between 0.1 and 2 μg/ml, was used to examine whether the upregulation of VEGF and VEGFR-2 influences tumour cell proliferation. As shown in Fig. 3, this antibody did not inhibit control LNCaP-IL-6- cells. In contrast, in LNCaP-IL-6+ cells the neutralising anti-VEGFR-2 antibody caused a 31% (after 48 h; Fig. 3a) or 25% (after 72 h; Fig. 3b) inhibition of growth. Maximal inhibition was observed with 1 μg/ml of the neutralising antibody. The effect was specific, since there was no inhibition of proliferation after treatment with the control antibody. Furthermore, the downregulation of proliferation was reversed by the addition of 10 ng/ml of VEGF (Fig. 3). These findings indicate that continuous treatment with IL-6 facilitates the establishment of a VEGF autocrine loop in prostate cancer cells.

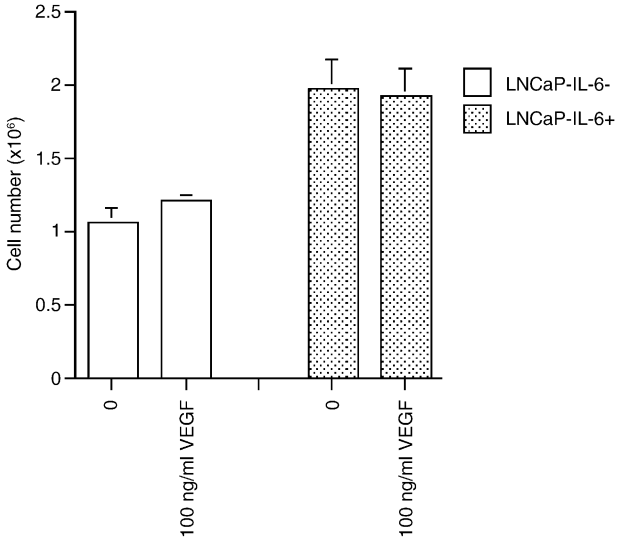

Fig. 1. Proliferation of LNCaP-IL-6- and LNCaP-IL-6+ cells in response to vascular endothelial growth factor (VEGF) (100 ng/ml). The cells were grown on 6-well plates in serum-free medium for 72 h before counting ($n=4$, mean±SEM). IL-6, interleukin 6.

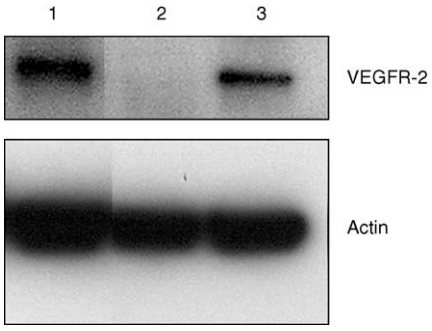

Fig. 2. Expression of VEGF receptor 2 (VEGFR-2) in the LNCaP sublines. A representative Western blot is shown ($n=3$); lane 1, PC-3 cells; lane 2, LNCaP-IL-6- cells; lane 3, LNCaP-IL-6+ cells. IL-6, interleukin 6.

4. Discussion

Growth-promoting effects of IL-6 have been demonstrated in primary cultures of prostate tumour cells [5], a cell line derived from prostate intraepithelial neoplasia [20], and PC-3 and DU-145 cells [21]. For this reason, therapeutic strategies aimed at antagonising the effects of IL-6 in prostate cancer have been proposed. An anti-IL-6 antibody inhibited the growth of PC-3 xenografts and this effect was associated with the stimulation of apoptosis [22]. However, proliferation studies with IL-6 in LNCaP cells have yielded equivocal results. There are divergent findings on the growth regulation of LNCaP cells by IL-6, most probably because of differences in the sensitivity of the various cell passages used in different laboratories [2,3,5,23]. We established the LNCaP-IL-6+ cells used in the present study in order to obtain a model relevant to changes in IL-6 responsiveness and signal transduction in patients with prostate cancer [4]. These changes might occur because IL-6 and its receptor are increased in tissue extracts from patients even in the early stages of prostate carcinogenesis [5]. Thus, prostate cancer cells are continuously exposed to IL-6, the serum concentrations of which become elevated in patients with metastatic disease [6].

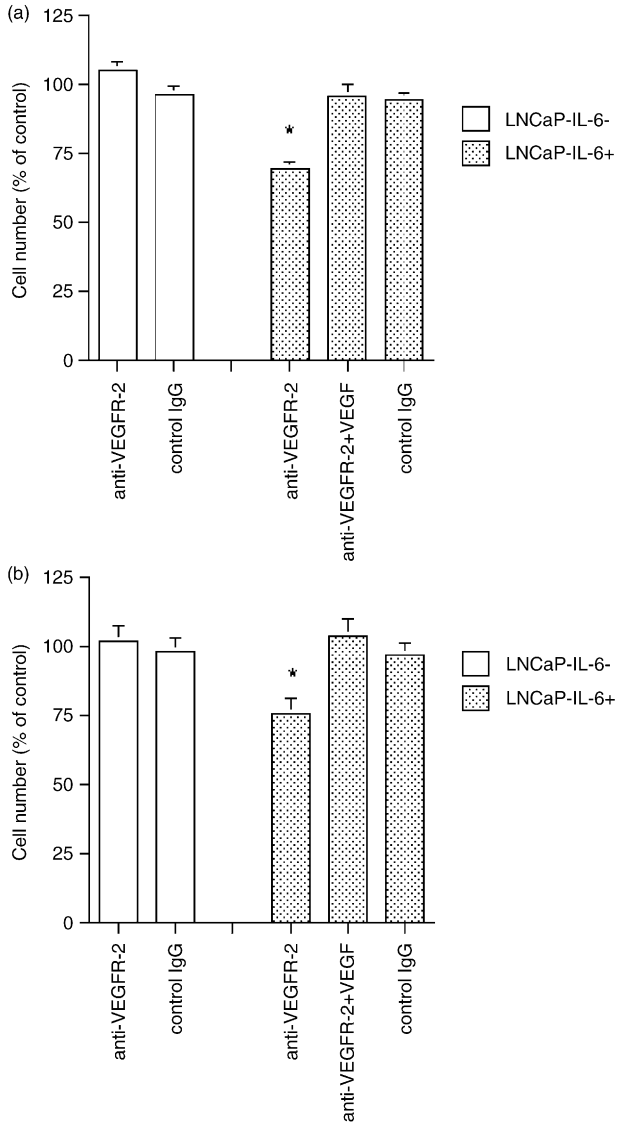

Fig. 3. Effect of a neutralizing anti-VEGFR-2 antibody on proliferation of LNCaP-IL-6- and LNCaP-IL-6+ cells. The cells were incubated with either anti-VEGFR-2 or control IgG antibody (1 μg/ml) for (a) 48 h and (b) 72 h and counted afterwards. Three to seven independent experiments were performed. The results are expressed as a percentage in relation to untreated control; bars, ±SEM (*$P<0.05$ anti-VEGFR-2 treatment versus untreated control; Mann-Whitney *t*-test). VEGFR-2, VEGF receptor 2; IL-6, interleukin 6.

LNCaP-IL-6+ cells acquire a growth advantage both *in vitro* and *in vivo* [4,7]. We earlier demonstrated marked alterations in the expression of molecules that regulate the G1/S transition in the cell cycle [7]. Our present results show that prostate cancer cells selected in the presence of IL-6 upregulate the expression of VEGF and VEGFR-2. It is possible that other growth factors are also induced during the continuous exposure of prostate cancer cells to IL-6. Our results are supported by those of Chen and colleagues, who found elevated VEGF in androgen-independent tumour cells that express IL-6 [12]. The use of the LNCaP-IL-6+ tumour model allowed us to investigate the role of IL-6 in the regulation of VEGF in prostate cancer. From our experiments, it is evident that the secretion of VEGF in the cell line selected in the presence of IL-6 and in the control subline is regulated by the PI 3-K pathway. The expression of VEGF in rat pituitary tumour cells is also mediated through the PI3-K pathway [24]. In control cells the JAK/STAT pathway is responsible for the upregulation of VEGF by IL-6. Our results reveal a novel aspect of JAK/STAT3 signalling in prostate cancer cells: although activated STAT3 is associated with inhibition of proliferation of LNCaP-IL-6-cells, it is, in the same cell line, involved in the upregulation of VEGF. We earlier showed that there is no phosphorylation of STAT3 in LNCaP-IL-6+ cells, in contrast to controls [7]. This finding is further supported by the present results demonstrating that the JAK-selective inhibitor AG 490 is efficient in blocking the IL-6-induced increase in VEGF in controls but not in cells generated after long-term treatment with IL-6. The induction of VEGF by androgen in LNCaP-IL-6+ cells is in agreement with our previous demonstration of the androgenic responsiveness of these cells [4]. The proliferation of both sublines was stimulated by lower concentrations of androgen, whereas the effect of R1881 on VEGF was clearly dose-dependent. However, the relative increase in VEGF in culture supernatants was lower in LNCaP-IL-6+ than in LNCaP-IL-6- cells, most probably because of a marked elevation of basal concentrations of VEGF in the selected subline. Enhancement of VEGF expression by androgens is one of the mechanisms by which these hormones control the growth of normal and malignant prostate tissues [16].

A lack of a proliferative response to exogenous VEGF was observed in LNCaP-IL-6+ cells and controls. VEGF failed to stimulate the proliferation of LNCaP-IL-6- cells, most probably because of absent expression of VEGFR-2. For parental LNCaP cells, both the presence and the absence of VEGFR-2 are reported [10,25]. Jackson and associates, who were able to detect VEGFR-2 in LNCaP cells, showed that the growth of these cells *in vitro* is stimulated by VEGF. Although the hormonal responsiveness of LNCaP-IL-6- is similar to that of the parental LNCaP cells, there is a difference in the expression of VEGFR-2. A previous study showed that the expression of VEGFR-2 does not change after treatment with androgens [26]. In this context, we should note that there are contradictory results on VEGFR-2 expression in prostate cancer tissues. VEGFR-2 was variable in carcinoma tissues in one study [25]; low- and moderate-grade tumours stained positively for the receptor. These results were supported by those of Jackson and colleagues [10], who detected VEGFR-2 in two out of six cancer tissues by immunoblot; there was a trend towards lower VEGFR-2 expression in poorly differentiated prostate cancers. In

contrast, the presence of VEGFR-2 was reported in all prostate cancers in another study, with increased intensity of immunostaining in poorly differentiated tumours [27].

The proliferation of LNCaP-IL-6+ cells is influenced by the VEGF autocrine loop and therefore exogenous VEGF could not further stimulate their growth. The presence of the VEGF autocrine loop in LNCaP-IL-6+ cells was postulated on the basis of experiments with the neutralising VEGFR-2 antibody that yielded a partial inhibition of cell growth. In the study by Jackson and associates the VEGFR-2 antibody decreased the proliferation of LNCaP cells by 20% [10]. PD 98059, the inhibitor of MAPK kinase, had a similar effect on the growth of LNCaP-IL-6+ cells [7]. An association between the activation of the MAPK pathway by IL-6 and LNCaP proliferation has also been demonstrated by Qiu and associates [3]. We hypothesise that the accelerated growth of LNCaP-IL-6+ cells is a consequence of multiple changes in the signalling pathways that control proliferation and apoptosis. The expression of VEGF in specimens of prostate cancer tissue is well documented [10,28]. The observation that VEGF colocalises with the receptors in cancer foci supports the idea that the VEGF autocrine loop is clinically significant [10].

The potential therapeutic utility of anti-VEGFR-2 or anti-VEGF antibodies has demonstrated *in vivo* [29,30]. Those antibodies reduced prostate tumour weight, microvessel density and tumour cell proliferation, and enhanced apoptosis. On the basis of the observed upregulation of VEGF in LNCaP-IL-6+ cells, we expect that angiogenesis has a substantial role in the growth of these tumours. The therapeutic use of antibodies against VEGF or its receptor in the LNCaP-IL-6+ tumour model is a subject of current investigations.

The action of VEGF on tumour cells is not restricted to prostate cancer. The expression of VEGF mRNA is elevated in ovarian carcinoma and VEGFR-2 has been detected in those tumour cells [31]. In breast cancer, VEGF is an autocrine survival factor for cells that express one of its receptors, neuropilin [32]. VEGF and its receptors were detected in pancreatic carcinoma cells, in which VEGF antagonists and a dominant-negative VEGFR-2 significantly inhibited tumour growth [33].

In summary, we have demonstrated that the VEGF autocrine loop in prostate cancer cells is established during prolonged exposure to IL-6. Our findings contribute to an understanding of the complexity of IL-6 effects in carcinoma of the prostate and emphasise an important role for this pleiotropic cytokine in prostate carcinogenesis.

5. Conflict of interest statement

The authors do not have a conflict of interest that could inappropriately influence their work.

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