RAR-β₁ OVEREXPRESSION IN CHROMOPHOBE RENAL CELLCARCINOMA: A NOVEL TARGET FOR THERAPEUTIC INTERVENTION?

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Aim: Retinoic acid (RA) has proven to possess modest but distinct activity in metastatic renal cell carcinoma (RCC), at least in a subgroup of patients. However, the exact molecular mechanisms leading to success or failure of RA application in individual patients are still unknown. As earlier studies have indicated that in RCC the RA receptor (RAR) beta might play a central role in RA signaling, we investigated the expression of the isoforms RAR-β₁+₂, in primary conventional and chromophobe RCC. Methods: We used quantitative RT-PCR methodology to study RAR-β₁ and RAR-β₂ expression in ten primary conventional RCC samples (clear cell type), in two chromophobe RCC specimens, and the respective corresponding normal kidney tissues. The housekeeping genes RPS9 and RPLP0 were applied to normalize differences in mRNA quality and quantity. Results: In contrast to conventional RCC samples, RAR-β₁ was significantly overexpressed in both chromophobe tumors compared to the adjacent normal kidney tissue (p = 0.03). On the contrary, RAR-β₂ expression did neither differ significantly between conventional and chromophobe RCC (p = 0.91) nor between malignant and normal kidney tissue (p ≥ 0.47). Conclusion: We demonstrate for the first time a significant and specific overexpression of RAR-β₁ in chromophobe RCC. In future we will have to confirm this result within a larger number of samples.

Key Words: retinoids, retinoid receptors, RAR, kidney cancer.

Metastatic renal cell carcinoma (RCC) remains one of the most treatment-resistant human malignancies; long-term survival is limited to a minority of patients. Being inspired by the successful application of retinoids in the treatment of various hematologic and solid malignancies as well as encouraging preclinical data, several groups have included vitamin A derivates and their active metabolites in the treatment protocols of metastatic renal cancer. But even though early results had been promising, until today, the addition of retinoids to established therapeutic cytokine based regimens has failed to improve response or survival in the majority of patients with metastatic RCC [1–4].

In general, the antiproliferative and differentiative effects of retinoids are mainly mediated through cellular retinoic acid binding proteins (CRABP-I and -II) and specific nuclear retinoid receptors. The CRABP have been proposed to regulate the intracellular concentration of free retinoic acid (RA) [5] and act as intracellular transporters for RA [6]. The nuclear retinoid receptors can be subdivided into two major classes, the retinoic acid receptors (RAR) and retinoid X receptors (RXR) [7]. These receptors are ligand-activated transcription factors acting by binding to retinoic acid response elements (RARE) located in the promoter regions of RA-target genes [8].

The RAR-β₁ gene generates at least four mRNA isoforms using two different promoters (P1 and P2) and as a consequence of alternative splicing [9, 10]. There is strong evidence that RAR-β₁ plays a central role in growth regulation and tumor genesis. Its gene maps on the short arm of chromosome 3, a region which is frequently deleted in cancer [11]. Recent studies have demonstrated that in cell lines derived from various malignant tumors the level of RAR-β₁ mRNA is decreased or undetectable [12–16] indicating that the loss of RAR-β₁ expression may be an important event in tumor genesis of certain cell types. The hypothesis was supported by an observation that RAR-β₁ can function as a tumor suppressor gene in lung, breast, and ovarian cancer cell lines [11, 15, 17]. Moreover, RAR-β₁ is poorly expressed in a number of malignant solid tumors including lung [12, 18, 19], colon [20], head and neck [21, 22], breast [15], cervix [23], ovary [16], and prostate cancer [13].

Motzer, Hoffman et al. [24, 25] reported that in contrast to the majority of solid tumors, in renal cell carcinoma the expression of the RAR-β₁ isoform might play a crucial role for RA-depended growth control.

To expand our knowledge about RA-signaling and the heterogeneous efficacy of RA in the treatment of advanced RCC, we focused our investigations on the expression of the most important isoforms of the RAR-β₁ receptor (RAR-β₁ and -β₂) in primary renal cell carcinoma and adjacent non-malignant kidney tissue.

MATERIALS AND METHODS

Patients. Written informed consent was obtained from all patients before entry into the study. The study was approved by the ethics committee of the University of Marburg. RCC and adjacent normal kidney tissue samples were collected from 12 patients immediately after radical tumor nephrectomy. 10 patients (N 1–10) presented with conventional RCC (clear cell type), two patients (N 11 and 12) suffered from chromophobe RCC; all patients were treated at the Philipps-University in Marburg between April and December 2003. Patient characteristics are summarized in the Table.
RT-PCR. Both kidney cancer and adjacent non-malignant kidney tissue samples were stored in RNAlater (Qiagen, Germany) right after nephrectomy. RNA was extracted using peqGold TriFast FL reagent (Peqlab, Germany) according to the manufacturer’s instructions. Subsequently, a DNA-digestion step using DNase was added (Ambion, USA). In the following, total RNA was transcribed with M-MLV reverse transcriptase (RT) (Gibco, Scotland) and oligo-dT primers (Gibco, Scotland) for 45 min at 42 °C. RT was stopped by heat-inactivation for 5 min at 90 °C and the addition of 40 μl Tris-EDTA(TE)-buffer (pH 8). The following primers (MWG-Biotech, Germany) were used for the subsequent PCR: RAR-β1 (sense: 5’ CAT TTG CCT CCC TCA CT TGG TTT 3’; antisense: 5’ ACG GGT AGG GTG TGG CTG GTG CAT AGT 3’; 162 bp), RAR-β2 (sense: 5’ TGG CAG CAT CGC CAG ACT 3’; antisense: 5’ TGG CAG ACG AAG CAG GTT TG 3’; 122 bp). mRNA integrity and reverse transcription were checked with primers for the human ribosomal protein S9 (RPS9)-gene (sense: 5’ CGG AGG AGC AGA CGG TGG AAG C 3’; antisense: 5’ CGA AGG GTC TCC GCG GGG TCA CAT 3’; 92 bp), and the human ribosomal protein (large) P0 (RPLP0)-gene (sense: 5’ GCT GCT GCC CGT GCT GGT G 3’; antisense: 5’ TGG TGG GCC TGG AGA TTT TAG TGG 3’; 130 bp), which are common “housekeeping genes” [26]. We used two independent housekeeping genes to minimize the risk that observed differences in gene expression are due to the regulation of a specific housekeeping gene itself. PCR was performed with AmpliTaq Gold polymerase (Applied Biosystems, USA) following the manufacturer’s instruction. Cycling conditions: 10 min at 95 °C, 35 cycles of 20 s at 94 °C, 30 s at 58 °C, 45 s at 72 °C and, finally, 10 min at 72 °C. PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Specificity of the amplified products was established by restriction enzyme digestion.

To precisely quantify RAR-β1 and RAR-β2 expression, we applied a real-time RT-PCR assay. The distribution of tumor/kidney ratios for RAR-β1 and RAR-β2 gene expression was calculated by real-time RT-PCR and corrected for RPS9 and RPLP0 differences ([RAR-β1/RPS9]normal kidney/[RAR-β1/RPS9]tumor) and [RAR-β2/RPLP0]normal kidney/[RAR-β2/RPLP0]tumor, respectively. In contrast to the ten conventional RCC samples tested, RAR-β1 was significantly overexpressed in both chromophobe tumors (T11+T12) compared to the adjacent normal kidney tissue. The median RAR-β1 tumor/kidney (T/N) ratio for patients with conventional RCC (N 1–10) and those with chromophobe RCC (N 11+12) were 1.1 and 36.87 using RPS9, and 0.84 and 31.26 using RPLP0, respectively (p = 0.03; Fig. 2). Only one conventional RCC sample (T10) also expressed considerably elevated amounts of RAR-β1 mRNA compared to its adjacent normal kidney tissue (T/N ratio, 28.43 and 23.57, corrected for RPS9 and RPLP0, respectively). Nevertheless, the RAR-β1 mRNA expression of differences in tumor:normal kidney mRNA expression ratios between conventional and chromophobe RCC were evaluated with the Mann-Whitney-u-Test.

RESULTS
RAR-β1, RAR-β2, RPS9 and RPLP0 mRNA expression were determined by conventional semi-quantitative RT-PCR in tumor and corresponding normal kidney tissue from 12 patients with sporadic RCC. The latter two genes were used as independent “housekeeping genes”. We were able to reveal that RAR-β1 was highly expressed in those tumor samples that had been identified as chromophobe RCC (T11+T12; Fig. 1). In contrast, out of the ten conventional RCC samples tested, only one tumor (T10) expressed high levels of RAR-β1. Unlike RAR-β1, RAR-β2 did not appear to be differently expressed between malignant and corresponding normal kidney tissue, independent of the histological tumor type (data not shown). However, both RAR-β1 and RAR-β2 expression levels seemed to vary among individual patients suffering from conventional RCC (see Fig. 1).

Table. Patients’ characteristics

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex / Age</th>
<th>Tumor classification</th>
<th>Tumor characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F / 64</td>
<td>conventional</td>
<td>pT3a, pNx, GII</td>
</tr>
<tr>
<td>2</td>
<td>M / 71</td>
<td>conventional</td>
<td>pT3a, pNx, GII</td>
</tr>
<tr>
<td>3</td>
<td>M / 63</td>
<td>conventional</td>
<td>pT2, pN0, GII</td>
</tr>
<tr>
<td>4</td>
<td>M / 66</td>
<td>conventional</td>
<td>pT1b, pNx, GII</td>
</tr>
<tr>
<td>5</td>
<td>F / 70</td>
<td>conventional</td>
<td>pT1a, pN0, GII</td>
</tr>
<tr>
<td>6</td>
<td>M / 61</td>
<td>conventional</td>
<td>pT1a, pNx, GII</td>
</tr>
<tr>
<td>7</td>
<td>M / 66</td>
<td>conventional</td>
<td>pT2, pNx, GII</td>
</tr>
<tr>
<td>8</td>
<td>M / 50</td>
<td>conventional</td>
<td>pT1b, pNx, GII</td>
</tr>
<tr>
<td>9</td>
<td>M / 73</td>
<td>conventional</td>
<td>pT3b, pN2, GII</td>
</tr>
<tr>
<td>10</td>
<td>F / 75</td>
<td>conventional</td>
<td>pT3a, pN0, GII</td>
</tr>
<tr>
<td>11</td>
<td>M / 66</td>
<td>chromophobe</td>
<td>pT1a, pNx, GII</td>
</tr>
<tr>
<td>12</td>
<td>M / 69</td>
<td>chromophobe</td>
<td>pT1b, pNx, GII</td>
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M — male; F — female. Tumor classification according to the Heidelberg Classification [31].
in this tumor sample (T10) was still 15 (RPS9 corrected) and 17 (RPLP0 corrected) times lower compared with the median RAR-β expression of the chromophobe tumor samples (T11–T12), confirming the results obtained by conventional RT-PCR (see Fig. 1, 2). Even though there were no significant differences of RAR-β expression between tumor and normal kidney samples among the ten patients with conventional RCC (N 1–10; \( p = 0.48 \) and \( p = 0.89 \) using RPS9 and RPLP0, respectively), we could disclose distinct inter-individual differences (see Fig. 1, 2).

![Graph 1](image1)

**Fig. 2.** RAR-β expression in primary RCC using real-time RT-PCR. The RAR-β mRNA expression in tumor (T) and corresponding normal non-malignant tissue (N) samples from 12 patients with RCC is demonstrated as a ratio corrected for that of RPS9 and RPLP0 expression. The median RAR-β, tumor/kidney (T/N) ratio for patients with conventional RCC (N 1–10) and those with chromophobe RCC (N 11–12) were 1.1 and 36.87 using RPS9, and 0.84 and 31.26 using RPLP0, respectively, displaying a significant overexpression of RAR-β, in chromophobe RCC compared to conventional RCC and normal kidney tissue (\( p = 0.03 \)).

On the contrary, RAR-β expression did not differ significantly between conventional and chromophobe RCC (median T/N ration, 1.05 and 2.33, and 0.75 and 2.39 applying RPS9 and RPLP0, respectively; \( p = 0.91 \)) nor between malignant and normal kidney tissue (\( p = 0.47 \) (RPS9 corrected), \( p = 0.85 \) (RPLP0 corrected)).

**DISCUSSION**

Three highly interesting randomized phase III trials which included the combination of RA and cytokines in patients with advanced RCC have recently been published. Motzer et al. [2] randomized 284 patients to treatment with IFN-α plus 13-cis RA or treatment with IFN-α alone. Even though there was no difference in median overall survival between either group, duration of response and progression-free survival at two years were significantly longer in patients who received combination therapy. Aass et al. [3] randomly assigned 320 patients with progressive metastatic RCC, who had all previously undergone radical nephrectomy, to treatment with IFN-α plus 13-cis RA or IFN-α alone. They could show that progression-free and overall survival for patients treated with the combination were significantly longer compared with patients on IFN-α alone.

Taken together, RA seems to have modest but certain activity in RCC, at least in a subgroup of patients. However, which molecular mechanisms are crucial for success or failure of RA application in patients with metastatic RCC? Motzer et al. were the first to indicate that the expression of the RA-receptor (RAR-β) might play an important role for the RA-response in RCC cells [24]. Using Northern blot analysis, only one out of twelve RCC cell lines tested (SK-RC-06) expressed RAR-β and showed a > 90% growth inhibition after RA treatment. Hoffman et al. [25] from the same team applied an isoform specific RT-PCR and revealed that low levels of RAR-β were expressed in all twelve RCC cell lines. However, the isoform RAR-β, was detected in SK-RC-06 cells, only. Thus they concluded that RAR-β, was primarily responsible for RA signaling in RCC. Finally, Berg and co-workers reported that up-regulation of RAR-β mRNA expression in tumor tissue of patients with metastatic RCC following treatment with 13-cis RA and IFN-α significantly correlated with major response to this therapeutic regimen [28].

Less unambiguous results were gained by a European group around van der Leede and co-workers [29]. They examined the expression of RAR-β in 12 primary kidney tumors and matched normal controls as well as 11 RCC cell lines. Only three cell lines (DUE RC-3, SK-RC-48, SK-RC-56) expressed at least low levels of RAR-β mRNA but were RA-insensitive. On the contrary, two other RCC cell lines (SK-RC-58, SK-RC-59) were sensitive to RA even though they did not express RAR-β at all. Concerning primary RCC tissue, RAR-β mRNA levels were found to be reduced in 5/12 and increased in 1/12 kidney tumors as compared to the expression levels in normal kidney tissue. Finally, Gerharz et al. [30] were not able to demonstrate a correlation between the RAR status and RA response in any of seven RCC cell lines tested.

In contrast to van der Leede et al. [29], we evaluated the expression of the two most important isoforms of the RAR-β receptor (RAR-β, RAR-β, and RAR-β) in primary renal cell carcinoma and adjacent non-malignant kidney tissue. Applying real-time RT-PCR we were able to show that neither RAR-β, nor RAR-β, were differ-
ently expressed in primary conventional RCC tissue compared with the adjacent normal kidney tissue. In contrast, RAR-β was significantly overexpressed in chromophobe RCC. The median tumor/ kidney ratio for patients who suffered from chromophobe RCC was 34 and 37 fold higher than for patients with conventional RCC using RPS9 and RPLP0 to correct for differences in mRNA quantity and quality, respectively. Currently, we are expanding our tissue collection to confirm these results within a greater number of samples and to analyze RAR-β isoform expression in patients with papillary and collecting duct tumors. In addition, we would like to ask the authors of the cited phase III trials to take a look at their databases and check whether patients with metastatic chromophobe RCC were included and responded better to RA treatment compared to those who suffered from conventional RCC. Provided that they did, the specific application of RA could be an interesting therapeutic option for patients with metastatic chromophobe RCC.

REFERENCES
25. Hoffman AD, Engelstein D, Bogenrieder T, Papan- drou CN, Steckelman E, Dave A, Motzer RJ, Dmitrovsky E, Albino AP, Nanas D. Expression of retinoic acid receptor beta in human renal cell carcinomas correlates with sensitiv-
ГИПЕРЭКСПРЕССИЯ RAR-β₁ ПРИ ХРОМОФОБНОМ РАКЕ ПОЧКИ: НОВАЯ МИШЕНЬ ДЛЯ ТЕРАПИИ?

Цель: как установлено, ретиноевая кислота (RA) обладает невысокой, но выраженной активностью при раке почки (RCC), как минимум у части больных. Однако молекулярные механизмы, определяющие эффективность применения RA у отдельных больных, по-прежнему не установлены. В связи с тем, что в более ранних исследованиях было продемонстрировано, что рецептор RA (RAR) бета может выполнять основную роль в передаче сигнала от RA, мы исследовали экспрессию изоформ RAR-β₁ в клетках первичного обычного и хромофобного RCC. Методы: методология количественного RT-PCR была использована для исследования экспрессии RAR-β₁ и RAR-β₂ в 10 образцах первичного обычного RCC (светлоклеточный тип), в двух образцах хромофобного RCC и в соответствующих образцах нормальной ткани почки. Для нормализации различий качества и количества мРНК оценивали экспрессию генов RPS9 и RPLP0. Результаты: в отличие от конвентциональных образцов RCC, в обоих хромофобных опухолях наблюдалась значительная гиперэкспрессия RAR-β₁ по сравнению с нормальной тканью почки (р = 0.03). Наоборот, уровень экспрессии RAR-β₂ не имел существенных различий между конвентциональной и хромофобной RCC (р = 0.91), равно как и между опухолевой и нормальной тканью (р ≥ 0.47). Выводы: впервые продемонстрирована значительная и специфическая гиперэкспрессия RAR-β₁ при хромофобной RCC. Ключевые слова: ретиноиды, рецепторы ретиноидов, рецептор ретиноевой кислоты, рак почки.