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Oncogenic Activation of Androgen Receptor

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Summary

There is considerable evidence implicating the aberrant activation or "reactivation" of androgen receptor in the course of androgen-ablation therapy as a potential cause for the development of castration-resistant prostate cancer. Several non-mutually exclusive mechanisms including the inappropriate activation of androgen receptor (AR) by non-steroids have been postulated. The present work is aimed to understand the role of neuropeptides released by neuroendocrine transdifferentiated prostate cancer cells in the aberrant activation of AR.

Objectives—The study was designed to study how neuropeptides such as gastrin-releasing peptide activate AR and to define the crucial signal pathways involved, in the hope to identify therapeutic targets.

Methods and Materials—Androgen-dependent LNCaP cell line was used to study the effects of bombesin/gastrin-releasing peptide on the growth of the cell line and the transactivation of AR. The neuropeptide was either added to the media or introduced as a transgene in LNCaP cells to study its paracrine or autocrine effect on LNCaP growth under androgen-deprived conditions. The activation of AR was monitored by reporter assay, chromatin immunoprecipitation (ChIP) of AR, translocation into the nucleus and cDNA microarray of the AR response genes.

Results—Bombesin/gastrin releasing peptides induces androgen-independent growth of LNCaP in vitro and in vivo. It does so by activating AR, which is accompanied by the activation of Src tyrosine kinase and its target c-myc oncogene. The bomesin or Src-activated AR induces an overlapping set of AR response genes as androgen, but they also a unique set of genes. Intriguingly, the Src-activated and androgen-bound ARs differ in their binding specificity toward AR response elements, indicating the receptors activated by these two mechanisms are not conformationally identical. Finally, Src inhibitor was shown to effectively block the activation of AR and the growth effects induced by bombesin.

Conclusion—The results showed that AR can be activated by neuropeptide, a ligand for G-protien coupled receptor, in the absence of androgen. The activation goes through Src-tyrosine kinase pathway and tyrosine kinase inhibitor is a potentially useful adjunctive therapy during androgen – ablation.

Keywords

neuroendocrine differentiation; neuropeptides; Src tyrosine kinase; androgen receptor activation; tyrosine kinase inhibitor; hormone refractory prostate cancers

Introduction

Prostate cancer (PCA) represents the most frequently diagnosed malignancy of men in the United States. PCA is a hormonally regulated malignancy and AR plays an important role in disease progression. One of the most troubling aspects of PCA progression is the conversion from an androgen-dependent to independent (AI) state, which at present defies any effective treatment. In the majority of end-stage, hormone-refractory (HR) tumors, AR continues to be expressed and appears to be activated by castration levels of androgen and adrenal androgens [1-3]. Thus a required step towards solving the clinical problem of prostate cancer, androgen independence, becomes one of understanding how AR is inappropriately activated and how to inhibit such aberrant signals.

AR and androgen-independence

AR plays a vital role in the development of male reproductive organs. Genetic defects in AR results in the failure to develop a prostate gland, a notion corroborated by recent AR knockout experiments in mice [4,5]. In normal development, androgen is primarily required for differentiation functions. By contrast, during the development of PCA, androgen becomes a growth and survival factor for tumor cells. At the early stage of localized and metastatic PCA, proliferation depends on androgen and androgen-ablation therapy is highly effective in controlling the disease. Treatment success however is only short-lived, as AI or HR clones eventually grow out, resulting in clinically unmanageable metastasis and mortality. Analysis of clinical AI PCAs revealed that over 90% express AR and androgen-response genes, indicating that the AR remains active and suggesting that AR is inappropriately activated in the absence of or at castration levels of testicular and adrenal androgens. Four mechanisms have been postulated to account for aberrant AR activation in AI tumors: 1) activation of AR by non-steroids via deregulated signals, 2) genetic mutations of AR, rendering the receptor hyperactive, 3) amplification or overexpression of AR and its coactivators, which sensitizes cells toward a low level of androgen, and 4) the increase of intracrine androgen. These four mechanisms are not mutually exclusive, and indeed it is likely that they work in concert to induce HR PCA. The fourth mechanism is a provocative new concept presented in this meeting (see the articles by Dr. P. Nelson and Dr. S. Balk). In this article, we will be elaborating on the first mechanism with recent results from our lab.

Androgen-independent AR activation by non-steroids

AR mediates androgen action by being a transcriptional factor that binds specific DNA sequences and recruits RNA polymerase II and a basal transcriptional complex for efficient transcription of cellular genes. The transcriptional activity of AR is mediated by coregulators (coactivators and corepressors) [6,7] which, in response to androgen's binding to AR and nuclear translocation, are assembled in a dynamic way at different response elements along the genome. The best recognized coactivators are the histone acetylases, such as p300/CBP [8,9] and the p160 SRC (steroid receptor coactivator) family [10-15]. These coactivators drive transcription by remodeling chromatin via histone acetylation and by recruiting RNA polymerase to the promoter, as we recently demonstrated [16]. The molecular basis for AR activation by androgen is a conformational change of AR induced by androgen binding, allowing the coactivators to associate. This process, however, is modulated—and in some cases, overridden—by phosphorylation, which has been postulated to be an underlying reason for AI activation of AR by non-steroidal agonists. Examples include interleukin-6 (IL-6) activation of AR via ERK phosphorylation of SRC-1 [17], EGF activation of AR via ERK phosphorylation of SRC-2 [18], IGF-1 activation of AR via AKT phosphorylation of AR [19], and neuropeptide activation of AR via the ERK pathway [20]. Indeed, *in vitro* phosphorylation and activation of AR by serine/threonine kinases ERK [21,22], AKT [23],

PKA [24,25] and PKC [26,27] have been reported. Other studies further implicate casein kinase 2 (CK2) in the androgen response and growth of PCA [28-30]. Additional posttranslational modifications of AR such as sumoylation, acetylation and protease cleavage, which affect AR activity have also been identified [31-33]. While serine/threoinine kinases are direct modulators of AR and its transcriptional machinery, they are not the immediate effectors of growth factors, cytokines, or chemokines. Indeed, we and others showed that IL-6, EGF and neuropeptide all activate tyrosine kinases in PCAs [20,34,35]. Our studies identified a tyrosine kinase complex involving Src/Etk/FAK to be a common target for all the above-described non-steroid ligands and for the case of neuropeptides, we present evidence that inhibitors of these kinases may have therapeutic value in the treatment of AI tumors.

Neuroendocrine differentiation and the development of hormone-refractory PCA

Our interest in neuropeptide and its possible role in HR PCA stems from the well documented observation that increased neuroendocrine cells accompany the development of HR PCA. We first demonstrated that IL-6, a progression factor of HR PCA, induced neuroendocine differentiation of LNCaP [35], which is now supported by several studies reported in the literature [36-39]. Others reported that androgen-deprivation and forskolin also induced neuroendocrine differentiation of LNCaP, indicating the propensity of this cell line to undergo neuroendocrine differentiation [40-42]. The connection of neuroendocrine differentiation to androgen withdrawal was subsequently confirmed in the in vivo xenograft systems [43,44], and had strong clinical implications. We found that the neuroendocrine cells have acquired strong apoptosis resistance, but are growth arrested; they themselves are thus not malignant, yet they are endowed with the potential to release cytokines, chemokines and growth factors, which fuel the surrounding undifferentiated prostate cancer cells to grow, migrate and survive, especially under the harsh conditions of androgen withdrawal). The “neurokines” released by these cells include gastrin-releasing peptide (GRP, the homolog of bombesin), neurotensin, PTHrP, IL-8, relaxin, VEGF, factors implicated in chemotaxis, survival, angiogenesis and bone metastasis [45]. Significantly, the work by Deeble et at [46] and by Jin et al [47] showed that neuroendocrine cells when coinjected with CaP xenograft in a paracinre fashion enhance tumorigenesis, in support of the above hypothesis.

Inappropriate activation of AR by neuropeptides

A major effort of our lab was directed toward studying of the effect of neuropeptide bomebsin (and its human homolog GRP) in the induction of androgen-resistance of prostate cancer cells. Our results [48] showed that GRP/bombesin induces LNCaP growth under androgen-free or low-androgen conditions. It activates Src/Etk/FAK tyrosine kinase complex, effectively translocates AR into the nucleus and activates AR transcription activity. Based on microarray analysis, bombesin was found to regulate a large number of androgen-response genes, including *KLK2-4*, *NKX3.1*, and *SARG*, confirming the ability of bombesin to activate AR. Indeed, close to 50% of the bombesin-regulated genes overlapped with androgen-response genes. At the same time, bombesin activated a unique set of genes, amongst which is *c-myc*, a target gene of Src kinase and about 20% of bombesin target genes bear *c-myc* target gene signature. C-myc is an oncogene strongly implicated in prostate carcinogenesis, as it is often amplified in advanced PCAs and is involved in androgen independent growth of PCAs. Transgenic animal bearing overexpressed c-myc develop prostate tumors [49-52]. These data suggest that Src activation may turn on both AR and c-myc pathways, resulting in more aggressive phenotypes.

To study the functional role of the Src complex in AR activation induced by bombesin, we used multiple Src inhibitors (PP2, SU6656 and AZD0530) as well as shRNA against Src. We

found PP2 effectively reduces Src activity, the growth, and AR transactivation of LNCaP induced by bombesin, but has little effect on androgen-activated AR [53]. Src inhibitor also suppresses the translocation of AR, indicating a possible role of Src phosphorylation to control the nuclear import of AR. In complete agreement with our finding, recent studies showed that AR can be directly phosphorylated by Src and that the tyrosine residue targeted by Src when mutated to phenylalanine affected the translocation of AR [54,55]. Using chromatin-immunoprecipitation (ChIP) assay, we found that the cofactor assembly of bombesin-activated AR is distinct from that of the androgen-bound AR. For instance, bombesin, but not DHT, induces the recruitment of c-myc to the PSA promoter. At the same time, androgen-bound AR assembles CBP and DAXX to the transcriptional complex, which is not shared by bombesin activated AR. Src kinase inhibitor effectively abolishes bombesin-mediated recruitment of coactivators to the AR complex; it has little effect on androgen-induced assembly. These data together suggest that Src kinase plays an obligatory role in neuropeptide-induced AR activation. We have extended this analysis to IL-8, another ligand for GPCR released by neuroendocrine cells [53]. The other interesting observation was that bombesin (and hence Src) activated AR has different target-specificity as compared to DHT. For instance, in the PSA promoter, there are two sets of AREs: AREIII (the distal enhancer, located around -6Kb) and AREI/II (the proximal enhancer, located at -0.1Kb). Src activated AR binds and transactivates only the proximal enhancer, whereas DHT-bound AR is recruited to and transactivates both sites. IL-8 and EGF activated ARs behave exactly like the bombesin activated one [53], consistent with Src being a common mediator of AR activation. These data, for the first time, suggest that Src or signal activated AR is conformationally different from DHT-activated one; targeting Src may provide added benefits to hormone therapy.

Inappropriate activation of AR by other ligands engaging GPCR

Bombesin engages G-protein coupled receptor and channels its signal through Src/Etk/FAK tyrosine kinase complex [20,48]. Since many of the neurokines released from neuroendocrine cells are ligands for GPCR, and GPCR activation is associated with HR PCA [56], we decided to study whether the above results can be extended to other GPCR ligands such as IL-8 [53] and Relaxin [57]. We found indeed both ligands are able to aberrantly activate AR and induce androgen-independent growth, via similar signal pathways (e.g., Src and beta-catenin). Thus the aberrant AR activation by deregulated GPCR/Src/beta-catenin/c-myc pathway may represent a general mechanism underlying the development of HR PCA, and inhibitors which block this common pathway may be considered as an adjunctive therapy to androgen ablation.

In Vivo Neuorpeptide Model

To explore Src as a target, we hypothesized that NE cells are androgen-independent and secrete neuropeptides that further support androgen sensitive cell proliferation in the absence of androgens. We developed an in vitro and in vivo model by stable overexpression of the GRP in LNCaP cells (LNCaP-GRP) through transfection and selection. LNCaP-GRP cells demonstrated androgen- and anchorage-independent growth and enhanced cell motility via Src activation. LNCaP-GRP cells developed orthotopic tumors in castrated nude and SCID mice and metastasized to regional lymph nodes in the SCID mice. The tumors expressed GRP, PSA and demonstrated nuclear translocation of the androgen receptor. These xenografts were re-cultured and provided paracrine growth support and migratory stimulation to wild-type LNCaP cells under androgen-deprived conditions in vitro and in vivo. This model is relevant to test targeted therapies against GRP, Src or related targets.

Clinical Implications

Using a novel and specific Src kinase oral inhibitor AZD0530, we demonstrated inhibition of growth and metastases in vivo. The relationship of Src to FAK appears very important in that there was 100% inhibition of lymph node metastases in AZD0530 treated mice. The inhibition of FAK was through its complex to Src. In clinical samples, Src hyperactivation correlates with aberrant androgen receptor activation of high-grade prostate cancer cells and also androgen-independent disease. Furthermore, Src plays a role in the environment of prostate cancer bone metastases as it is not only expressed by prostate cancer cells, but also by osteoclasts and is involved in the vicious cycle of bone degradation. These observations have resulted in AZD0530 being tested in patients with hormone refractory prostate cancer, and prostate and breast cancer patients with bone metastases. It is an example where mechanism of oncogenic activation of AR translates into model development and clinical application. Due to the increased expression of a variety of oncogenes by androgen deprivation, inhibitors to these targets may prove useful as adjunctive therapy to castration.

Summary and Discussion

In this article, we described the mechanism whereby neuropeptides such as bombesin or GRP activates androgen receptor in an androgen-independent fashion. Although our studies were carried out in charcoal-stripped conditions, our results are directly applicable to castration conditions where a low level of androgen is still present. We and others showed that the effects of neuropeptides and androgen on AR activation are synergistic. Perhaps the most provocative finding of this study is the demonstration that Src tyrosine kinase is involved. Src's effects on AR appears to be multiple: 1) it facilitates the translocation of AR, likely due to direct phosphorylation of AR at tyrosine residues in the cytosol, 2) it activates beta-catenin and c-myc, both co-activators of AR, which in turn augment the transcriptional function of AR, and 3) it activates other kinase pathway such as ERK and AKT leading to phosphorylation of co-activators of AR. In addition, Src's engagement with FAK and Etk, two kinases involved in invasion, migration and survival, promotes metastasis. The finding that Src/Etk/FAK complex is a common target for several neurokines released from neuroendocrine transdifferentiated PCA cells, suggest that they present potential targets for intervention. Our bias is that the functions of neurokines and hence activated Src kinase are to sustain the growth survival of PCAs during the androgen-ablation crisis, providing opportunities for these cells to divide and to further mutate into hormone-refractory state. Application of inhibitors of these kinases should be considered at early stage of or in conjunction with hormone therapy.

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