Acta Oncologica, 2005; 44: 299–303

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ORIGINAL ARTICLE

Alteration of gonadotropin-releasing hormone receptor expression with the progression of prostate cancer in the Dunning rat adenocarcinoma sublines

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Abstract

Inhibitory effects of GnRH analogues on tumour growth in vitro suggests that such direct effects may be of importance also in vivo. However, the role of GnRH receptors (GnRH-R) in prostate tumour progression is largely unknown. The aim was therefore to investigate the variation of GnRH-R expression with prostate tumour progression using Dunning rat adenocarcinoma sublines representing different prostate tumour grades. GnRH-R levels were quantified in the rat dorsolateral (DLP) and Dunning sublines (PAP, AT-1, AT-2, AT-3, MatLyLu) using competitive RT–PCR and Western blot. The results showed that all Dunning sublines had significantly elevated GnRH-R mRNA expression levels compared with DLP. Comparison of GnRH-R mRNA levels between different tumour grades revealed no difference in mRNA expression. However, the anaplastic and highly metastatic AT-3 and MatLyLu tumours displayed a tendency for lower GnRH-R mRNA values than the non-metastatic tumour sublines. Our data demonstrate the expression of GnRH-R in normal rat DLP and in different Dunning sublines. However, GnRH-R seems not to be involved in tumour progression.

Introduction

Gonadotropin-releasing hormone (GnRH) analogues are currently used to treat hormone-dependent prostate cancers (PCa), but the cellular targets and mechanisms affected by this treatment are partly unknown. Evidence is now accumulating that GnRH analogues may inhibit tumour growth, not only by suppressing the pituitary–testicular axis but also by acting directly at the level of the tumour [1,2]. Specific binding sites for GnRH (GnRH-R type I and II) have been characterized in a number of extrapituitary tissues including the prostate [3–5]. GnRH-like immunoreactive material has been isolated from the human PCa cell lines LNCaP and DU 145 and from specimens of human malignant prostate tumours [1,2,6]. Furthermore, it has been shown that LNCaP cells produces and secretes an enzyme that degrades GnRH [7]. Taken together these results imply a functional GnRH system in the prostate that might have auto- or paracrine effects on the regulation of prostate growth. However, the significance of the presence of such a local GnRH system is not known.

Prostate cancer is usually androgen dependent in its early stages but during tumour progression androgen dependence is generally lost [8,9] and the therapeutic options are now limited. The molecular and cellular mechanisms behind prostate tumour progression are largely unknown. The role of GnRH-Rs in tumour progression is unknown. It was, however, recently demonstrated that the concentration of GnRH-R was negatively correlated with Gleason score, i.e. higher Gleason sores correlated with lower amount of GnRH-R [10]. On the other hand, Straub et al. [11] demonstrated increased GnRH-R gene expression in hormone-refractory PCa compared with hormone-dependent PCa and benign prostatic hyperplasia (BPH).

In order to study GnRH-R variation with prostate tumour progression, different Dunning rat adenocarcinoma sublines representing different prostate tumour grades were analysed with a previously

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(Received 11 August 2004; accepted 1 December 2004)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.1080/02841860510007512

developed competitive reverse transcriptase–polymerase chain reaction (RT–PCR) and Western blot.

Material and methods

Dunning rat adenocarcinoma tissue

The tumour sublines were chosen to represent different prostate tumour grades, and included Dunning R3327 PAP; slow growing, androgen sensitive, well differentiated and non-metastasizing [12], AT-1 and AT-2; fast growing, androgen insensitive, anaplastic and with low to moderate metastatic capacity [13], AT-3 and MatLyLu; fast growing, androgen insensitive, anaplastic, and highly metastatic [13]. Since the tumours originated from a spontaneous tumour in the rat dorsolateral prostate (DLP) [14], the GnRH-R expression levels in the tumour sublines were compared with the expression level in the DLP. Pieces of tumour and DLP tissue were obtained from a previous animal experiment conducted by Häggström et al. [15]. Tumour and DLP material was stored at -70°C or fixed in formalin and embedded in paraffin.

Total RNA preparation and competitive RT–PCR

Total RNA was isolated using the TRIzol reagent (Life Technologies, Täby, Sweden) according to the manufacturer's instructions. RNA concentrations were quantified spectrophotometrically at 260 nm and RNA integrity was verified by ethidium bromide staining of 28 S and 18 S ribosomal RNA after agarose gel electrophoresis.

The mRNA levels for GnRH-R and the housekeeping gene cyclophilin (CF) were quantified by using a competitive RT–PCR procedure and PCR primers as described previously [16,17]. Truncated RNA of GnRH-R and CF were used as internal standards (IS) and competitively amplified with GnRH-R and CF in the RT–PCR reaction. Each RNA sample was titrated with three amounts (double samples) of IS, ranging from 1.43 amol–0.29 fmol for GnRH-R and 1.33 fmol–26.7 fmol for CF. After 35 rounds of amplification (95°C, 30 s; 57°C, 30 s; 72°C, 45 s) the resulting PCR products were analysed and mRNA levels were calculated from the linear regression by extrapolating at equivalent template to IS signals as previously described [16,17].

Protein extraction and Western blot

Total cellular proteins were extracted from snap frozen pieces of rat pituitaries and prostate tissue by homogenisation, using a Micro Dismembranator U (B.Braun Biotech International GmbH, Melsungen, Germany) at 2000 rpm for 45 s in lysis buffer containing 0.5% NP-40, 0.5% Na-Doc, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris, pH: 7.5; 1 mM EDTA, 1 mM NaF and Complete® protease inhibitor (Boehrinher Mannheim, Germany) according to the manufacturer's instructions. Samples were incubated on ice for 20 min and lysates were cleared by centrifugation at 13000 $\times$ g, at 4°, for 30 min. Protein content was measured in the supernatant using BCA protein assay (Pierce Chemical Co., IL, USA). For Western blotting, 15 μg of protein sample was mixed with electrophoresis sample buffer containing 2% SDS and 5% 2-mercaptoethanol and boiled for 10 min and separated under reducing conditions on 10% SDS-polyacrylamid gel. Fractionated proteins were electro blotted onto nitro-cellulose membrane (Hybond ECL, Amersham, Sweden AB), and membranes were stained with 1% Poncaue-red, 0.05% acetic acid, to ascertain equal loading of proteins. Filters were blocked in 5% dry milk in PBS, 0.1% Tween-20 (PBS-T) for 3 h before incubation with primary antibodies for 1 h at room temperature. GnRH-R was detected with a mouse monoclonal antibody that reacts with GnRH-R of mouse, rat and human origin (Ab-3, NeoMarkers, Union City, CA, USA), diluted 1:100, while actin was detected with a rabbit polyclonal antibody to actin (anti-actin, no. A2066, Sigma, Stockholm, Sweden), diluted 1:7000. After washing of the filters with PBS-T, membranes were incubated with peroxidase conjugated secondary antibodies for 1 h and proteins were then detected using Pierce superignal west dura kit (Boule Nordic AB, Huddinge, Sweden). Molecular size of the protein bands was determined by the parallel electrophoresis of molecular weight markers (Bio-Rad Laboratories AB, Sweden).

Statistical analysis

The statistical significance of differences between Dunning tumour sublines and DLP was compared using the Kruskal Wallis H test and if significance was reached, followed by the Mann–Whitney U test. A p-value less or equal to 0.05 was considered significant. Data are presented as mean $\pm$ SEM of 5–7 observations in each group.

Results

GnRH-R mRNA expression

In order to study GnRH-R variation with prostate tumour progression, different Dunning rat adenocarcinoma sublines representing different prostate tumour grades were analysed with a competitive RT–PCR reaction. By competitively amplifying a

truncated RNA standard with each sample the relative amount of GnRH-R and CF mRNA was estimated. The specificity of the RT–PCR reaction has previously been determined by sequencing of the observed products and comparison with the reported GnRH-R sequence. Negative control samples without the addition of any RNA did not give rise to any detectable products, ruling out cross-contamination between samples (results not shown).

The mRNA for the housekeeping gene CF was amplified in parallel with GnRH-R mRNA in order to correct the GnRH-R mRNA levels for RNA degradation. The mRNA levels for CF were found not to be constant between different tumour sublines and therefore no correction for CF was made in the analysis of GnRH-R mRNA in this study. Instead RNA concentration and integrity was further verified by ethidium bromide staining of 28 S and 18 S ribosomal RNA after agarose gel electrophoresis.

All Dunning tumour sublines showed significantly elevated GnRH-R mRNA expression levels compared to DLP ($p = 0.003$, Figure 1). Comparison of GnRH-R mRNA levels between different tumour grades revealed no major differences. A tendency to lower GnRH-R mRNA values was, however, observed for the anaplastic and highly metastatic AT 3 and MatLyLu tumours ($p = 0.125$, Correction of GnRH-R mRNA for CF mRNA gave principally the same results as the uncorrected data) (Table I).

GnRH-R protein expression

By using the Western blot technique, GnRH-R protein was detected in tissue extracts of normal DLP and the Dunning tumour sublines (Figure 2). The GnRH-R antibody recognized a protein with the approximate weight of 60 kDa in all prostate and

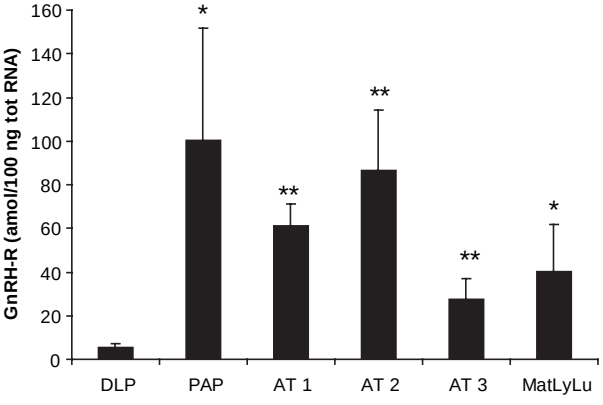

Figure 1. Relative GnRH-R mRNA levels in 100 ng total RNA from rat DLP and Dunning tumour sublines as quantified by competitive RT–PCR. Values are expressed as means ± SEM of 5–7 rats in each group;. *$p < 0.05$, when compared with the DLP group, **$p < 0.01$, when compared with the DLP group.

Table I. Summary of GnRH-R, CF and corrected GnRH-R mRNA data in 100 ng total RNA from rat Dunning sublines and DLP, as quantified by competitive RT–PCR.

Tissue	GnRH-R	CF	Corr CF ($\times 10^{-3}$)
DLP	5.27 ± 1.69	6110 ± 319	0.83 ± 0.25
PAP	100.46 ± 51.44	3359 ± 451	27.26 ± 9.98
AT 1	61.30 ± 9.70	12280 ± 2428	6.61 ± 2.19
AT 2	86.57 ± 27.47	8790 ± 2500	11.72 ± 2.57
AT 3	27.51 ± 9.68	8760 ± 995	3.26 ± 1.00
MatLyLu	40.16 ± 21.86	7773 ± 1022	5.97 ± 2.97
Pituitary[1]	725 ± 22	2500 ± 160	290 ± 8

[1]Levels of GnRH-R in pituitary tissue from Sprague Dawley rats, from another experiment, for comparison of GnRH-R mRNA levels with normal DLP and Dunning tumour sublines.

prostate tumour tissues. This molecular mass corresponds to that reported for the rat pituitary GnRH-R I [18]. A positive signal was detected in pituitary tissue, suggesting specific detection of the expected 60–64 kDa GnRH-R protein.

Discussion

Therapy with GnRH agonists is currently a standard treatment for men with advanced PCa [19,20]. The clinical application of GnRH analogues in the treatment of hormone-dependent malignancies is primarily based on their ability to inhibit the hypophyseal–gonadal axis through the desensitization of gonadotropes and hence the suppression of circulating levels of sex steroids [21,22]. In addition, specific membrane receptors for GnRH, at least type I, have been found in a high percentage of human prostate tumours and various rat and human PC cell lines [1,2,4,10,16,17]. These receptors mediate inhibiting effects of GnRH analogues in vitro suggesting that direct inhibitory effects on the tumour may be of importance also in vivo. The mechanism behind this effect is unknown, but it has been speculated that the anti-proliferative signal is mediated by a pertussis toxin-sensitive G-protein α_I coupled to phosphotyrosine phosphatase, which counteracts the growth factor induced tyrosine kinase activity [23,24]. GnRH analogues also reduce the expression of locally produced growth factors, i.e. epidermal growth factor (EGF) and insulin-like growth factor (IGF) I and II [25,26]. We have shown that prostate cancer patients treated with a GnRH agonist for 3 months were positive for immunoreactive GnRH-R in the prostate, suggesting that local effects of GnRH may also be of importance for long-term local tumour responses and may offer a target for new therapeutic strategies [27].

As androgen ablation therapies only provide a remission of limited duration and most patients with

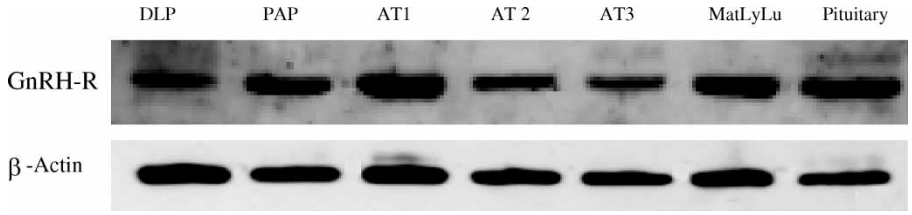

Figure 2. Western blotting of GnRH-R in rat DLP and Dunning tumours; PAP, AT-1, AT-2, AT-3, MatLyLu and rat pituitary by 10% SDS-PAGE under reducing conditions.

metastatic PCa eventually die from androgen-independent PCa it is of interest to examine factors related to tumour progression. In this study, the expression of GnRH-R in different Dunning rat adenocarcinoma sublines representing different prostate tumour grades was investigated, in order to evaluate alterations in the local GnRH system during tumour progression. GnRH-R expression was found to be elevated in all Dunning tumour sublines investigated, when compared with normal rat prostatic tissue. These results are in line with our previous observation that the GnRH-R mRNA levels are increased in tumour vs. benign prostate tissue [19]. However, comparison of GnRH-R mRNA levels between different tumour grades, previously in man now in the rat, revealed no difference in mRNA expression, suggesting that the GnRH-R is not involved in tumour progression. Some studies have suggested that the expression of GnRH-R is lost or down-regulated in prostatic tumours during the progression to a hormone-independent phenotype [13] while others have demonstrated an increased incidence of GnRH-R gene expression in hormone-refractory PCa compared with hormone-dependent PCa and benign prostatic hyperplasia (BPH) [14]. The reason for these discrepancies is not evident.

In conclusion, our data demonstrate the expression of GnRH-R in normal rat DLP and in different Dunning rat adenocarcinoma sublines, including the Dunning R3327 PAP; slow growing, androgen sensitive, well differentiated, and non-metastatic, the AT-1 and AT-2; fast growing, androgen insensitive, anaplastic, and with low to moderate metastatic capacity, and the AT-3 and MatLyLu; fast growing, androgen insensitive, anaplastic, and highly metastatic prostate tumours. GnRH-R does not seem to be involved in tumour progression since there was no significant correlation between the expression of GnRH-R mRNA and tumour grade but GnRH-R may on the other hand serve as a therapeutic target in all types of prostate tumours.

Acknowledgements

This work was supported by grants from the Swedish Cancer Society (project no. 1760), the Maud and Birger Gustavsson Foundation, the Nilssons Foundation and the Lions Research Foundations, Umeå University, Sweden.

The authors wish to thank Ms Åsa Skytt for skilfully contributing to this paper through her technical assistance.

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