Antitumor Efficacy of Recombinant Human Interleukin-2 Combined with Sorafenib Against Mouse Renal Cell Carcinoma

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Received December 17, 2008; accepted February 20, 2009; published online March 31, 2009

Objective: Recombinant human interleukin-2 (rhIL-2) has been clinically used in the treatment of renal cell carcinoma (RCC). Sorafenib, a multi-targeted kinase inhibitor, has been approved for RCC as well as IL-2. The purpose of this study was to evaluate the antitumor efficacy of IL-2 combined with sorafenib in three different murine renal cancer models using Renca cells.

Methods: We established the subcutaneous tumor model by inoculating wild-type Renca cells into the backs of BALB/c mice, the pulmonary metastatic tumor model by an intravenous injection of luciferase-expressing Renca cells into the tail vein and the orthotopic tumor model by injecting luciferase-expressing Renca cells into the renal subcapsule. These tumor-bearing mice were treated intra-peritoneally with rhIL-2 and/or per os with sorafenib. The antitumor efficacy was evaluated by measuring the tumor size of the subcutaneous tumor or photon intensity of the pulmonary metastatic tumor and the orthotopic tumor.

Results: When rhIL-2 was combined with sorafenib, the antitumor efficacy was significantly augmented in comparison with either rhIL-2 or sorafenib alone in all the models. Sorafenib did not inhibit rhIL-2-induced natural killer cell expansion and rhIL-2 had no effect on the anti-angiogenic activity of sorafenib.

Conclusions: The results suggest that the combination of rhIL-2 and sorafenib may offer significant potential as a novel therapeutic approach for patients with RCC.

Key words: IL-2 – sorafenib – combination therapy – renal cell carcinoma

INTRODUCTION

Recombinant human interleukin-2 (rhIL-2) has been a useful agent for advanced renal cell carcinoma (RCC) therapy over the past two decades. This cytokine enhances a host immune response against tumor cells, stimulating natural killer (NK) cells and lymphokine-activated killer (LAK) cells. The rhIL-2 therapy has demonstrated a response rate of approximately 15–20% and has also led to complete and durable clinical response for patients with RCC (1–3). Recently, low-dose IL-2 administration in combination with interferon-α showed clinical outcomes with response rates of 30–40% for patients with only lung metastasis (4,5).

Sorafenib is a novel molecular-targeted agent approved for the treatment of advanced RCC in 2007 in Japan. It has multi-kinase inhibitory activity for Raf-1, mutant B-RAF and p38 with additional activity against VEGFR, PDGFR-β, FLT-3 and c-Kit. Sorafenib inhibits tumor cell proliferation and reduces tumor angiogenesis in a number of animal models with kidney, breast, lung and colon cancer cells (6,7). The rationale for anti-angiogenic therapy for RCC is based on the target mechanism (8). According to clinical trials with RCC patients who had previously been treated with first-line therapy, sorafenib has shown a good ability to stabilize the disease and has doubled the extension of progression-free survival when compared with data from placebo groups (9). However, incidents of severe side effects, short interval of a durable response and drug failure have been reported as limitations to anti-angiogenic therapy (10,11).

Combination therapy by agents with different mechanisms of action may help enhance antitumor activity. The efficacy of a standard immunotherapy combined with some anti-angiogenic agents has been investigated in several clinical studies for RCC (12). Recently, the combination of sorafenib
and interferon α-2b has been confirmed to have a positive antitumor effect in advanced RCC patients (13,14).

To investigate the efficacy of rhIL-2 combined with sorafenib and its mode of action in in vivo models, which reflect the pathologic conditions of RCC patients, we established a pulmonary metastatic model and an orthotopic model of mouse RCC, which have been used to demonstrate the antitumor efficacy of rhIL-2 or sorafenib (7,15). We showed that the growth inhibitory activity of rhIL-2 combined with sorafenib was more potent than that of each monotherapy and the independent mechanisms of these agents may contribute to the antitumor efficacy of this combination therapy.

MATERIALS AND METHODS

REAGENTS

Commercial formulation of rhIL-2 (Imunace®35) was produced in Shionogi & Co. Ltd (Osaka, Japan). It has $35 \times 10^4$ Japan reference units (JRU)/vial; one JRU corresponds to one international unit. Sorafenib (Nexaver®; Bayer Pharmaceuticals Corporation, West Haven, CT, USA) was suspended in 0.5% (w/v) methylcellulose 400 cP solution (Wako Pure Chemical Industries, Osaka, Japan) and the concentration of sorafenib was adjusted in view of the mass content. Rabbit anti-mouse AsGM1 antibody was purchased from Molecular Probes, Alexa Flour 488 coupled to Alexa Flour 488. For nucleic acid staining, the content. Rabbit anti-mouse AsGM1 antibody was purchased from Molecular Probes, Inc. (Eugene, OR, USA). FITC-labeled rat anti-mouse CD4 antibody, PE-labeled rat anti-mouse CD8 antibody and rat anti-mouse CD31 antibody were obtained from BD Pharmingen (San Diego, CA, USA).

CELL LINES

Renca, murine renal cancer, cells were generously provided by Dr Tomoaki Fujioka (Iwate Medical University School of Medicine, Iwate, Japan). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO2. Stable transfection of Renca cells with pUSEamp® (Upstate, Lake Placid, NY, USA) inserted luciferase gene was performed according to the Lipofectamin® (Invitrogen, Carlsbad, CA, USA) method. Briefly, the transfection mixture was prepared by adding 30 μl of Lipofectamin to 500 μl of Optimem containing 10 μg of pUSEamp-Luc. After 20 min of incubation at room temperature, this mixture was used to transfect cultured Renca cells. After 24 h, medium was completely removed and substituted with 10% FCS-containing DMEM supplemented with 1 mg/ml G-418 (Sigma-Aldrich Co., St Louis, MO, USA). Surviving clones were screened for bioluminescence using the In Vivo Imaging System® (IVIS) 200 (Xenogen, Alameda, CA, USA) and luciferase-expressing Renca (Renca-Luc) cells were maintained with 10% FCS-containing DMEM supplemented with 1 mg/ml G-418.

MURINE MODELS OF RENAL CANCER (RENCA)

Female BALB/c mice were obtained from Charles River Laboratories Japan. The mice were used at the age of 5–9 weeks. All animal procedures were approved by the Institutional Animal Care and Use Committee in our laboratory, and mice were given food and water ad libitum. In the experiments, rhIL-2 and sorafenib were used at doses without producing severe body weight loss due to drug-related toxicity.

The subcutaneous tumor model was established by inoculating wild-type Renca cells ($3 \times 10^6$) subcutaneously (s.c.) into the right side of the backs of mice. Tumor-bearing mice were subjected daily to systemic administration of $2 \times 10^4–8 \times 10^4$ JRU/mouse rhIL-2 intra-peritoneally (i.p.) and/or 10 mg/kg sorafenib per os (p.o.) from the next day after tumor inoculation for 14 days. Tumor size was measured with an electric caliper, and tumor volume was calculated using the following formula: tumor volume = (length × width × width)/2. On Day 15, which was the day after the last administration, the growth inhibitory effect was estimated using the treated/control ratio (T/C).

The pulmonary metastatic tumor model was established experimentally by an intravenous injection of $2 \times 10^5$ cells/0.1 ml/mouse Renca-Luc cell suspension into the tail vain of BALB/c mice on Day 0. The mice were administered with rhIL-2 (i.p., $2 \times 10^4$ JRU/mouse) and/or sorafenib (p.o., 10 mg/kg) from Day 10 for 14 days. The photons produced by luciferase activity within Renca-Luc cells were acquired quantitatively using IVIS 200 with injection of luciferin to mice. On Day 24, which was the next day after the last administration, the growth inhibitory effect was evaluated.

The orthotopic tumor model was established by injecting $3 \times 10^4$ cells/3 μl of Renca-Luc cell suspension with Matrigel® (BD Biosciences, NJ, USA) into the renal subcapsule of the left kidney of BALB/c mice under anesthesia. The administration of rhIL-2 and/or sorafenib was started after the implanted tumor cells were in the growth phase.

IMMUNOHISTOCHEMICAL STAINING AND QUANTIFICATION

After the antitumor efficacy had been evaluated with the orthotopic implantation model, the tumor tissue specimens were used for immunohistochemical staining. The paraffin sections of tumors were stained immunohistochemically with a 1:100 dilution of primary antibody (rat anti-mouse CD31) followed with a 1:100 dilution of goat anti-rat IgG antibody coupled to Alexa Flour 488. For nucleic acid staining, the slides were incubated in 300 nM DAPI (Invitrogen) for 4 min and mounted in DABCO mounting medium (Sigma). The tissue sections were viewed at ×100 magnification and the images were captured with a digital camera. Eight fields per section were analyzed, and the areas of CD31-positive objects were quantified using Lumina Vision version 2.2.2.
decreased the photon level in the lung after 14 days of
As shown in Fig. 2B, all agent-treated groups significantly
inhibited the re-growth of cancer cells on Day 7. After ascertaining
the tail vein on Day 0. Monitoring of the photons revealed
(Fig. 2A). The bioluminescence activity expressed as a
tumor progression in lung was monitored with IVIS 200
Renca-Luc cells were injected into the tail vein and the
TUMOR MODEL
Cytometry
Peripheral blood was obtained in the pulmonary metastasis
model mouse treated with drugs. The cells were centrifuged
in mouse Ficol–Isopaque gradient regents. Peripheral blood
mononuclear cells (PBMCs) were stained with FITC-labeled
rat anti-mouse CD4 antibody, PE-labeled rat anti-mouse CD8 antibody or rabbit anti-mouse AsGM1 antibody with
secondary antibody (donkey anti-rabbit IgG coupled to
Alexa Flour 488) and analyzed on Epics flow cytometer
(Beckman–Coulter, Fullerton, CA, USA).

RESULTS
COMBINATION THERAPY IN SUBCUTANEOUS IMPLANTATION MODEL
We evaluated the in vivo antitumor efficacy of rhIL-2 com-
bined with sorafenib in subcutaneous tumor-bearing murine
model using Renca cells. The treatment with rhIL-2
(20 000, 40 000 and 80 000 JRU/mouse, i.p.) combined
with sorafenib (10 mg/kg/day, p.o.) was initiated on Day 1
which was the day following tumor inoculation. As shown
in Fig. 1, rhIL-2 inhibited in vivo tumor growth in a dose-
dependent manner. The antitumor efficacy with sorafenib at
10 mg/kg/day was almost equivalent to that of rhIL-2 at
80 000 JRU/mouse in this model. The combination
of rhIL-2 (40 000 or 80 000 JRU/mouse) and sorafenib
strongly inhibited the tumor growth between Day 4 and
Day 15. Tumor growth in all treated mice groups was sig-
ificantly inhibited in comparison with that in the control
mice. Next, we analyzed the T/C values on Day 15, which
were pooled in independent experiments (Table 1). The T/
C values of combined treatments with rhIL-2 plus sorafenib
were significantly reduced compared with those of either
either rhIL-2 or sorafenib. In particular, 40 000 JRU/mouse
rhIL-2 combined with 10 mg/kg sorafenib led to no body
weight loss compared with either rhIL-2 or sorafenib. In
particular, 40 000 JRU/mouse
rhIL-2 combined with 10 mg/kg sorafenib led to no body
weight loss compared with the control (Table 1), while
maintaining almost complete inhibition of tumor growth
between Day 4 and Day 15 (Fig. 1).

COMBINATION THERAPY IN PULMONARY METASTATIC
TUMOR MODEL
Renca-Luc cells were injected into the tail vein and the
tumor progression in lung was monitored with IVIS 200
(Fig. 2A). The bioluminescence activity expressed as a
photon in the lung on Day 4 was reduced after injection into
the tail vein on Day 0. Monitoring of the photons revealed
the re-growth of cancer cells on Day 7. After ascertaining
tumor growth in the lung, the therapy was started on Day 10.
As shown in Fig. 2B, all agent-treated groups significantly
decreased the photon level in the lung after 14 days of

Figure 1. Anticancer efficacy of recombinant human interleukin-2 (rhIL-2) combined with sorafenib in s.c. Renca model. The next day after s.c. injection with Renca cells (3 × 10^6 cells/mouse), Balb/c mice were treated daily with rhIL-2 (i.p., 2 × 10^4, 4 × 10^4 and 8 × 10^4 JRU/mouse) and/or sorafenib (p.o., 10 mg/kg) for 14 days. Tumor length and width were measured with an electronic caliper and tumor volume was calculated from the follow-
ing formula: tumor volume = (length × width × depth)/2. Data represents the mean ± SD of six mice. *P < 0.05 and **P < 0.01 for control by Dunnett’s multiple test.

Table 1. Anticancer efficacy of rhIL-2 combined with sorafenib in s.c. Renca model

<table>
<thead>
<tr>
<th>Group</th>
<th>rhIL-2 (JRU/mouse)</th>
<th>Sorafenib (mg/kg)</th>
<th>n</th>
<th>T/C (mean ± SD)</th>
<th>Maximum body weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1.00</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>rhIL-2 alone</td>
<td>20 000</td>
<td>0</td>
<td>4</td>
<td>0.90 ± 0.24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>40 000</td>
<td>0</td>
<td>3</td>
<td>0.69 ± 0.26</td>
<td>2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>80 000</td>
<td>0</td>
<td>3</td>
<td>0.49 ± 0.28</td>
<td>0</td>
</tr>
<tr>
<td>Sorafenib alone</td>
<td>0</td>
<td>10</td>
<td>4</td>
<td>0.44 ± 0.11</td>
<td>3.3 ± 3.9</td>
</tr>
<tr>
<td>Combination</td>
<td>20 000</td>
<td>10</td>
<td>4</td>
<td>0.38 ± 0.17</td>
<td>1.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>40 000</td>
<td>10</td>
<td>3</td>
<td>0.22 ± 0.12</td>
<td>1.3 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>80 000</td>
<td>10</td>
<td>3</td>
<td>0.17 ± 0.04</td>
<td>7.3 ± 4.2</td>
</tr>
</tbody>
</table>

*Renca cells (3 × 10^6 cells/mouse) were injected s.c. into the right side of the backs of the Balb/c mice on Day 0. Treatment with recombinant human interleukin-2 (rhIL-2) and/or sorafenib was initiated on Day 1 for 14 days. Each experiment was performed on six mice per group and T/C was calculated from the tumor volume on Day 15. One mouse of the total of 24 mice treated with rhIL-2 (20 000 JRU/mouse) died accidentally.

Maximum body weight loss is shown as the percent of the initial weight and represents the mean ± SD.

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COMBINATION THERAPY IN ORTHOTOPIC TUMOR MODEL

Renca-Luc cells were injected into the renal subcapsule and the tumor progression in the kidney was monitored by its bioluminescence activity. The administration of rhIL-2 (40,000 JRU/mouse, i.p.) and/or sorafenib (10 mg/kg/day, p.o.) was started on Day 11 for 10 days. The strongest antitumor efficacy was observed with the combination therapy through the tumor re-growing phase treatment in the orthotopic implantation model; this was the same as the results from the pulmonary metastasis model (Fig. 3A). Next, to assess the degree of angiogenesis, immunohistochemical staining for anti-CD31 antibody was done in the tumor samples obtained from all mice after measuring bioluminescence activity. The microvessels in the tumors on Day 21 were stained green color (Fig. 3B). The tumor sections from mice treated with sorafenib alone or sorafenib plus rhIL-2 showed equally significant decrease in the percentage of CD31-positive area (Fig. 3C). Therefore, it was clear that rhIL-2 did not interfere with the anti-angiogenic efficacy of sorafenib when rhIL-2 was combined with sorafenib.

INFLUENCE OF THE COMBINATION THERAPY ON A HOST IMMUNE RESPONSE

To investigate whether the combination therapy affects a host immune response, we evaluated the populations of leukocytes in PBMCs by flow cytometry. The PBMCs were collected from pulmonary metastasis model mice treated for 11 days. As shown in Fig. 4A, the population of AsGM1 (NK cell marker)-positive cells was significantly increased in the groups treated with rhIL-2 alone or rhIL-2 plus sorafenib. However, there was no change of the populations of CD4- and CD8-positive cells by treatment with rhIL-2 alone, sorafenib alone or the combination of these agents (Fig. 4B). Thus, it was obvious that sorafenib did not affect the rhIL-2-induced NK cells' augmentation when sorafenib was combined with rhIL-2.

DISCUSSION

Conventional treatment consists of cytokine therapy using rhIL-2 and interferon-α. In recent years, however, the efficacy of molecular-targeted drugs for treatment of RCC has been demonstrated, and improved therapeutic responses are expected with combined treatment of this class of drugs and cytokine therapy. The objective of this study was to develop an effective clinical therapy for patients with RCC to further improve efficacy.

In this study, the effect of combined therapy with rhIL-2 and sorafenib was evaluated in three animal models of RCC. The results showed a greater antitumor efficacy of the combined treatment in all three models, compared with treatment with either drug alone. We therefore expected synergistic antitumor effects by a combination of these agents acting on the independently different modes.

In the experiment using the subcutaneous murine model of renal cancer, each monotherapy significantly inhibited the increase in tumor volume in comparison with the control. This result was consistent with previous findings in this common model (7,16). Then, the combination therapy of rhIL-2 added to sorafenib represented more effective inhibition than each treatment individually. When the T/C value of rhIL-2 alone was multiplied by the T/C value of sorafenib alone, the T/C value of the corresponding dose of rhIL-2...
combined with sorafenib showed a tendency to be less than the multiplied T/C value in three dosing cases, suggesting that the combination therapy brought about synergistic efficacy.

As for the mechanisms of action, sorafenib inhibited angiogenesis and rhIL-2 produced augmentation of NK cell activity when administered alone, as reported previously.
in growth, since sorafenib inhibits Raf and p38. In view of this, sorafenib is likely to have a direct inhibitory effect on tumor growth, anti-angiogenesis by sorafenib

(7,17), whereas rhIL-2 had no effect on T cells. Similar findings were also observed for rhIL-2 in the tumor; immunostaining of the tissue specimens showed the accumulation of NK cells in the areas surrounding the tumor following the administration of rhIL-2, but CD4- and CD8-positive cell infiltration was inconspicuous with no difference between any treated group and the control group. Many studies have investigated the effect and mechanism of action of rhIL-2, but the results vary with the mouse strains, transplantation method, dosing protocol and type of organs examined. Previous studies have shown that NK activity could be generated from a variety of mouse strains (18,19). Some studies have demonstrated the effect of rhIL-2 on T cells; however, the present study did not detect any such effects. This might be attributable to the fact that the Renca model is immunosuppressive, so that a dramatic induction of T lymphocytes can hardly occur despite the administration of rhIL-2 (15,20). It would be necessary to investigate the effect of the drug on cytotoxic T lymphocyte (CTL) activity and other responses in another type of model.

There was no effect of rhIL-2 on tumor blood vessels and no significant difference was observed between the response to sorafenib alone and that to sorafenib in combination with rhIL-2. Therefore, it was determined that the involvement of rhIL-2 is limited on tumor angiogenesis. In addition, sorafenib had no effect on NK cells that are dramatically induced with rhIL-2. It remains unclear whether sorafenib acts upon NK cells or not, although the available clinical trial data indicate mild hematotoxicity of sorafenib (9,10). More experiments will be required to understand the tumor killing activity of NK cells induced by rhIL-2 stimulation.

Considering the rationale for combination efficacy of rhIL-2 with sorafenib, different independent modes of the action of rhIL-2 which induces high activity and proliferation of NK and LAK cells, and the action of sorafenib which causes anti-angiogenesis may act synergistically to kill RCC cells in vivo (Fig. 5). Activated NK and LAK cells are able to kill RCC cells effectively during the suppression of tumor growth progression in cause of anti-angiogenesis by sorafenib. That is to say, sorafenib has a role in supporting the actions of NK and LAK cells. When the tumor volume is very small and little angiogenesis is needed for tumor growth, anti-angiogenic agents cannot be expected to cause a great deal for antitumor efficacy. However, the host immune response enhanced by rhIL-2 will be potent against such as small tumors. Such reciprocal help from combination therapy may lead to synergistic antitumor efficacy in vivo. Recent clinical trials of combination therapy with sorafenib and cytokines showed additive antitumor efficacy and no particular unexpected adverse reaction (12,13,21). Our findings suggest that the combination therapy of rhIL-2 with sorafenib can be a novel therapeutic approach for patients with RCC including metastasis.

Acknowledgments

We are grateful to Dr Tomoaki Fujioka (Department of Urology, Iwate Medical University School of Medicine, Japan) for providing the Renca cells and his helpful discussions.

Conflict of interest statement

M. Iguchi, M. Matsumoto, K. Hojo, T. Wada, Y. Matsuo, A. Arimura and K. Abe are employed by Shionogi & Co., Ltd.

References


