Immunohistochemical detection of retinoic acid receptor-α in prostate carcinoma: Correlation with proliferative activity and tumor grade

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Abstract. Background: The use of retinoids as differentiation therapy is a novel approach to prostate cancer. Retinoids act via their own nuclear receptors, RARs and RXRs, modulating gene activity, cell growth and differentiation. This study provides new data on the content and cellular distribution of RARα protein in prostate carcinoma specimens, in correlation to tumor grade and proliferative activity. Material and methods: A total of 84 cases of primary prostate carcinoma, divided into 3 subgroups according to tumor grade, were immunohistochemically evaluated for retinoic acid receptor-α (RARα) and Ki67 using the streptavidin/peroxidase method on formalin fixed, paraffin embedded tissues. Results: RARα positivity was detected in all cases of prostatic carcinoma, with a more profound expression in well differentiated cancers. A statistically significant correlation between RARα staining and tumor grade was found (ANOVA, p < 0.031). Ki67 immunoreactivity was present in 35.7% of cases, but no correlation with tumor grade was found. When RARα staining was correlated with Ki67 positivity, a statistically significant correlation was present (unpaired t-test, p < 0.003). Conclusions: These findings indicate that RARα expression is correlated to some extent with tumor grade and its presence is more profound in highly proliferative tumors. Further studies are needed to establish the possible clinical value of the immunohistochemical evaluation of RARα content in tumor specimens.

Key words: immunohistochemistry, Ki67, prostate carcinoma, retinoic acid receptor-α

Introduction

Prostate cancer is the second leading cause of death and now the most common cancer among men in Europe and the United States [1, 2]. Its incidence has risen dramatically over the last decade. Unfortunately, despite the use of the most efficient diagnostic procedures available, prostate cancer has already migrated outside the prostate at the time of the first diagnosis in approximately 50% of cases [3]. Androgen ablation therapy remains the mainstay of endocrine manipulation of advanced stage disease but the observed unresponsiveness in the 25% of cases and the time-limited effectiveness of androgen blockade clearly underline the need for alternative treatments.

The evolving role of retinoids in cancer treatment and prevention is being extensively studied in many malignancies, prostate cancer included. Retinoids are metabolites of Vitamin A and are considered to be important signaling molecules in the modulation of growth and differentiation of normal and neoplastic cells [4]. Retinoids can prevent the formation of chemically induced cancers in experimental animals and inhibit proliferation of a large variety of normal and neoplastic cells in vitro. Their effects are known to be mediated by the nuclear receptors for all-trans (RARs) and 9-cis-retinoic acid (RXRs). These receptors are members of the steroid/thyroid receptor superfamily and act like transcription factors, binding as dimers to hormone response elements in the promoter regions of target genes to enhance or repress transcription. Different response elements and possible interactions between RARs and RXRs greatly increase the number of RA-dependent responses, leading to a molecular interpretation of the multiple actions of retinoids. The RAR content of certain tumors seems to be important for potential therapeutic employment of retinoids [5, 6]. On the other hand, proliferative activity is an
important, independent prognostic factor of the malignant potential of many tumors [7–9]. Assessment of proliferative activity is usually performed with Ki67 immunostaining, which is most commonly detected by use of the MIBI antibody. Ki67 is a 395-kDa antigen encoded by a single gene on chromosome 10 whose expression is tightly associated with the entry of cells into the cell cycle.

The aim of this study was to confirm the presence of RARα receptors in prostatic carcinoma by using immunohistochemical methods on formalin-fixed, paraffin-embedded tissue and to examine any possible correlation of RARα content with tumor grade. Moreover, we examined the relationship of RARα expression with the proliferative activity of the tumors.

**Materials and methods**

**Tissue samples.** A total of 84 cases of primary prostate carcinoma from men between 52 and 85 years old (mean 71 years) were included in the study. Human prostatic tissue was obtained from transurethral resections and radical prostatectomies performed in our department from 1991–1997. Tumors were graded according to the Gleason grading system [10] and further divided into 3 subgroups (Grade I: Gleason score 2–4, Grade II; Gleason score 5–7, Grade III; Gleason score 8–10). The distribution of cases in each group was as follows: Grade I = 24, Grade II = 41, Grade III = 19. Tissues were routinely fixed in formalin and embedded in paraffin blocks. At the review of the hematoxylin and eosin stained sections, paraffin blocks and serial sections were cut at a thickness of 4 μm. The prepared slides were used within 2 days from preparation, in order to avoid the recently reported phenomenon of loss of expression of steroid receptors in immunohistochemistry, due to time-related degradation [11, 12].

**Immunohistochemistry.** The commercially available antibodies for RARα (C20, Santa Cruz Biotechnology Inc., Santa Cruz CA) and Ki67 (Dako A/S, Denmark) were used. The immunostaining was performed according to the Streptavidin-Biotin Complex Peroxidase method using the commercially available KWIK™ kit (IMMUNON™, Pittsburgh, PA). Specimens of RARα positive breast carcinoma and Ki67 positive prostate cancer were used as positive controls. Primary antibody was replaced with non-immune sera for negative controls. After deparaffinisation and rehydration through a series of xylene and alcohol, sections were treated with the Microwave antigen unmasking buffer technique [13]. Slides were immersed in a jar with sodium citrate buffer pH: 6 and heated 3 times for 5 min in a microwave oven at 700 W. After each 5 min interval the buffer was inspected for vaporization loss and the oven was reactivated for 5 min. Once the 3 cycles were completed, the slides were allowed to cool down at room temperature for 20 min and then rinsed in TBS pH: 7.6 for 10 min. Incubation with IMMUNON™ Protein blocking agent was then performed for 20 min. Sections were then incubated with primary antibody. Ki67 antibody was used in 1:80 dilution and left overnight in moist chambers at room temperature while RARα was used in 1:250 dilution and incubated for 2 hours at 25 °C. Slides were then washed in TBS for 3 × 5 min, first washing in TBS+TWEEN 0,1% and the Kwik™ Biotinylated Universal Secondary AB Reagent was applied for 20 min. Slides were then washed again in the previously described manner and Kwik™ Streptavidin Peroxidase Reagent was applied for 20 min. Further washing with TBS was followed by incubating the sections with Chromogen-Substate Solution (DAB) for 5 min. The slides were then rinsed in running tap water for 5 min and counterstained with Mayer hematoxylin for 1 min. After dehydration slides were cleared with xylene and mounted with Entellan (Merck, Darmstadt, Germany).

**Analysis of staining.** Morphological assessment of immunostained tissue and manual counting of immunolabeled tumor cells were performed with the aid of a graticule. Glandular epithelium was assessed in 10 representative high power fields (40×) per case and stained nuclei were expressed in percentage. Only tumor cells with unequivocal nuclear staining were counted as positive.

**Statistical methods.** Overall variations in the percentages of nuclear staining for RARα and Ki67 and their relationship with tumor grade were evaluated by analysis of variance (ANOVA) and Tukey’s Multiple Comparison Test was used for comparisons between grade subgroups. Relationships between the percentages of RARα labeled cells and Ki67 positivity were evaluated by Student t-test. A p value less than 0.05 was considered to be significant.
Results

Immunohistochemical staining with the anti-RARα antibody revealed the presence of RARα in all prostate carcinoma specimens, although the degree and the cellular distribution of staining varied among tissue specimens. Overall, RARα staining was detected in the nuclei of prostatic epithelial cells (Figures 1, 2). Occasionally, cytoplasmic perinuclear staining with a microgranular appearance was present. When the immunohistochemical expression was analyzed for each grade group by analysis of variance, a statistically significant difference was present ($p < 0.0031$, mean values 42.21, 25.26 and 30.01 for Grade I, II and III respectively). A somewhat unexpected increase of RARα expression was observed in the poorly differentiated (Grade III) tumors and, interestingly, Tukey’s multiple comparison test revealed no statistically significant difference between Grade II and Grade III. Mononuclear inflammatory infiltrate was largely RARα positive. Spindle cells, mainly fibroblasts, within the tumor stroma were occasionally positive.

Ki67 reactivity was strong, localized in the nuclei of tumor cells but limited at 30 of 84 cases (35.7%). Immunoreactive nuclei were randomly scattered across the tumor areas (Figure 3). The positivity range for Ki67 varied from 0 to 50%. Mean values in each grade group were 8.4, 11.2 and 8.9 for group Grade I, II and III respectively. Analysis of variance between grade groups did not reveal any statistically significant difference ($p > 0.05$).

When RARα expression was correlated to Ki67 positivity, a statistically significant difference was found in the RARα content between Ki67 positive and Ki67 negative tumors (unpaired $t$-test, $p < 0.003$, mean values 52.50 and 32.87 respectively).

Discussion

We investigated the cellular expression of RARα in prostate carcinoma specimens of several degrees of differentiation by immunohistochemical staining of formalin-fixed, paraffin-embedded tissue using the streptavidin-biotin-peroxidase method. This technique provided the possibility to exactly localize the in vivo expression of RARα protein. Similarly, determination of Ki67 expression of the examined tissues was performed, providing results that were used to correlate the expression of these two markers with each other as well as with tumor grade.

RARα expression was present in all tissues examined but a variable degree of staining was noted. A modest inverse relationship between the percentage of RARα positive tumor cells and histological grade was found, especially for Grade I and Grade II tumors, suggesting a differentiation-dependent trend. The unexpected moderate increase in RARα content in Grade III tumors is also noteworthy. A possible explanation to this phenomenon could be that the combination of factors regulating RARα gene transcription is altered in poorly differentiated tumors. Other hypotheses proposed include: (1) loss of mechanisms of RARα down regulation in these tumors, and (2) in situ retinoid levels that are possibly insufficient to achieve down regulation, thus leading to overexpression of RARα [5]. Further investigation is needed in order to elucidate the role of such factors in RARα gene transcription at the promoter level.

The use of immunohistochemistry in the in situ detection of RARs and RXRs in several tissues is not yet widespread, due to several reasons. The commercially available antibodies towards specific subtypes of the retinoid acid receptors are quite limited. Their performance on chemically fixed tissues is also not well defined [6]. However, staining attempts in several tissues, prostate included, have given satisfactory results [5]. Although performance of immunohistochemical studies on formalin-fixed, paraffin-embedded tissue has been previously criticized, the use of the microwave antigen unmasking technique seems valuable and attains high quality staining results.

Ki67 has been extensively used as an indicator of the proliferative state in cells, since it is expressed only in cycling cells and bears a short half-life. The estimation of the proliferative activity has prognostic implications in many neoplasias [14–16]. Although studies are limited, the expression of Ki67 in prostate carcinoma is considered an independent prognostic factor [17, 18]. Recent advances in antigen retrieval techniques allow for measurements of Ki67 in formalin fixed, paraffin embedded tissues.

In the present study Ki67 immunoreactivity was detected in 35.7% of cases. The pattern of immunoreactivity and the number of immunoreactive tumor cells correlate well with the findings in other studies. The limited proportion of tumor cells that exhibit positive staining for Ki67 indicates that proliferative activity is low in prostate cancer. Still, controversy exists on
Figure 1. RARα immunostaining in prostate carcinoma grade III. Counterstaining by hematoxylin (×20).

Figure 2. RARα expression in prostate carcinoma grade II (×40).
whether Ki67 expression is related to tumor grade. Although Ki67 index and tumor grade correlation has been reported, other studies suggest that no correlation exists between proliferation index and Gleason score [19, 20]. In our study analysis of variance failed to yield any statistically significant results between different grade groups.

Although the gold standard in measuring proliferation rate in tissues remains incorporation of the thymidine analogue bromodeoxyuridine and subsequent immunohistochemical quantification of labeled cells [16], Ki67 immunoreactivity may well be one of the simple and reliable markers associated with proliferation at hand to date. Correlation of RARα receptors and Ki67 in prostate cancer is extremely limited in the literature. Still, there is good evidence to suggest that prostate cancer behaves similarly to other endocrine-related cancers, like breast and ovarian tumors. It is possible that prostatic cells exploit similar signaling pathways for growth and differentiation with the above mentioned steroid hormone-dependent neoplasms. It has been reported that in breast carcinoma RARα expression is directly correlated with Ki67 positivity [5]. In our study, although no direct correlation between RARα levels and Ki67 immunopositivity was present (linear regression analysis, \( p > 0.05 \)), when RARα expression was compared between Ki67 positive and negative specimens, a statistically significant difference was found between the two groups. Similar findings have been reported for lung cancer, for which stronger staining for RARα also occurred in lung carcinomas with high proliferative activity [21]. Previous studies have suggested the important role of RARα in growth inhibition of cancer cell [22, 23]. The apparent uncoupling of RARα expression and proliferation inhibition could be explained by altered transcriptional regulation during malignant progression [5]. It is possible that the composition of factors regulating RARα gene transcription from both promoters (RARα1 and RARα2) is different in highly proliferative tumors from tumors with low proliferative activity. Another possible explanation for the high levels of RARα in tumors with great proliferative activity is that mechanisms to down-regulate RARα are lost in these tumors [22].

Due to the time-limited value of androgen ablation in the treatment of advanced prostate cancer it is becoming increasingly apparent that the future lies
in the development of other, alternative treatments. The remarkable potential of retinoids to be active at most stages of the carcinogenic process as well as their unique action as differentiation inducers in established neoplasms has led to an initial enthusiasm for their potential role in the treatment or even prevention of prostate cancer. Still, past and current attempts of RA-based treatments of patients with prostate cancer have yielded confusing results [24]. The in vitro use of Vitamin D3 analogs and synthetic retinoids (e.g. CD271 and CD 437) on human prostatic carcinoma cell lines has given promising results, showing cell growth inhibition, increased cell differentiation and induction of apoptosis [25–27]. Similar results were obtained with retinamide, a 4HPR synthetic retinoid that modulates the expression levels of some apoptosis-related genes [28, 29]. However, despite preclinical rationale, retinoids have failed to prove efficient in the clinical management of prostate cancer [24]. Early clinical experience with Liarozole, a drug increasing the cellular levels of RA, indicated only modest response in patients with progressive prostate cancer [30]. Real progress in the design of RA-based treatment will depend on further studies that will elucidate their action mechanisms and improve their clinical usefulness.

This study demonstrates the ability to immunohistochemically evaluate the retinoic acid receptor-α content in formalin-fixed, paraffin embedded prostatic carcinoma tissue, along with the proliferative activity status, estimated through Ki67 immunohistochemical evaluation. A relationship between the RARα content and the highly proliferative tumors was present. Further studies with other RAR or RXR subtypes may help to the thorough understanding of the role of retinoic acid receptors in the prevention and treatment of prostate cancer. At present, it may be useful to immunohistochemically evaluate the RARα content of prostate cancer specimens in order to define the subset of patients that will probably benefit from RA-based treatment.

References


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