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"HORMONE-REFRACTORY" PROSTATE CANCER. A PUTATIVE NEW MECHANISM: THE UPSIDE-DOWN RESPONSE TO ANDROGENS

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RESUM

En aquest article volem revisar la diversitat dels mecanismes moleculars suposadament responsables del creixement independent d'andrògens del càncer de pròstata. Es demostra que alguns càncers de pròstata que escapen de la teràpia endocrinològica estan compostos per cèl·lules sensibles als andrògens.

Ens centrarem en els resultats del nostre laboratori i en els d'altres grups de recerca que suggereixen el mateix concepte nou: el comportament del càncer de pròstata refractari als andrògens està associat a una resposta invertida de les cèl·lules als andrògens. Hem observat un alentiment paradoxal en el creixement de diverses línies cel·lulars induït pels andrògens. Aquestes línies cel·lulars provenen de les cèl·lules LNCaP, ja sigui per evolució espontània o per cultiu crònic en un medi sense andrògens. La línia ARCaP (*androgen-reverted carcinoma of the prostate*) va ser establerta a partir de l'ascitis d'un pacient amb càncer de pròstata avançat. Els tumors que varen créixer a partir d'aquestes cèl·lules reverteixen, encara que transitòriament, en el tractament androgènic. Volem suggerir que la castració podria permetre la proliferació de les cèl·lules que eren paradoxalment alentides pels andrògens i que aquesta reacció invertida als andrògens podria ser el possible mecanisme pel qual el càncer de pròstata deixa de respondre a la teràpia hormonal. Aquests resultats aportarien unes bases racionals per a comprendre el tractament antiandrogènic intermitent.

Paraules clau: pròstata, andrògens, antiandrògens, resistència, inhibició.

SUMMARY

In this paper we survey the diversity of the molecular mechanisms suspected to be responsible for the androgen-independent growth of prostate cancer. It has been shown that some prostate cancers, which escape endocrine therapy, are composed of androgen-sensitive cells.

We focus on the results from our laboratory and from a few others that suggest a new concept: that the androgen-refractory behavior of prostate cancer may be associated with an inverted response to androgens by cells. The proliferation of several cell lines was paradoxically slowed by androgens. In the afore-mentioned studies, a series of these cell lines arose from the LNCaP cell line, either spontaneously or after culturing them chronically in androgen-poor culture medium. The ARCaP (androgen-reverted carcinoma of the prostate) was established from the ascites of a patient with advanced prostate cancer. Usually, tumors grown from such cells regress, albeit transiently, under androgen treatment. It has been suggested that castration could allow the proliferation of cells that are paradoxically slowed by androgens and that the inverted response to androgens could possibly be a mechanism, by which prostate cancer escapes from endocrine therapy. These results provide the rationale for intermittent treatment.

Keywords: prostate, androgen, anti-androgen, resistance, inhibition.

PROSTATE CANCER CELLS AS ANDROGEN TARGETS

In industrialized countries, prostate carcinoma is the second most prevalent malignant tumor in men after skin cancer. It accounts for up to 33% of all male cancers (Carducci and Carroll, 2005). At age 85, the cumulative risk of malignancy ranges from 0.5% to 20% worldwide (Gronberg, 2003). In the year 2000, the mortality rates were 15 and 19 per 100,000 men in Spain and France, respectively (Crawford, 2003). Moreover, its incidence is expected to rise with the advancing age of the population. It is encouraging to know that screening and appropriate treatments are able to decrease prostate cancer mortality (Etzioni *et al.*, 2003; Labrie *et al.*, 2004).

The prostate depends on endogenous androgens, mainly DHT, for growth and differentiation. Growth stimulation results from the activation of cell proliferation and the inhibition of apoptosis. Androgens also play a central role in prostate cancer development (DeMarzo *et al.*, 2003; Feldman and Feldman, 2001; Kokontis and Liao, 1999), in conjunction with diet, lifestyle and genetic background (Nelson *et al.*, 2003). The chemical prevention of prostate cancer has been attempted with some success by blocking the last step of DHT production at the level of testosterone reduction using 5-alpha reductase. However, it is not currently recommended, since the trial has raised some doubts about the risk of increasing the incidence of high-grade tumors (Lieberman, 2003; Scardino, 2003; Thompson *et al.*, 2003). The adjuvant treatments most currently used for advanced prostate cancers are aimed at blocking androgen effects through androgen deprivation and/or the administration of androgen receptor (AR) antagonists. Although many tumors initially regress, most of them relapse, i.e., they cease to respond and proliferate, in spite of continued treatment. Such tumors are referred to as androgen-independent prostate cancer (AIPC), hormone-refractory, hormone-insensitive, hormone-relapsed or hormone-resistant. However, it must not be forgotten that such tumors are composed of cells that are indeed responsive to androgens and anti-androgens (see below).

A large majority of the biological effects of androgens require classical AR that belongs

to a wide family of ligand-dependent transcription factors (Heinlein and Chang, 2004). These receptors also bind various compounds and, as a result, exhibit androgen-type effects. Ligand binding changes AR conformation, similar to the way in which a hand changes the shape of a glove. The resulting AR activation includes the dissociation from heat shock protein, homodimerization, phosphorylation, and finally, the binding to chromatin. The homodimer, charged with ligand, binds to short sequence(s) of DNA, called the androgen-response element (ARE), which is located upstream of the initiation site of transcription or the DNA-binding proteins, located in the region. In both cases, the dimer recruits specific co-factors. Some of them display enzyme activity that controls the opening / condensation of chromatin, in order to facilitate or prevent the formation of the initiation complex for transcription. The transcription of androgen-responsive genes is up- or down-regulated, according to the set of co-factors that have been recruited and are already located on the promoter. Pure antagonists bind to AR and do not lead to gene transcription, because the co-inhibitors are also recruited. Mixed agonist-antagonist ligands, called SARM —Specific Androgen Receptor Modulator, (Negro-Vilar, 1999)—, behave as agonists in some cells or tissues and as antagonists in others, depending on the DNA alterations (mutations and epigenetic modifications) and the cell signaling, triggered by membrane receptors. As a result, all of the following contribute to the type of reaction observed in response to endogenous or exogenous compounds: the AR concentration, the AR-ligand induced fit, the concentration, the activity and combination of co-factors, and the membrane signals. Non primary genomic actions are also known. They involve classical AR and interactions with proteins, involved in the intracellular signaling triggered at the membrane level. Androgens can also modulate the SHBG signaling from its membrane receptor, thought to be a G-protein coupled receptor (Rosner *et al.*, 1999). The modification of any compound involved in androgen action is potentially a determinant for hormone resistance.

MECHANISMS OF TUMOR "RESISTANCE"

Five main types of pathways for the development of AIPC have been categorized (Edwards and Bartlett, 2005*a*, *b*; Feldman and Feldman, 2001; Grossmann *et al.*, 2001). 1) The *hypersensitive pathway* refers to situations in which the response to androgens by cells is higher than expected, when taking into account the quantity of androgens added to the experimental model or measured in the blood. Cells with such a pathway can grow in a low androgen environment, such as that encountered in castrated patients. This pathway could result from the following alterations: an increase in AR concentration, which is sometimes the consequence of AR gene amplification (Koivisto *et al.*, 1997), the presence of high-affinity AR encoded by a mutated AR gene (somatic mutation), the expression of a particular hyper-active set of co-factors (Gregory *et al.*, 2001), and a local augmentation of DHT production, brought about by an increase of 5-alpha reductase activity. It is of interest to note that a modest increase in AR is a molecular determinant for resistance to anti-androgen therapy (Chen *et al.*, 2004; Isaacs and Isaacs, 2004). 2) The *promiscuous pathway* is mainly observed in cells with particular missense mutations of the AR gene (Linja and Visakorpi, 2004; Veldscholte *et al.*, 1990). These mutations increase the affinity for various ligands and broaden the list of compounds able to bind and activate AR. Hence, adrenal androgens, glucocorticoids, progestins, and the antagonist flutamide bind to different mutated AR and stimulate cell proliferation. The so-called promiscuous AR

can explain both the rise of PSA in patients treated with flutamide and glucocorticoids, as well as explaining the improvement (transient) sometimes observed following the arrest of treatment. Co-factor alterations may be involved in these unexpected biological responses. 3) The *outlaw pathway* is AR dependent, but AR-ligand independent. In this case, the AR-dependent gene expression is mainly enhanced via the phosphorylation of AR and its co-factors. Cytokines such as IL6 (Culig *et al.*, 1996; George *et al.*, 2005), growth factors such as IGF-1, EGF, KGF, and heuregulin (Gioeli, 2005; Gregory *et al.*, 2005), neuropeptides and adrenomedullin (Rocchi *et al.*, 2001), and the over-expression of HER2/neu can induce androgen effects. The fact that antiandrogens inhibit the effects of cytokines and growth factors, but not those resulting from the over-expression of HER2/neu, suggests that different domains of AR are involved in the cross-talk between AR and membrane receptor cell signaling. HER2/neu activates the MAP kinase and the protein kinase B (AKT) pathways. The AKT pathway is downregulated by a PIP3 phosphatase, encoded by PTEN. Therefore, a loss-of-function mutation of PTEN enhances the AR-dependent pathway. 4) The *by-pass pathway* is independent of AR and AR ligands. When tumors escape treatment, there is more cell division than cell death, e.g., by apotosis. The low apoptotic activity is in correlation with the increase of the anti-apoptotic proteins, bcl-2, bcl-x, clusterin (So *et al.*, 2005), and c-FLIP (Gao *et al.*, 2005). In pathways 1 to 4, cells are supposed to become resistant under treatment by various mechanisms: the accumulation of mutations, a change in the epithelium/stroma ratio (Lee and Tenniswood, 2004), a change in the local concentrations of hormones, cytokines, and growth factors, etc. 5) The *lurker cell pathway* is clearly different from the four previous pathways. The lurker pathway postulates that true androgen-resistant cells may be present in the tumors before treatment has started. This could occur when stem cells, known to be androgen resistant, are transformed. Several pathways can function simultaneously, since prostate cancer cells are very heterogeneous (Shah *et al.*, 2004). Other mechanisms may exist, e.g., increased angiogenesis (Gustavsson *et al.*, 2005) and an inversion of the response to androgens (see below). A set of genes that may be involved in the progression to androgen independence has recently been described (Pfundt *et al.*, 2005; Shi *et al.*, 2004).

A NEW PUTATIVE MECHANISM OF "TUMOR RESISTANCE": THE AR-DEPENDENT UPSIDE-DOWN PATHWAY

The proliferation of several cell lines was paradoxically slowed by androgens. First, a series of such cell lines arose from the LNCaP cell line, found in a lymph node of a patient treated for advanced prostate cancer (Horoszewicz *et al.*, 1983). The switch occurred either spontaneously as LNCaP-R/R2 (Joly-Pharaboz *et al.*, 1995), or it occurred after being cultured chronically in androgen-poor culture medium, such as LNCaP 104-R (Kokontis *et al.*, 1994) and MOP (Joly-Pharaboz *et al.*, 2000) or JAC cells (unpublished). These cells expressed the promiscuous T877A mutated AR. Second, another source of androgen-repressed cells (ARCaP, for androgen reverse carcinoma of prostate) was the ascites fluid of a patient with advanced prostate cancer (Zhau *et al.*, 1996). The pathway that sustained these paradoxical effects may be referred to as the upside-down pathway in Feldman's terminology, which points out that androgens, instead of stimulating, as shown in wild-type LNCaP, inhibit cell proliferation. In fact, the differences between wild-type LNCaP and LNCaP variants are not so simple. The dose-dependent response was bell-shaped in the wild-type, with a maximum of around 0.1 nM R 1881, while there was a dose-

dependent inhibition of proliferation in other cells (ED_{50} = 0.1 nM R1881). Concerning the dose-dependent inhibition, DHT was nearly as potent as R 1881, indicating that the response occurred in the physiological range of the androgens. Simultaneously, the cell cycle was blocked at G0-G1 (Joly-Pharaboz *et al.*, 2000, 1995; Zhau *et al.*, 1996), and small spherical cell fragments appeared around the MOP cells. This fragmentation has been confirmed by flow cytometry (not published). In addition, a ligand-induced apoptosis was shown in MOP cells, but not in R2 cells. Surprisingly, androgen treatment lead to a frank cell hypertrophy, as the cell content of proteins and RNA was increased. Several molecular determinants of the paradoxical response to androgens were sought. We did not find any quantitative or qualitative differences in AR between androgen-stimulated and androgen-inhibited cells. There was no increase, even modest, in AR concentration. MOP and R2 cells contained the same mutated AR as LNCaP cells. The same poly CAG polymorphism in exon 1 was found in the AR gene of MOP cells and LNCaP cells. No new mutation was found in the last seven exons of the AR gene (Joly-Pharaboz *et al.*, 1995, 2000;). The same pattern of ligand affinities for AR was shown in R2 and LNCaP cells (Joly-Pharaboz *et al.*, 1995), and there was a rather good correlation between the affinity of the ligands for AR (R 1881, DHT, cyproterone acetate, estradiol, progesterone, and R 5020) and their efficacy in inhibiting cell proliferation. The paradoxical response to androgens did not appear to be associated with any change in the levels of mRNA encoding the following co-factors: ARA 70, SRC-1, CBP, TIF-2 RAC3/ACTR, and SMRT (not published). The androgen regulation of several genes was not modified when cells switched from the androgen-positive to the androgen-negative control of proliferation. For example, androgens increased the PSA and VEGF secretions by MOP cells dose-dependently (Joly-Pharaboz *et al.*, 2000; Kalach *et al.*, 2005) and unpublished results). Androgens down-regulated c-myc RNA in the three cell lines LNCaP, MOP and R2 dose-dependently (Foury *et al.*, 1998). More recently, we have shown that estradiol, but not the synthetic estrogen DES, exerts androgen-like effects through mutated AR binding and not through ER binding (Kalach *et al.*, 2005).

Androgens inhibit the proliferation *in vivo* of those cells that are also inhibited in culture. This conclusion was drawn from experiments performed on nude mice grafted sc. with various cells. The tumors developing from such cells regressed transiently under androgen treatment (Joly-Pharaboz *et al.*, 2000; Umekita *et al.*, 1996). In our study, testosterone enanthate slowed the take of the graft, and more interestingly, led to tumor regression, without any exceptions out of more than one hundred tumors treated to date. Since the tumors regressed under treatment (some tumors became non-palpable under the skin of the nude mice), the androgens clearly increased cell death. However, due mainly to the fact that the tumors presented large zones of necrosis, we were unable to demonstrate any increase in cell apoptosis *in vivo*. As observed in cells in culture, the intact up-regulation of several genes (PSA, VEGF) by androgens was also intact in the animal tumors. All of the MOP tumors escaped androgen treatment; therefore, the androgen used was not a magic bullet against MOP-like cells. TE cells, cultured from tumors that escaped treatment, were less responsive to androgens than the MOP cells, which had given rise to the tumor. However, the cells recovered a response to androgens, similar to that of parental MOP cells after ten subcultures.

CONCLUSION

From this short survey, it appears that cells, used as models for studying the molecular determinants for the resistance of advanced

prostate cancer to endocrine therapy, do contain functional AR. Although the altered pathway did involve frequent abnormalities in AR-dependent signaling, the sole increase in anti-apoptotic proteins may by-pass the consequences of an androgen blockade. True androgen-resistant AR-negative cells, such as PC3 and DU-145, have been established from metastases of human prostate cancer; and, prostate stem cells may be a source of lurker cells. The participation of androgen-resistant cells in the phenomenon of tumor escape from hormone therapy remains to be specified.

The molecular determinants for the inversion of response to androgens remain to be found. This information may provide us with an answer as to why the paradoxical effects of androgens have not been mentioned so far, to the best of our knowledge, in any of the reviews on androgen and anti-androgen resistance. It is our opinion that such an inversion offers an explanation for tumor escape from treatment. Although androgen-repressed cells also escape from androgen treatment in an experimental model, the androgen-induced inhibition of cell proliferation is a further rationale for intermittent therapy, i.e., the succession of periods of androgen privation and androgen or estrogen administration (Peyromaure *et al.*, 2005; Rashid and Chaudhary, 2004). Since the benefits of endocrine therapy on advanced prostate cancer are transient, new drugs or new schedules of treatments are required. Encouraging results from chemotherapy have been recently reported (Hussain *et al.*, 2005; Petrylak, 2005; Petrylak *et al.*, 2004). We would like to stress that reverted hormone action is not as strange as it may appear at first glance. Indeed, in a follow-up to some work done with José Sáez, we were able to show that estradiol, known to induce the hypertrophy of the anterior pituitary, inhibited the growth of some rat pituitary tumor cells (Morel *et al.*, 1982). In addition, the growth of several cell lines isolated from the human breast cancer MCF-7 cells, was inhibited by estradiol at concentrations that stimulated the growth of the parental MCF-7 (Lewis *et al.*, 2005; Uchiumi *et al.*, 1991).

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