Mechanisms of endocrine therapy-responsive and -unresponsive prostate tumours

Z Culig, H Steiner, G Bartsch and A Hobisch¹

Department of Urology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria

¹Department of Urology, General Hospital Feldkirch, Cariuagasse 45, A-6800 Feldkirch, Austria

(Requests for offprints should be addressed to Z Culig; Email: zoran.culig@uibk.ac.at)

Abstract

Several options for the endocrine treatment of non-organ-confined prostate cancer are available. They include surgical or medical removal of androgenic hormones or administration of non-steroidal anti-androgens. However, tumour progression after a period of remission of the disease inevitably occurs in virtually all patients. The androgen receptor (AR) is, in various tumour models, implicated in the development of therapy resistance but molecular mechanisms that by-pass the receptor have also been described. Adaptation mechanisms relevant to tumour recurrence include up-regulation of AR mRNA and protein, overexpression of AR coactivators, increased activation of mutated receptors by steroids and anti-androgens, and ligand-independent activation. For research studies, sublines that respond to but do not depend on androgen for their proliferation were generated. Coactivators SRC-1, TIF-2, RAC3, p300, CBP, Tip60, and gelsolin are highly expressed in endocrine therapy-resistant prostate cancer. AR point mutations are increasingly detected in relapsed cancers and contribute to the failure of endocrine therapy in a subgroup of patients. Ligand-independent activation of the AR by HER-2/neu and interleukin-6 is associated with activation of the signalling pathway of mitogen-activated protein kinase. Increased activity of intracellular kinases may affect cellular events in both an AR-dependent and -independent manner. Mitogen-activated protein kinases are strongly phosphorylated in endocrine therapy-resistant prostate tumours. Similarly, activation of the AR by phosphorylated protein kinase B, Akt, has also been reported in prostate cancer. Activation of the Akt pathway contributes to increased survival of prostate tumour cells.

Endocrine-Related Cancer (2005) 12 229–244

Introduction

Responsiveness of normal and malignant prostate to androgenic stimulation is determined by the presence of the androgen receptor (AR) in epithelial and adjacent stromal prostate cells. Malignant prostate tumours respond to androgen ablation by retardation of growth (Huggins & Hodges 1941). This recognition has greatly influenced therapeutical concepts in human prostate cancer over decades. Androgen ablation therapy was introduced for treatment of non-organ-confined prostate cancer and its effectiveness in stabilization of the disease has not been surpassed by any other treatment. Removal of androgen may be achieved either by castration or administration of luteinizing

hormone-releasing hormone (LHRH) analogues. These drugs lead to desensitization of luteinizing hormone receptors and subsequent inhibition of testosterone production. Besides androgen ablation, endocrine approaches aimed to control prostate cancer growth include blockade of the AR, a transcription factor responsible for regulation of genes involved in control of proliferation, apoptosis, angiogenesis, and differentiation in prostate. At present, the non-steroidal AR antagonists hydroxyflutamide and bicalutamide are widely used in prostate cancer endocrine therapy. The AR structure is similar to that of other steroid receptors; it consists of well-conserved DNA- and ligand-binding domains and the N-terminal region that contains a variable number of polyglutamine and

polyglycine repeats. A more detailed review of basic aspects of AR action has been published elsewhere (Gobinet *et al.* 2002). AR antagonists prevent acquisition of a transcriptionally active conformation of the AR and are commonly administered as a monotherapy or during initial phases of treatment with LHRH analogues. However, there is a need to obtain more results from large clinical trials to compare the effectiveness of non-steroidal anti-androgens with that of androgen ablation. With regard to the quality of life of the patients, anti-androgen monotherapy offers advances over androgen withdrawal.

In the last decade, the number of available models relevant to human carcinoma of the prostate has increased and the complexity of pathways implicated in tumour progression have become obvious. The responsiveness of prostate cancers to steroid hormones varies at different stages of prostate carcinogenesis. Novel findings as to how steroid and peptide hormones control prostate growth have opened new possibilities for the development of therapeutic agents to target selected molecules. However, it has also become clear that the heterogeneity of prostate cancers requires an individualized approach in order to achieve a prolonged stable phase of the disease.

The purpose of this review is to delineate the most common mechanisms underlying the progression of prostate cancer towards therapy resistance. It will therefore focus on the role of androgenic signalling as well as on kinase pathways that by-pass steroid receptors.

Heterogenous expression of AR in prostate cancer cells and clinical specimens

The presence of the AR was clearly demonstrated in prostate tissues obtained from patients who failed therapy, lymph node, and distant metastases (Van der Kwast *et al.* 1991, Hobisch *et al.* 1995, 1996). High AR expression in epithelium and concomitant reduced expression in stroma are associated with high tumour grade (Henshall *et al.* 2001). However, the mechanistic link between growth advantage and loss of stromal AR in prostate cancer requires further investigations.

AR expression in prostate cancers is influenced by post-translational and epigenetic modifications. *In vitro*, AR expression has frequently been studied in LNCaP cells derived from a lymph node metastasis from a patient who failed endocrine therapy (Horoszewicz *et al.* 1983). From those studies, it became clear that there are differences in androgenic regulation of AR mRNA and protein (Fig. 1) (Krongrad *et al.* 1991).

The inhibitory effect on mRNA is compensated by the stabilization of the AR protein by androgen. AR expression in LNCaP cells is inhibited by epidermal growth factor (EGF) and transforming growth factor (EGF)- α at the mRNA level (Henttu & Vihko 1993). It is important to note however that growth factors might exhibit opposite effects on the expression and activity of the AR. Inhibition of AR protein expression in prostate cancer cells was observed in conditions in which proliferation is down-regulated by chemopreventive compounds, such as vitamin E, resveratrol, the non-steroidal anti-inflammatory drugs flufenamic acid and exisulind, and selenium (Mitchell *et al.* 1999, Zhu *et al.* 1999, Zhang *et al.* 2002, Lim *et al.* 2003, Dong *et al.* 2004) (Fig. 1). This treatment results in a diminished expression of androgen-target genes, such as the prostate-specific antigen (PSA) gene. Thus, the treatment outcome is similar to that reported after administration of AR antisense oligonucleotides or neutralizing antibodies to LNCaP cells (Eder *et al.* 2000, Zegarra-Moro *et al.* 2002). The AR is implicated in the development of endocrine therapy resistance by various non-exclusive mechanisms. Several studies on the role of the AR in prostate cancer progression were carried out with steroid-deprived cells generated to mimic the clinical situation. During long-term androgen ablation, the levels of AR mRNA and protein become up-regulated in LNCaP and MDA PCa2b cells (Kokontis *et al.* 1994, Culig *et al.* 1999, Hara *et al.* 2003a). AR levels increase in recurrent prostate cancer, in some cases as a result of protein stabilization (Gregory *et al.* 2001a). This change facilitates the development of receptor hypersensitivity and aberrant reaction to anti-androgens. Because of a decreased threshold for receptor stimulation in patients who are subjected to androgen withdrawal, the presence of adrenal precursors of testicular androgens may be relevant to the regulation of prostate cancer growth and apoptosis. In concordance with data obtained with LNCaP cells *in vitro*, genetic profiling of a series of prostate cancer xenografts revealed that the most consistent change during transition to the endocrine therapy-insensitive stage is AR up-regulation, associated with agonism of AR blockers and changes in the relative abundance of coactivators or corepressors assembled on the promoters of AR target genes (Chen *et al.* 2004). In a subline of LNCaP cells derived during continuous androgen withdrawal *in vitro*, stimulation of reporter gene activity by bicalutamide was also evident (Culig *et al.* 1999). Although it did not reach the same levels as transcriptional activity measured after incubation with androgen, it was sufficient for induction of the stimulation of growth of the androgen-ablated

Figure 1 Compounds that up- or down-regulate the AR expression in human prostate cancer. The AR consists of a well-conserved DNA- and ligand-binding domain that are separated by a hinge region, and the N-terminal region. Several cytokines implicated in the regulation of prostate cancer growth modulate AR levels.

subline *in vitro* and *in vivo*. In a recently published study in which tissues from patients with relapsed cancer were obtained, AR mean optical density was similar to that in benign prostate (Mohler *et al.* 2004). Those data indicated that the AR in therapy-resistant tumours is present at levels that allow stimulation by androgens, such as androstenediol, which is persistent at high concentrations in prostate tissue after castration (Mizokami *et al.* 2004). Evidence for reactivation of the androgen signalling cascade was also obtained in a gene profiling study in human prostate cancer (Holzbeierlein *et al.* 2004). Data on increased AR protein expression are complemented by those obtained in studies investigating AR gene copy number in recurrent prostate tumours. AR gene amplification occurs in a subgroup of patients who present with tumour progression after endocrine treatment (Visakorpi *et al.* 1995, Linja *et al.* 2001). However, there is no conclusive evidence that AR amplification is causally associated with failure of endocrine therapy. There is no difference in time to relapse between patients who present with AR amplification compared with those without change in AR gene copy number (Edwards *et al.* 2003). Interestingly, on the basis of data from a group of 77 patients, Palmberg *et al.*

(2000) proposed that AR amplification is linked to a favourable response to complete androgen blockade. One possibility to explain these findings is an involvement of the AR in the regulation of prostate differentiation. Endocrine therapy could be more efficient in well-differentiated tumour tissue.

AR expression in clinical samples is heterogenous and its lower levels in some cells could be explained by hypermethylation of the promoter island CpG in the AR gene (Jarrard *et al.* 1998). This phenomenon was initially observed in the DU-145 cell line, in which treatment with the demethylating agent 5-aza-2'-deoxycytidine led to the re-expression of the AR and PSA. The AR gene is hypermethylated in about 30% of cases of recurrent prostate cancer (Nakayama *et al.* 2000). The findings obtained with DU-145 cells therefore have clinical significance.

The relevance of prostate cancer sublines derived in androgen-depleted conditions for understanding the mechanisms of tumour progression

Sublines generated from prostate cancer cells that respond to androgen but do not require it for their

Table 1 Overview of cell lines and their derivatives commonly used in research on androgenic responsiveness

Cells	Characteristic features
LNCaP	Mutated AR Thr868Ala, promiscuous activation, hydroxyflutamide agonist (Veldscholte <i>et al.</i> 1990)
Androgen-deprived LNCaP	Hypersensitive AR, some sublines stimulated by bicalutamide PSA expression diminished, AR mutation Thr868Ala (Kokontis <i>et al.</i> 1994, Culig <i>et al.</i> 1999)
LNCaP C4-2	High basal PSA levels Androgen-independent recruitment of the Tip60 coactivator AR mutation Thr868Ala (Wu <i>et al.</i> 1994)
CWR22-Rv1	Mutated AR His874Tyr and in-frame duplication of exon 3 androgen-independent expression of AR downstream genes (McDonald <i>et al.</i> 2000, Tepper <i>et al.</i> 2002)
LAPC 4	Ligand-independent induction of AR by HER-2 (Craft <i>et al.</i> 1999)
MDA PCa 2a	Double AR mutant Leu701His and Thr877Ala (Zhao <i>et al.</i> 1999a)
AR CaP	Androgenic inhibition of tumour cell growth (Cinar <i>et al.</i> 2001)
PC-3 AR	Androgenic induction of growth arrest and apoptosis, wild-type AR (Heisler <i>et al.</i> 1997)

growth are suitable models for studies on changes in androgen signalling (Table 1). In general, LNCaP cells are sensitive to acute androgen ablation to which they respond by growth retardation. However, various adaptation mechanisms are operative during chronic maintenance in a steroid-depleted environment. One of the most frequently used LNCaP sublines is C4-2 cells, derived during *in vivo* propagation in castrated animals (Wu *et al.* 1994). They acquire a higher invasive and metastatic potential and up-regulate PSA baseline levels (Thalmann *et al.* 1994). This PSA up-regulation is in contrast to that observed in cells selected by long-term androgen ablation or interleukin (IL)-6 treatment *in vitro*, in which PSA expression is considerably reduced (Gao *et al.* 1999, Hobisch *et al.* 2001). PSA gene silencing in a subline derived during continuous androgen withdrawal *in vitro* is due to hypermethylation (Wang *et al.* 2001). Thus, in some prostate cancer patients, the expression of PSA might be low or undetectable despite AR expression. It is therefore not surprising that there is a considerable heterogeneity in expression of these proteins when metastatic lesions from various sites from the same patient are compared

(Shah *et al.* 2004). In fact, there is no strong correlation between their expression in metastatic lesions. Down-regulation of AR protein by antisense oligonucleotides in an androgen-deprived subline was associated with an increase in the levels of the cell cycle inhibitor p21WAF1 and reacquisition of the androgen-dependent phenotype (Wang *et al.* 2001). In the CWR22 xenograft model system, sublines with variable growth phenotypes emerged 80 to 400 days after androgen withdrawal (Agus *et al.* 1999). In the relapsed prostate cancer xenograft CWR22, there is an increased expression of multiple AR-regulated genes (Gregory *et al.* 1998, Kim *et al.* 2002). The data from Gregory *et al.* (1998) and Kim *et al.* (2002) demonstrated that the AR signalling pathway is functional in hormone therapy-refractory prostate cancer. In fact, this conclusion has been reached with different methodological approaches in various models. The cell line CWR22-Rv1 was used for studies on hormonal responsiveness in advanced prostate cancer (Attardi *et al.* 2004). These authors showed that the cells become stimulated by a wide range of steroid hormones. This aberrant stimulation might occur as a result of an in-frame tandem duplication of exon 3 that encodes the second zinc finger of the DNA-binding domain of the AR (Tepper *et al.* 2002). In contrast to models representative of chronic androgen ablation, the regulation of AR expression does not correlate with proliferative changes during intermittent androgen ablation. We have recently established a series of LNCaP sublines after intermittent androgen withdrawal *in vitro* (Hobisch *et al.* 2004). In that model system, AR protein expression is up-regulated during prolonged androgen deprivation and inhibited after subsequent addition of androgen. However, these cells acquired a growth advantage and the proliferation was not inhibited in sublines that down-regulate the AR. Thus, the AR signalling pathway might be by-passed during the progression of prostate cancers treated with intermittent androgen ablation. It could be speculated that an increased expression of several cytokines contributes to activation of intracellular kinase pathways and growth advantage in sublines that mimic intermittent androgen withdrawal.

Sublines derived under androgen-depleted conditions are reliable models with which to study interaction with AR cofactors under pathological conditions. AR coactivators, proteins with histone acetyltransferase activity, interact with one or more domains of the AR, thus leading to remodelling of the chromatin structure through the acetylation of histones. Not only expression, but also functional interactions of coregulatory proteins, may be altered in the late stages of prostate

cancer. The coactivator Tip60 could be recruited in the absence of androgen to the promoter of the PSA gene in an androgen-deprived LNCaP subline but not in the parental cells (Halkidou *et al.* 2003). This mechanism might be operative with other coactivators in prostate cancer thus enhancing expression of AR target genes in late tumour stages.

It should however be mentioned that PC-3 cells stably transfected with AR cDNA have been used in prostate cancer biology studies. In that cellular context, AR re-expression was clearly associated with a less malignant phenotype. Androgen treatment of AR-expressing PC-3 cells blocks progression through the cell cycle leading to growth inhibition and apoptosis (Heisler *et al.* 1997). Similar observations were made with the ARCaP cell line which is derived from ascites from a prostate cancer patient and which expresses endogenous AR (Cinar *et al.* 2001). Although the relevance of these models needs to be studied further, the results raise some questions about the limitations of therapeutic intervention focused on the AR.

AR coactivator alterations occur in endocrine therapy-resistant prostate cancer

Although a large number of AR-interacting proteins have been detected in androgen-target tissues, the relevance of most of them for the development of prostate cancer and progression towards therapy resistance remains to be determined. Recently, increasing numbers of cofactor antibodies have become available and the expression of a subgroup of them has been demonstrated at different stages of prostate carcinogenesis (Table 2). It was demonstrated that increased expression of SRC-1 and TIF-2 coactivator proteins contributes to prostate cancer progression (Gregory *et al.* 2001b). As a consequence of over-expression of these cofactors, there is a reduced threshold for androgenic steroids needed to activate the AR. However, regulation of SRC-1 mRNA and protein levels in prostate cancer is not completely understood. In a group of patients with therapy-resistant prostate cancer, there was a lower expression of SRC-1 mRNA compared with that in untreated patients (Linja *et al.* 2004). Amplification and over-expression of SRC-1 mRNA were seen only in the LuCaP 70 xenograft but not in specimens obtained from patients who failed endocrine therapy. It is, however, difficult to compare both studies without caution since Linja *et al.* (2004) investigated mRNA

Table 2 Selected AR coactivators and their functional importance in prostate cancer

Coactivator	Expression and function in prostate cancer
SRC-1	Up-regulation in therapy-refractory tumours (Gregory <i>et al.</i> 2001b) Implicated in ligand-independent activation by IL-6 (Ueda <i>et al.</i> 2002b)
TIF-2	Up-regulation in therapy-refractory prostate cancer (Gregory <i>et al.</i> 2001b) Involvement in synergistic activation by androgen and EGF (Gregory <i>et al.</i> 2004)
CBP	Potential of agonistic effects of hydroxyflutamide (Comuzzi <i>et al.</i> 2003) Up-regulation by androgen ablation (Comuzzi <i>et al.</i> 2004)
p300	Required for ligand-independent activation by IL-6 (Debes <i>et al.</i> 2002)
RAC3	Correlation with prostate cancer grade and stage (Gnanapragasam <i>et al.</i> 2001)
ARA70	Enhancement of AR activation by several steroids and non-steroidal AR antagonists (Yeh <i>et al.</i> 1998, Miyamoto <i>et al.</i> 1998)
ARA55	AR coactivation in the presence of either androgen, oestradiol, or hydroxyflutamide (Fujimoto <i>et al.</i> 1999)
ARA54	Enhancement of transcriptional activity of the LNCaP AR in the presence of oestradiol or hydroxyflutamide (Kang <i>et al.</i> 1999)
ART 27	Very low expression in prostate cancer (Taneja <i>et al.</i> 2004)
Cdc25B	Overexpressed in prostate cancer (Ngan <i>et al.</i> 2002)
Tip60	Nuclear accumulation in advanced prostate cancer Up-regulation by androgen ablation (Halkidou <i>et al.</i> 2003)
Gelsolin	Potential of agonistic effects of hydroxyflutamide up-regulated by androgen ablation (Nishimura <i>et al.</i> 2003)

expression. SRC-1 is involved in ligand-independent activation of the AR by IL-6, a cytokine that regulates proliferation, apoptosis, and angiogenesis in prostate cancer (Ueda *et al.* 2002). The AR-associated protein RAC3, a member of the p160 family of coactivators, is expressed in LNCaP cells at a higher level than in PC-3 or DU-145 cells respectively (Gnanapragasam *et al.* 2001). Most AR cofactors also have a regulatory function in AR-negative prostate cancer cell lines. In clinical samples, RAC3 expression correlates with prostate tumour grade and stage and poorer disease-specific survival. An inhibitory effect of androgen on the expression of AR cofactors was described in the case of

Tip60, cAMP Response Element-Binding Protein-Binding Protein (CBP), or gelsolin (Halkidou *et al.* 2003, Nishimura *et al.* 2003, Comuzzi *et al.* 2004). A marked potentiation of AR activity by hydroxyflutamide was observed in the presence of CBP or gelsolin (Comuzzi *et al.* 2003, Nishimura *et al.* 2003). High expression levels of these coactivators were found in samples obtained from patients who failed endocrine therapy. These findings suggest that one of the reasons for failure of prostate cancer hormonal therapy is up-regulation of AR coactivator proteins which may be linked to a hypersensitive androgen signalling pathway. The coactivators may thus contribute to the anti-androgen withdrawal syndrome, which is characterized by a time-limited improvement in the condition of the patients after withdrawal of an anti-androgen. In patients who underwent radical prostatectomy, the levels of expression of the functional homologue of CBP, p300, correlated with proliferation, tumour volumes, extraprostatic extension, and seminal vesicle involvement (Debes *et al.* 2002). The main constituent of caveolae membranes, caveolin, associates with the AR thus leading to enhancement of its transcriptional activity in a ligand-dependent manner (Lu *et al.* 2001). Targeting interactions between these coactivators and the AR may thus improve the effectiveness of endocrine therapy.

It has been proposed that alterations in the expression or function of several cofactors that are more specific for the AR occur in prostate tumours. In this context, the cofactor ARA70, which potentiates AR activation in the presence of testicular and adrenal androgens, oestradiol, and anti-androgens (Heinlein & Chang 2004), has been studied extensively. Li *et al.* (2002) found a decreased expression of ARA70 in prostate cancer tissues by *in situ* hybridization. Consistent with these results, proliferation of LNCaP cells and colony formation were inhibited after overexpression of ARA70, thus suggesting its role as a tumour suppressor. There is also evidence that expression levels of the coactivator AR trapped clone-27 (ART-27) are substantially decreased in prostate cancer tissues (Taneja *et al.* 2004).

In contrast, data from Hu *et al.* (2004) showed an increase in ARA70 protein in high-grade prostate cancers and cells cultured in androgen-deficient conditions. More research is needed to clarify the reasons for these contrasting results. The use of a dominant-negative mutant of ARA70 led to inhibition of LNCaP proliferation; however, the contribution of inhibition of an individual AR coactivator to a potential anti-tumour effect should be further investigated *in vivo* (Rahman *et al.* 2003). Due to interactions between the

AR and its various partner proteins, several other cofactors could compensate for the loss of one of them. AR transcription activation function is potentiated by cyclin E through interaction with the receptor N-terminal region (Yamamoto *et al.* 2000). This interaction is likely to be relevant to the mitogenic effect of androgenic hormones in prostate cancer. Overexpression of cdc25B, a dual-specific phosphatase which activates cyclin-dependent kinases and enhances AR activation, was reported in prostate cancer patients (Ngan *et al.* 2002). Conversely, the tumour suppressor retinoblastoma also potentiates AR activation (Yeh *et al.* 1998). Similarly, coactivation of the AR by the breast cancer susceptibility gene BRCA1 is associated with induction of apoptosis (Yeh *et al.* 2000).

Alterations in AR corepressors may also be associated with therapy failure in prostate cancer. The corepressor Silencing Mediator of Retinoid and Thyroid Hormone Receptors (SMRT) decreased induction of AR activity by androgen through inhibition of N/C interactions (Liao *et al.* 2003a). AR activation is also inhibited by cyclin D1 (Petre-Draviam *et al.* 2003). At present, there are no data available on corepressor expression alterations in clinical prostate cancer material.

Impact of mutated AR for prostate cancer progression towards resistance to hormonal therapy

AR point mutations were intensively studied in prostate cancer cell lines and clinical specimens. Although some controversies regarding their frequency in prostate tumors still exist in the literature, there is a consensus that they contribute to failure of endocrine therapy in a subgroup of patients (Taplin *et al.* 2003). Interestingly, no correlation between AR mutations and survival or the anti-androgen withdrawal syndrome was found. Detection of mutations in prostate cancer tissue was improved when material obtained by laser capture microdissection was used (Marcelli *et al.* 2000). Mutations are more common in patients with high-grade cancer as well as in those with distant metastases. In these tumour stages, several other genetic alterations occur and this could be a reason why the correlation with patient survival has not been established. Mutations in the N-terminal region of the AR were detected in patients treated with orchiectomy and estramustine, whereas in individuals treated with orchiectomy alone somatic AR mutations were located predominantly in the ligand-binding domain (Hyytinen *et al.* 2002). A mutation in the hinge region of the AR detected in a patient with high-grade prostate cancer (Ser646Phe) leads to an increased functional activity of

the AR (Thompson *et al.* 2003). Several AR mutations were characterized in the mouse transgenic adenocarcinoma of the prostate model (Buchanan *et al.* 2001). Mutations at the boundary of the hinge and ligand-binding domain yield increased transactivation. AR mutations that broaden the activation spectrum are present in human cell lines LNCaP, MDA PCa 2a, and the xenograft CWR22. The mutated LNCaP AR was characterized in a number of research studies that yielded consensus regarding its activation by oestrogenic and progestagenic steroids, adrenal androgens, glucocorticoids, and the anti-androgen hydroxyflutamide (Veldscholte *et al.* 1990, Chang *et al.* 2001). Bicalutamide is the only anti-androgen which retains antagonistic properties in LNCaP cells (Veldscholte *et al.* 1992). The AR mutant in MDA PCa 2a cells contains the two alterations, Leu701His and Thr877Ala (Zhao *et al.* 1999a). That receptor binds cortisol with a high affinity (Zhao *et al.* 2000). Activation of the MDA PCa 2a AR by cortisol is associated with induction of PSA expression and stimulation of growth. The CWR22 AR contains a mutation at codon 874 (His replaced by Tyr) and shows an increased activation by adrenal androgens and hydroxyflutamide (Tan *et al.* 1997). Adrenal androgens are also potent activators of the mutated AR Val715Met and Val730Met (Culig *et al.* 1993, Peterziel *et al.* 1995). The question whether the treatment with anti-androgens itself has an influence on frequency of point mutations was addressed *in vitro* and *in vivo*. Hara *et al.* (2003b) described mutations in a subline of LNCaP cells which were chronically treated with bicalutamide. The AR mutations detected led to an exchange of Trp at position 741 to Cys or Leu. Most interestingly, these were the same mutations as those previously discovered in tissues of prostate cancer patients who received bicalutamide therapy (Haapala *et al.* 2001). Mutations at AR codon 741 result in acquisition of agonistic properties of bicalutamide and hydroxyflutamide antagonism, in contrast to parental LNCaP cells. Conversely, a possible role for bicalutamide as a 'second-line' treatment was proposed for mutated receptors that increasingly respond to hydroxyflutamide (Joyce *et al.* 1998, Taplin *et al.* 1999).

The most rapid approach in the analysis of functional properties of mutated AR is the use of a colorimetric yeast reporter assay (Shi *et al.* 2002). In that study, it was demonstrated that there might be different consequences of AR mutations; loss or reduction of function (48%) were observed as well as wild-type (7%) and gain of function (45%). A subgroup of patients with prostate cancer presented with mutations that led to the inhibition of transcription

activation function. The mutated AR Ala748Thr was rapidly degraded and expressed at a lower level in cells than in the wild-type receptor (James *et al.* 2002). Androgens dissociate from that mutated receptor five times faster than with the wild-type AR. Similarly, a Cys619Tyr mutation is associated with inactivation and mislocalization of the receptor (Nazareth *et al.* 1999).

AR responsiveness through activation by non-steroidal compounds in prostate cancer and the role of the mitogen-activated protein kinase (MAPK) signalling pathway

Among compounds that activate AR function in the absence of ligand or in a synergistic manner with low androgen doses, there is an important role of the EGF receptor-related molecule HER-2. Its overexpression resulted in enhanced growth of prostate cancer xenografts and up-regulation of PSA (Craft *et al.* 1999). However, HER-2 overexpression in prostate cancer is limited to a subgroup of patients and cannot account for the development of resistance in the majority of cases. Activation of the AR by HER-2 and related growth factors requires the functional pathway of MAPK (Yeh *et al.* 1999). Treatment of cells with the MAPK kinase inhibitor PD98059 substantially reduced PSA levels. The ability of anti-androgens to antagonize AR activation is reduced in the presence of activated MAPK (Craft *et al.* 1999). Prostate cancer cells engineered to express a constitutively active Ras, which up-regulates MAPK activity, became more sensitive to stimulation with lower androgen doses (Bakin *et al.* 2003a). In the C4-2 LNCaP subline, dominant-negative Ras restored their sensitivity to bicalutamide (Bakin *et al.* 2003b). This finding suggests that inhibition of Ras reverts a malignant phenotype and has a potential benefit in patients with advanced carcinoma of the prostate. MAPK activity increased in tumour specimens obtained during androgen ablation therapy (Gioeli *et al.* 1999). Phosphorylation of MAPK in prostate cancer cell lines correlates with their aggressiveness; PC-3 and DU-145 cells show a higher MAPK phosphorylation than LNCaP cells which have a lesser metastatic potential (Chen *et al.* 1999, Putz *et al.* 1999). High constitutive MAPK activity in DU-145 is caused by an autocrine loop involving the EGF receptor. In LNCaP and DU-145, but not in PC-3 cells, treatment with growth factors that signal through receptor tyrosine kinases and agents that elevate intracellular

cAMP levels has a co-operative effect on MAPK activation. Results obtained with LNCaP cells suggest that elevated MAPK phosphorylation in prostate cancer occurs due to stimulation by various cytokines, one of which is TGF- β . MAPKs are involved in a mitogenic switch of TGF- β in cancer (Park *et al.* 2000). TGF- β is a well-known pleiotropic growth factor and the use of PD98059 restored a growth-inhibitory effect of TGF- β . Stimulatory effects of inhibitors of differentiation proteins on proliferation of LNCaP cells are associated with activation of the MAPK pathway (Ling *et al.* 2002). In an experimental model developed to monitor changes in IL-6 signalling in prostate cancer patients, a more malignant LNCaP subline shows activation of the MAPK pathway, in contrast to parental cells (Steiner *et al.* 2003). Thus, MAPK might be a common target for novel experimental therapies in human prostate cancer. In this context, MAPKs were inhibited by a flavonoid anti-oxidant silibinin, low doses of grape seed extract, the neuropeptide calcitonin, and kinin receptor antagonists (Segawa *et al.* 2001, Sharma *et al.* 2001, Tyagi *et al.* 2003, Barki-Harrington *et al.* 2003). Addition of PD98059 to treatment with docetaxel, an agent which suppresses growth by altering microtubule assembly, enhanced a growth-inhibitory effect (Zelivianski *et al.* 2003).

Ligand-independent activation of the AR by IL-6 is also a subject of major interest (Hobisch *et al.* 1998, Chen *et al.* 2000). IL-6 up-regulation in prostate cancer occurs as a result of a concerted action of signalling pathways of nuclear factor κ B, activating protein-1, and TGF- β (Park *et al.* 2003, Zerbini *et al.* 2003). In addition, there might be an impact of androgen ablation on the elevation of IL-6 levels in prostate cancers. IL-6 binds to the IL-6 receptor which is composed of ligand-binding and signal-transducing subunits. Multiple signalling pathways, in particular those of Janus kinases/signal transducers and activators of transcription (JAK/STAT), MAPK, and phosphatidylinositol 3-kinase (PI3-K) may transmit IL-6 signal in target cells, thus being responsible for either growth stimulation or inhibition. It is not possible to clearly associate phosphorylation of STAT3, which is a characteristic feature of clinical prostate cancer, to malignant transformation (Mora *et al.* 2002). Phosphorylation of STAT3 in response to IL-6 was reported in experiments in which growth arrest was observed but also in connection with the cytokine's induced cell proliferation (Spiotto & Chung 2000, Giri *et al.* 2001). These differences may occur because of different requirement of intermediary proteins, such as SHP-2, in IL-6 signal transduction. Functional implications of AR activation by IL-6 were

investigated in LNCaP cells in which there was an induction of expression of PSA mRNA and protein in association with growth retardation. It should, however, be studied as to how ligand-independent AR activation by IL-6 regulates cellular events in other tumour models. AR activation by IL-6 depends on the presence of coactivators p300 and SRC-1 (Debes *et al.* 2002, Ueda *et al.* 2002). It was possible to completely suppress this ligand-independent activation by treating cells with p300 small interfering (si)RNA (Debes *et al.* 2002). In addition to IL-6, which is produced by malignant prostate cells, other cytokines, such as oncostatin M, IL-4, and IL-8 also activate the AR (Godoy-Tundidor *et al.* 2002, Lee *et al.* 2003, 2004). In the presence of oncostatin M, acquisition of agonistic activity of hydroxyflutamide was observed (Godoy-Tundidor *et al.* 2002). The cytokine regulates the growth of prostate cancers by various autocrine and paracrine loops (Mori *et al.* 1999, Royuela *et al.* 2004). IL-6 is not a single agent that causes inhibition of proliferation and stimulation of PSA in LNCaP cells; similar observations were reported after treatment with the differentiation agent butyrate (Sadar & Gleave 2000).

In addition to classic induction of MAPK by growth factors or compounds that increase cAMP levels, these kinases are also elevated after short-term treatment with androgens. Either the AR or oestrogen receptor associate with Src thus stimulating the signalling pathway of Raf-1 and ERK-2 and leading to an increased proliferation of prostate cancer cells (Migliaccio *et al.* 2000). Surprisingly, anti-androgen hydroxyflutamide stimulated phosphorylation of MAPK in AR-negative DU-145 cells (Lee *et al.* 2002). This might represent another mechanism responsible for the anti-androgen withdrawal syndrome observed in prostate cancer patients. This activation led to up-regulation of cyclin D1 and enhanced cellular proliferation. AR ligand-independent activation was observed in response to β -catenin, a molecule that regulates intracellular adhesion. Neuropeptides whose expression is up-regulated in most advanced prostate tumours were shown to stimulate AR activity and proliferation (Lee *et al.* 2001, Dai *et al.* 2002).

Regulation of survival of prostate cancer cells and AR activity by protein kinase Akt

Protein kinase Akt is phosphorylated by the signalling pathway of PI3-K and overexpressed in a variety of human malignancies. AR expression and function in LNCaP cells are influenced by the PI3-K pathway

(Manin *et al.* 2002). Protein kinase Akt is implicated in prostate cancer progression in an AR-dependent and AR-independent manner. Activation of the AR by HER-2 is due to Akt phosphorylation at serine 213 and 791 (Wen *et al.* 2000). It was reported that AR activation by Akt is characteristic for higher LNCaP passages; in contrast, there is an inhibitory effect in low passages (Lin *et al.* 2003). Akt up-regulation in prostate cancer is in part due to overexpression of the AR coactivator SRC-3 (Zhou *et al.* 2003). Differences in activation of the AR by non-steroidal compounds may be explained by the relative predominance of a particular signalling pathway; it was found that the pathways of JAK/STAT and MAPK enhance AR activation by IL-6, whereas the PI3-K pathway is inhibitory (Yang *et al.* 2003). A downstream target of Akt is the Forkhead in Human Rhabdomyosarcoma (FKHR) transcription factor, which interacts with the AR thus protecting the cells from apoptosis (Li *et al.* 2003).

Activation of the PI3-K pathway is counteracted by the phosphoinositide phosphatase PTEN, a tumour suppressor that is commonly down-regulated in cancer. In prostate cancer, decreased PTEN expression is associated with high Gleason grade (McMenamin *et al.* 1999). Constitutive activation of Akt is observed in cells resistant to cytotoxic agents. LNCaP cells lost PTEN expression and showed a constitutive Akt activity (Carson *et al.* 1999). Consequently, PTEN re-expression in LNCaP cells leads to increased apoptosis. PI3-K activation is elevated in LNCaP cells after prolonged androgen ablation (Murillo *et al.* 2001). Androgen-independent proliferation of LNCaP cells occurred in parallel with loss of expression of p27 whose stability is down-regulated by PI3-K. Akt levels in the LNCaP model system correlate with tumour volume (Graff *et al.* 2000).

The Akt pathway is implicated in the regulation of a variety of cellular events. Constitutively active Akt is involved in androgen-initiated up-regulation of hypoxia-inducible factor-1, which is in turn stimulatory to vascular endothelial growth factor (Mabjeesh *et al.* 2003). Anti-apoptotic effects of insulin-like growth factor-binding protein-5, which potentiates the action of insulin-like growth factor-I, are mediated through Akt (Miyake *et al.* 2000). Phosphorylation of Akt is associated with $\alpha(V)\beta(3)$ integrin-induced migration of prostate cancer cells on vitronectin and osteopontin (Fornaro *et al.* 2003). Integrins increase the levels of survivin through the Akt pathway thus preventing tumour necrosis factor- α -induced apoptosis. Prostate cancer invasion and metastasis are regulated in part by androgens; the enzyme matrix metalloproteinase 2,

whose expression is elevated in aggressive prostate cancers and is involved in degradation of extracellular matrix, is up-regulated by androgens through the PI3-K pathway (Liao *et al.* 2003b).

In prostate cancer, expression of the key lipogenic enzyme fatty acid synthase is up-regulated by Akt. Treatment with the PI3-K inhibitor LY294002 or transfection of PTEN cDNA led to inhibition of fatty acid synthase expression (Van de Sande *et al.* 2002). LNCaP cells are resistant to the apoptotic inducer tumour necrosis factor-related apoptosis-inducing ligand due to high constitutive Akt activity (Chen *et al.* 2001).

All these findings suggest that therapeutic intervention aimed to inhibit Akt activation is justified in prostate cancer. Celecoxib, a compound that inhibits the enzyme cyclo-oxygenase-2, is considered a potentially useful chemopreventive agent in prostate cancer. Blockade of Akt activation was observed after treatment with celecoxib and is also characteristic for an inhibitory effect of neutral endopeptidase in prostate cancer (Kulp *et al.* 2004). Chemoprevention or therapy of prostate cancer is in several cases associated with inhibition of Akt. Examples include black tea polyphenols and 17allylamine-17-demethoxygeldanamycin (Solit *et al.* 2002, Siddiqui *et al.* 2004).

Conclusions

Androgenic hormones are responsible for proliferative and differentiation effects in prostate. These regulations occur through activation of AR. The outcome of androgen ablation therapy in prostate cancer might be influenced by the structure of the AR, changes in its sensitivity, and interaction with partner proteins. Although various cellular adaptation mechanisms that affect AR signalling have been identified in prostate cancer, their impact on time to progression and patients' survival has not yet been clarified. In endocrine therapy-resistant prostate cancer, the signalling pathway of the AR is functional. Novel treatment options that interfere with androgen signalling have been proposed in experimental systems; however, their effectiveness in advanced prostate cancer remains to be investigated.

Acknowledgements

The authors are grateful to Mr Robert Schober for editorial assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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