Overexpression of Retinoic Acid Receptor α in Hepatocellular Carcinoma

Keiji Sano,1,2 Tadatoshi Takayama, Koji Murakami, Ikuo Saiki, and Masatoshi Makuuchi

Hepato-Biliary-Pancreatic Surgery Division, Artificial Organ and Transplantation Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan [K. S., T. T., M. M.], and Department of Pathogenic Biochemistry, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University, Toyama 931-0194, Japan [K. M., I. S.]

INTRODUCTION

HCC1 is one of the most common malignancies worldwide and causes almost 1 million deaths annually (1, 2). Although early diagnosis and surgical treatment improve survival (3), long-term survival is still unsatisfactory because of a high incidence of recurrence, which may be reduced by some novel options (4, 5). The prognosis of unresectable cases remains poor (6) because effective antitumor drugs are not yet available (7).

Retinoids, vitamin A analogues, strongly affect embryogenesis, differentiation, and carcinogenesis (8). The biological activity of retinoids is exerted through binding to specific nuclear receptors in the steroid/thyroid hormone family. Two major classes of retinoid receptors, RARs and retinoic X receptors, have been identified, each of which consists of three distinct receptor subtypes: α; β; and γ (8). Clinically, the successful treatment of patients with acute promyelocytic leukemia with ATRA, a natural retinoid, is well documented (9). However, the failure of long-term treatment has been reported due to the enhancement of plasma clearance (10) and to the induction of several metabolizing enzymes in the liver that inactivate retinoic acid (11). Moreover, in a Phase II trial, oral treatment with ATRA was reported to be ineffective against unresectable HCC (12).

The relationship among receptor subtype expressions has been investigated, for instance, in lung cancer (13), and some receptor-selective retinoids that bind to and transactivate specific retinoid receptors have been shown to be effective against non-small cell lung cancer (14) and HCC (4). RXR-α has been described as highly expressed in human HCC and possibly responsible for the aberrant growth of HCC (15, 16). As for RARs, we reported previously that an RXR-α-selective retinoid analogue may be useful for chemotherapeutic treatment of patients with acute promyelocytic leukemia with ATRA, a natural retinoid, is well documented (9). However, the failure of long-term treatment has been reported due to the enhancement of plasma clearance (10) and to the induction of several metabolizing enzymes in the liver that inactivate retinoic acid (11). Moreover, in a Phase II trial, oral treatment with ATRA was reported to be ineffective against unresectable HCC (12).

Conclusions: These results imply that RAR-α is the dominant receptor in HCC, which suggests that RAR-α-selective retinoid analogues may be useful for chemotherapeutic treatment.

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3 The abbreviations used are: HCC, hepatocellular carcinoma; RAR, retinoic acid receptor; ATRA, all-trans retinoic acid; RT-PCR, reverse transcription-PCR; AP-1, activator protein 1; TAC-101, 4-[3,5-bis(tri-methylsilyl)[benzamido]benzoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T/N, tumor versus nontumor intensity; TNM, tumor-node-metastasis.

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2 To whom requests for reprints should be addressed, at Hepato-Biliary-Pancreatic Surgery Division, Artificial Organ and Transplantation Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81-3-5800-8654; Fax: 81-3-5684-3989; E-mail: SANO-2SU@h.u-tokyo.ac.jp.
HCC to evaluate the potential for the clinical application of retinoic acid derivatives to treat HCC.

**MATERIALS AND METHODS**

**Patients.** Between September 1999 and February 2000, we performed hepatectomy in 32 consecutive patients with HCC. Fresh surgical specimens of HCC and paired nontumor liver tissue (5 mm in cube), more than 1 cm away from the HCC, were obtained.

**Cell Lines and Chemicals.** The HCC cell line JHH-7 was kindly provided by Dr. S. Nagamori (Jikei University School of Medicine, Tokyo, Japan), and primary-cultured hepatocytes were purchased from Cell Systems Co. (Kirkland, WA). Both were maintained as monolayer cultures in CS-C medium (Cell Systems Co.) at 37°C in a humidified atmosphere of 5% CO₂/95% air. TAC-101, which shows selective binding affinity for RAR-α, was provided by TAIHO Pharmaceutical Co. Ltd. (Tokyo, Japan). ATRA was purchased from WAKO Pure Chemical Industries Ltd. (Tokyo, Japan). Both reagents were dissolved in DMSO at a concentration of 20 mM before use. Antibody for RARs was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat antirabbit antibody was purchased from Amersham (Piscataway, NJ).

**RT-PCR.** RT-PCR was used to detect mRNA for RARs and GAPDH (17). Total RNA from HCC and nontumor tissue was extracted using Isogen (Nippon Gene Inc., Toyama, Japan) in 14 patients. In the other 18 patients, only HCC tissues were analyzed for more precise analysis. First-strand cDNA was prepared from RNA using 1 unit of avian myeloblastosis virus reverse transcriptase, 5 μg of total RNA as a template, and 10 ng/ml poly(dT)₁₂₋₁₈ oligonucleotide in the presence of 10 mM DTT and 0.5 unit of RNase inhibitor (Life Science Inc.). The reverse transcription reaction profile was 95°C for 3 min, 4°C for 10 min, and 42°C for 45 min. PCR amplification consisted of denaturation at 94°C for 30 s, annealing at 56°C (RAR-β, RAR-γ/H9251, RAR-β/H9252, and RAR-γ/H9253) or 60°C (RAR-α and RAR-γ), and extension at 72°C for 1 min and 30 s using template cDNA and a TAKARA Ex Taq PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan). The PCR products for RAR-α, RAR-β, RAR-γ, and GAPDH were amplified at 33, 36, 36, and 27 cycles, respectively. Each cycle was determined from preliminary experiments with various amplification cycles in each of the RARs extracted from the HCC cell line JHH-7 and hepatocytes. Each assay was performed in triplicate. The sequences of primers have been described previously (18). The PCR products were electrophoresed on 1.5% agarose gel and detected by ethidium bromide staining. The relative abundance of mRNA for RARs was expressed as intensity ratios relative to GAPDH, as determined at appropriate amplification cycles (exponential phase). RT-PCR analysis was semiquantified using a density analysis system (Scanalytics, Billerica, MA).

**Western Blot Analysis.** Nucleoprotein of HCC and nontumor specimens (5 mm in diameter) was extracted using a nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, IL). Extracted protein (5 μg) was electrophoresed on polyacrylamide gel (19). The gels were blotted onto nitrocellulose filters (Bio-Rad, Hercules, CA). The filters were developed with rabbit polyclonal antibodies specific for RAR-α, RAR-β, and RAR-γ (4°C, overnight), horseradish peroxidase-conjugated goat antirabbit antibody (room temperature, 2 h), and SuperSignal (Pierce) detection.

**Semiquantitative Analysis.** The intensity of mRNA for RARs in RT-PCR analysis was semiquantified using a density ratio to GAPDH at appropriate amplification cycles (exponential phase), determined with the Master Scan Gel Analysis System (Scanalytics). The intensity of each RAR protein was also semiquantitatively determined by analyzing electrophoresis product in Western blot analysis from each 5 μg of extracted nuclear protein.

**Clinical Correlation of RARs.** The intensities of mRNA for RARs and protein of RARs in 32 HCC tissues were compared with regard to several clinical factors: age; sex (male versus female); tumor size; α-fetoprotein; des-γ-carboxyprothrombin; hepatic virus infection (hepatic B virus infection versus hepatic C virus); stage grouping [stage I or II versus stage III or IVA (20)], histological differentiation (well differentiated versus moderately or poorly differentiated), vascular invasion (positive versus negative), and background liver (normal liver or chronic hepatitis versus liver cirrhosis).

**Assay for in Vitro Antiproliferative Activity.** JHH-7 cells and hepatocytes were seeded (4000 cells/well) into 96-well culture plates. After 24 h of incubation, various concentrations of TAC-101 or ATRA were added to the culture plates. After 72 h of incubation, crystal violet staining was performed as described previously with some modification (18).

**Statistical Analysis.** The significance of differences in the intensity, intensity ratio, and clinicopathological variables (sex, hepatic virus infection, stage grouping, histological differentiation, vascular invasion, and background liver) was assessed using the two-tailed Welch’s t test. Other clinicopathological variables (age, tumor size, α-fetoprotein, and PIVKA-II) were examined by Spearman’s rank correlation coefficient test. Differences at P < 0.05 were considered to be statistically significant.

**RESULTS**

**Intensity of mRNA for RARs.** Intensity for RAR-α in tumor tissue was significantly higher than that in liver tissue (1.021 ± 0.373 versus 0.534 ± 0.442; P = 0.002). There was no significant difference in the intensities for RAR-β and RAR-γ between the tumor and liver (Table 1).

<table>
<thead>
<tr>
<th>Intensity (RARs/GAPDH)</th>
<th>Non-tumor tissue (n = 14)</th>
<th>HCC tissue (n = 31)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAR-α</td>
<td>0.534 ± 0.442</td>
<td>1.021 ± 0.373</td>
<td>0.002</td>
</tr>
<tr>
<td>RAR-β</td>
<td>0.943 ± 0.458</td>
<td>0.845 ± 0.340</td>
<td>0.482</td>
</tr>
<tr>
<td>RAR-γ</td>
<td>0.824 ± 0.397</td>
<td>0.712 ± 0.483</td>
<td>0.418</td>
</tr>
</tbody>
</table>

*Two-tailed Welch’s t test was performed.*
There was a significant difference in the intensity of RAR-α between HCC and liver tissue (2027.5 ± 973.7 versus 1228.3 ± 645.0, P = 0.002), but not in RAR-β or RAR-γ (Table 2).

**Tumor versus Nontumor Intensity Ratio.** To assess the intensity ratio in individual patients, we calculated a T/N ratio of mRNA for RARs (Fig. 1) and RAR proteins (Fig. 2) in 14 patients. The T/N ratio of mRNA for RAR-α was significantly higher than those for RAR-β and RAR-γ (P = 0.02 for RAR-β and P = 0.006 for RAR-γ). The T/N ratio of RAR-α protein was also higher than those of the other two RARs (P = 0.04 for RAR-β and P = 0.007 for RAR-γ; Table 3).

**Clinical Correlation of RARs.** The correlations between expression levels of RAR-α, RAR-β, and RAR-γ and several clinical factors were analyzed. Only one significant difference was found in the intensity of RAR-β protein between the intensity in TNM stage I or II HCC and that in TNM stage III or IVA HCC (P = 0.01).

**In Vitro Antiproliferative Activity of Retinoids.** We used RAR-α-selective retinoid (TAC-101) and RAR panagonist (ATRA) to test in vitro antiproliferative activity. As shown in Fig. 3A, both TAC-101 and ATRA potently inhibited the proliferation of the HCC cell line JHH-7, which shows high levels of RAR-α expression. TAC-101 was more effective than ATRA at inhibiting the proliferation of this HCC cell line.

**DISCUSSION**

We found significantly higher expression levels of RAR-α (both mRNA and protein levels) in HCC tissue than in nontumor tissue, whereas there were no differences in the expression levels of RAR-β and RAR-γ between tumor and nontumor tissues. We first confirmed the preferential expression of RAR-α in resected tissues, as shown previously in cell lines (17, 18, 21).

Ligand-binding RARs form heterodimeric units with retinoid X receptors (22) and induce various biological effects including differentiation (23), apoptosis (24), and antimetastatic (25) and antiangiogenic activity (26). A tight link exists between the expression of specific retinoid receptors and preclinical or clinical retinoid responses. Some retinoids have been reported to induce apoptosis in human lung cancer cells via RAR-α (27). We showed previously that TAC-101, a novel benzoic acid derivative that shows selective binding affinity for RAR-α, induces apoptosis not only in a HCC cell line in vitro but also in vivo.

**Table 2** Expression levels of protein for RARs

Nuclear protein was extracted from HCC tissues and matched liver tissues. Western blot analysis was performed using extracted protein (0.005 mg/lane). The intensity of each proteins was determined using the Master Scan Gel Analysis System. Data are given as mean ± SD.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nontumor tissue (n = 14)</th>
<th>HCC tissue (n = 31)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAR-α</td>
<td>1228.3 ± 645.0</td>
<td>2027.5 ± 973.7</td>
<td>0.002</td>
</tr>
<tr>
<td>RAR-β</td>
<td>918.2 ± 435.6</td>
<td>936.1 ± 742.3</td>
<td>0.920</td>
</tr>
<tr>
<td>RAR-γ</td>
<td>412.6 ± 193.1</td>
<td>372.1 ± 123.0</td>
<td>0.559</td>
</tr>
</tbody>
</table>

* Two-tailed Welch’s t test was performed.

![Fig. 1 Expression of RAR proteins in the nuclear fraction of HCC tissues and matched liver tissues.](image)

![Fig. 2 Expression of RAR proteins in the nuclear fraction of HCC tissues and matched liver tissues.](image)

![Fig. 3 Antiproliferative activity of retinoids.](image)

**Table 3** T/N ratios of mRNA for RARs and RAR proteins

Nuclear protein was extracted from HCC tissues and matched liver tissues. Western blot analysis was performed using extracted protein (0.005 mg/lane). The intensity of each proteins was determined using the Master Scan Gel Analysis System. Data are given as mean ± SD.

<table>
<thead>
<tr>
<th>Protein</th>
<th>mRNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAR-α</td>
<td>3.35 ± 2.59&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.12 ± 2.58&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>RAR-β</td>
<td>1.27 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.46 ± 0.95&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RAR-γ</td>
<td>1.06 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91 ± 0.47&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P = 0.02.  
<sup>b</sup> P = 0.006.  
<sup>c</sup> P = 0.04.  
<sup>d</sup> P = 0.007.
Overexpression of RAR-\(\alpha\) in HCC tumors, therefore, RAR-\(\alpha\)-selective retinoids may induce apoptosis in HCC via RAR-\(\alpha\)-mediated signals to give an antitumor effect. TAC-101 also shows AP-1 antagonism, perhaps via RAR-\(\alpha\) (28, 29). Increased AP-1 activity is associated with carcinogenesis in some malignancies (30). Antagonism of AP-1 by retinoic acid has been reported to inhibit malignant transformation. In addition, RAR-\(\alpha\)-dependent antagonism of AP-1 inhibited the growth of a lung cancer cell line (31). AP-1 is a heterodimeric transcription factor and consists of the oncoprotein Jun and Fos families. Although hepatocarcinogenesis is considered to be caused by multistep processes (32), in an analysis of 290 HCC patients, Yuen et al. (33) revealed that there was a positive association between the expression of c-Fos and the expression of c-Jun in HCC tissues. Thus, a RAR-\(\alpha\)-selective retinoid, such as TAC-101, which shows potent AP-1 antagonism, may be beneficial not only for the therapeutic treatment of HCC but also for preventing secondary hepatocarcinogenesis.

HCC cells become well-differentiated to moderately or poorly differentiated as the tumor progresses (34, 35). In the human bronchus, the response to receptor-selective retinoids has been reported to vary among normal, premalignant, and malignant cells (36). In this study, the overexpression of RAR-\(\alpha\) in tumor tissues of HCC was not significantly correlated with various clinical factors including tumor cell differentiation, tumor size, tumor markers, or tumor staging. On the other hand, the intensity of RAR-\(\beta\) protein was significantly higher in early-stage HCC compared with advanced-stage HCC. RAR-\(\beta\) was also reported to play important roles in differentiation and antiproliferation in hepatoma cells (37). Therefore, to clarify the possible preventive effect on malignant transformation in hepatocarcinogenesis, additional studies are needed, because no premalignant tumors were examined in this study.

We have already demonstrated that an in vitro-established HCC cell line responded to RAR-\(\alpha\)-selective retinoids with regard to the inhibition of cell proliferation (17). Following that result, in vitro experiments in this study showed that the growth of a RAR-\(\alpha\)-elevated HCC cell line was potently inhibited by treatment with retinoids at concentrations that did not affect the growth of primary-cultured hepatocytes. Moreover, at the same concentration, TAC-101 was more effective than panagonist, ATRA at inhibiting the proliferation of the HCC cell line. Retinoid resistance may be due to decreasing plasma levels resulting from a consistent acceleration of ATRA metabolism (10). Therefore, TAC-101 can be expected to be effective without resistance for longer periods than ATRA. It may be possible to avoid the adverse effects associated with chronic administration of high doses of retinoids because the dosage of TAC-101 can be smaller than that of ATRA. However, this cannot be predicted with great certainty because synthetic retinoids, which may not have the same biological properties as vitamin A, may also have different adverse effects (38).

In conclusion, the present study revealed that RAR-\(\alpha\) was a dominant receptor for HCC in resected specimens, suggesting that RAR-\(\alpha\)-selective retinoic acid derivatives may be clinically useful for chemotherapy in HCC patients.

**Acknowledgments**

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**References**


4. Muto, Y., Moriwaki, H., Nominoya, M., Adachi, S., Saito, A., Takasaki, K. T., Tanaka, T., Tsurumi, K., Okuno, M., Tomita, E., Nakamura, T., and Kojima, T. Prevention of second primary tumors by retinoic acid has been reported to inhibit malignant transformation. In addition, RAR-\(\alpha\)-selective retinoid, such as TAC-101, which shows potent AP-1 antagonism, may be beneficial not only for the therapeutic treatment of HCC but also for preventing secondary hepatocarcinogenesis.


**Fig. 3** Antiproliferative activity of retinoids against HCC and primary-cultured hepatocyte. JHH-7, a hepatocellular carcinoma cell line (A), and primary-cultured hepatocyte (B) were treated with TAC-101 (●) or ATRA (○) for 72 h. The antiproliferative activity was determined by crystal violet colorimetric assay.


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