Title
Potent anti-tumor effects of combined therapy with a telomerase-specific replication-competent adenovirus (OBP-301) and IL-2 in a mouse model of renal cell carcinoma.

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Abstract

OBP-301 (telomerase-specific replication-competent adenovirus with hTERT promoter) was constructed in a previous study and it showed a strong anti-cancer effect by inducing cell lysis in human lung and prostate cancer cells. This study investigated the effectiveness of a combination therapy of OBP-301 and Interleukin-2 (IL-2) in a mouse model of renal cell carcinoma (RCC). The cell-killing effect of OBP-301 was confirmed in vitro in the RENCA cancer cells. In in vivo experiment, luciferase-expressing RENCA cells were implanted in the left kidney and lung of BALB/c mice to prepare the RCC metastatic model. The animals were randomly divided into 4 treatment groups: PBS, IL-2 alone, OBP-301 alone, and the combination. The analyses of orthotopic tumor weight, lung metastasis, and luciferin-stained tumor images 14 days after each treatment showed significant tumor growth inhibition in the combination group in comparison to the OBP-301 or IL-2 treated groups. In addition, the percentage of regulatory T cells (Tregs) in the combination group was significantly suppressed in comparison to that in the PBS and single agent treatment groups. The outcomes of this study suggest that tumor-specific oncolytic immunovirotherapy may become an attractive strategy for the treatment of human RCC.
Introduction

Renal cell carcinoma (RCC) has become the 4th most common genitourinary cancer with more than 51,000 new cases and about 13,000 cancer-specific deaths recorded per year in the United States.\textsuperscript{1} A considerable number of patients show distant metastasis at the time of diagnosis. Despite undergoing surgical treatment, chemotherapy, and radiotherapy, most of the RCC patients show disease progression and metastasis. Therefore, there is an urgent need for developing novel and effective therapies for RCC.

Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosome ends, and its activation is considered to play a crucial role in not only cell proliferation but also carcinogenesis.\textsuperscript{2,3,4,5} Previous studies have demonstrated an enhanced telomerase activity in more than 85% of human cancers, but only in a few normal somatic cells.\textsuperscript{3,4} Human telomerase reverse transcriptase (hTERT) positively regulates telomerase activity at the transcriptional level and is selectively overexpressed in proliferating neoplastic tissues and cells.\textsuperscript{2,3,4,5} Therefore, it seems reasonable to consider the hTERT promoter as a cancer-specific promoter. Previous study developed a unique strategy for oncolytic virotherapy based on the replicative capacity of the adenovirus by placing the envelope 1 (E1) gene under the control of a hTERT promoter.\textsuperscript{6,7,8,9,10} OBP-301 (Telomelysin\textsuperscript{©}) -a cancer-selective replication-competent oncolytic adenovirus- can induce cell lysis in human non-small-cell lung cancer and prostate cancer cells.\textsuperscript{6,9} OBP-301 has the ability to replicate within tumors, enter the blood circulation, and then replicate in distant metastatic tumors after the intratumoral administration.\textsuperscript{9}

Interleukin-2 (IL-2) is a member of the immuno-cytokine family and was approved by Food and Drug Administration (FDA) of the United States for the treatment of
metastatic RCC. However, the overall response rates of the immunotherapy have been shown to be 6.5%. Due to the limited efficacy of IL-2 treatment, an effective treatment strategy that includes a combination of other methods is required for increasing the efficacy of IL-2 immunotherapy. The present study investigated whether a combination therapy of OBP-301 and IL-2 has enhanced anti-tumor and anti-metastatic effects in a mouse model of orthotopic RCC in comparison to OBP-301 or IL-2 therapy alone.
Materials and Methods

Animals

Female BALB/c mice (6 to 8 week old) were purchased from SLC Inc., (Hamamatsu, JAPAN). They were maintained in a specific pathogen-free environment with free access to food and water at the laboratory animal center of Okayama University. They were allowed to adapt to their environment for more than 1 week before beginning the experiments. The animals were housed and handled in accordance with the Okayama University Animal Research Committee Guidelines.

Cell line

The mouse RCC cell line (RENCA) was provided by the American Type Culture Collection (Rockville, MD). The RENCA cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were cultured at 37°C in a 5% CO₂ atmosphere and routinely passaged by trypsin-EDTA treatment in 100 cm² flasks.

OBP-301 and recombinant IL-2 protein

OBP-301 (Telomelysin®, a tumor-specific replication-competent adenovirus) is a construct in which the hTERT promoter drives the expression of the E1A and E1B genes linked with an internal ribosome entry site. Before use, these viruses were purified by CsCl₂ step-gradient followed by CsCl₂ linear-gradient ultracentrifugation. Recombinant human IL-2 protein was kindly provided by Shionogi Co. (Osaka, JAPAN), and this protein was diluted in the culture medium just before use for the in vitro studies, and in phosphate-buffered saline (PBS) for the in vivo studies.
**In vitro cell number assay**

RENCA cancer cells were seeded onto a 96-well plate at a density of 1000 cells/well. After 24 hours of incubation, the cells were treated with OBP-301 added at a multiplicity of infection (MOI) of 0.1, 1 and 10, or with recombinant IL-2 protein at 10, 100 and 1000 units/ml. Further, RENCA cells were treated with 1 MOI of OBP-301 mixed with 10, 100 and 1000 U/ml of IL-2. The *in vitro* cell-killing effect was analyzed by determining the cell number at the indicated time points using a cell proliferation kit of sodium 3´-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay (Roche Diagnostics, Indianapolis, IN), according to the manufacturer’s instructions.

**Imaging of RCC tumors in mouse**

The tumors derived from luciferase- stably transfected RENCA cells were imaged by the luciferase expression in the tumors. Briefly, the animals were anesthetized with isoflurane before and during imaging and then were injected intraperitoneally with luciferin (a substrate for luciferase) at 150 mg/kg in a volume of 100 μl. The animals were imaged at a peak time of 20 min post-luciferin injection via an IVIS-200 instrument (Xenogen, Alameda, CA), using exposure times and sensitivity settings to avoid saturation. Image processing was done using the Living Image software program (Xenogen), by means of a region-of-interest analysis of the total photons per second for each tumor, with appropriate background subtraction.

**In vivo therapeutic experiments in the mouse RCC model**

The mouse model with orthotopic RCC tumors and metastatic lung tumors was
established in female BALB/c mice. Luciferase- stably transfected RENCA cells (5 × 10^4 cells/100 μl PBS) were orthotopically injected into the subcapsular space of the left kidney and then 1 × 10^4 of the cells (in 100 μl PBS) were intravenously injected via the tail vein. At 10 days after the cell injection, tumor imaging was done and the mice were divided into 4 treatment groups [namely, PBS, IL-2, OBP-301, and the combination (OBP-301 plus IL-2)] according to the tumor burden. The mice were administered 16000 units of recombinant IL-2 protein intraperitoneally or/and with OBP-301 (10^7 PFU/100 μl) via the tail vein, on day 0, day 2 and day 4 of the treatment. In the PBS treatment group, 100 μl of PBS was injected both intraperitoneally and intravenously as the vehicle control. On day 14 after each treatment, tumor imaging was done and the tumor volume was analyzed in each mouse. The mice were then sacrificed and the weight of the orthotopic tumor in the left kidney and the bilateral lung tissue specimens with metastases were measured.

**Flow cytometry**

For flow cytometry, 100 μl of blood sample from each mouse was collected in a plastic tube with ethylenediaminetetra-acetic acid (EDTA). The blood was then incubated with phycoerythrin-labeled anti-mouse CD4 antibody (eBioscience, San Diego, CA) and fluorescein isothiocyanate-labeled anti-mouse Foxp3 antibody (eBioscience) for 1 hour at 4°C. The labeled sample was washed twice with cold PBS. The sample was resuspended in 250 μl of cold PBS and analyzed using a fluorescence-activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA) by gating on lymphocytes.
**Histology and immunohistochemistry**

The mice were sacrificed for a histological analysis on day 14 after each treatment. The tissues were removed, fixed in formalin, embedded in paraffin, and sectioned. The 5 μm sections were stained with hematoxylin and eosin and examined for histological changes. Immunohistochemistry was performed using a primary antibody against adenovirus type 5 E1A (BD PharMingen, San Diego, CA) to identify the OBP-301-infected tumor cells, as previously described.14,15 Briefly, the tumor tissue was excised, placed in the optimal cutting temperature (OCT) compound, and snap frozen in liquid nitrogen. The frozen sections (10 μm) were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature (RT), washed, and permeabilized with PBS containing 0.1% Triton X-100. The endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 10 minutes. The sections were incubated with the primary antibody (1 : 50 dilution with PBS) at RT for 16 hours and then with Histofine Simple Stain MAX PO (Nichirei, Tokyo, JAPAN) for 30 minutes. Subsequently, the bound antibodies were detected using diaminobenzidine-tetrahydrochloride as the substrate and the sections were then counterstained with hematoxylin.

**Statistical analysis**

The data are shown as the mean ± standard deviation. Unpaired Student’s t-test was performed for analyzing the statistical difference between any 2 groups. Differences were considered to be significant if the P value was less than 0.05.
Results

In vitro anti-cancer efficacy of OBP-301 and IL-2 in RENCA cells

The RENCA cells were infected with OBP-301 at the indicated MOIs to determine whether OBP-301 infection induces cancer cell-killing. At a MOI of 10, a significant reduction in RENCA cell number to less than 20% was observed on day 3 and day 4 after OBP-301 treatment (Fig. 1a). Increasing concentrations of recombinant IL-2 protein (10-1000 units/ml) did not contribute any anti-proliferative effects on RENCA cells (Fig. 1b). When RENCA cells were treated with 1 MOI of OBP-301 mixed with 10, 100 and 1000 U/ml of IL-2, the addition of IL-2 did not enhance the cell-killing effect of OBP-301 (data not shown).

Potent anti-tumor effects of combined OBP-301 and IL-2 treatment on both orthotopic and metastatic tumors in the RCC mouse model

The therapeutic efficacy of OBP-301 plus IL-2 treatment on the growth of RENCA cells was assessed in vivo. Luciferase-expressing RENCA cells were injected orthotopically and intravenously into immunocompetent mice to produce a mouse RCC model with an orthotopic tumor and metastatic lung tumors. Ten days after the cell injection, tumor luciferase-signaling was typically observed in the area of left kidney (Fig. 2a, [Before treatment (Day 0)]) using the IVIS instrument. Next, the mice were treated with one of 4 treatments [PBS, IL-2, OBP-301, and the combination (OBP-301 plus IL-2)]. After 14 days of the treatment, the PBS treated mice showed a significant tumor growth, as estimated by IVIS (Fig. 2). OBP-301 administration induced more than a 50% reduction of tumor growth in comparison to the PBS group (Fig. 2b). The combination of OBP-301 plus IL-2 protein induced a potent tumor-inhibitory effect that
was statistically strong in comparison to that induced by the administration of each single agent. The tissue weight of the orthotopic tumor 14 days after the treatment revealed that the combination treatment induced a significant inhibition of the tumor growth in the kidney (Fig. 3a). The growth of metastatic lung tumors was also assessed in the treatment groups. After 14 days of treatment, the bilateral lungs were removed and the weight was measured. The PBS treated group, showed a large number of lung tumors on the surface (Fig. 3b). The increase of the lung weight was significantly suppressed in the OBP-301 and IL-2 combination group in comparison to the other groups, thus indicating the strongest inhibitory effects of the lung tumor growth (Fig. 3b). The tissue weight of orthotopic tumor and lungs with metastatic tumor was correlated with the level of luciferase expression analyzed by IVIS between the treatment groups.

*In vivo synergistic effect of OBP-301 treatment on the down-regulation of peripheral CD4⁺ Foxp3⁺ regulatory T cells by IL-2 administration*

The population of CD4⁺ Foxp3⁺ regulatory T cells (Tregs) is elevated in patients with progressive cancer and Tregs can thwart protective anti-tumor immunity. Recent human cancer trials suggest that depleting Tregs can be clinically beneficial. In order to explore the potential mechanisms underlying the potent anti-tumor effect elicited by the combination of OBP-301 and IL-2, the percentage of the CD4⁺ Foxp3⁺ regulatory T cells was assessed in the peripheral blood of each mouse. Blood was collected prior to initiating the treatment and on day 14 after the treatment. The percentage of CD4⁺ Foxp3⁺ T cells in the total lymphocytes was quantified by FACS analysis. The population of CD4⁺ Foxp3⁺ T cells was similar between all of the groups.
before the treatment (Fig. 4). After 2 weeks of treatment, the percentage of CD4⁺ Foxp3⁺ T cells in the IL-2 treated group was reduced in comparison to that of the PBS treated mice (Fig. 4b). Furthermore, the population of CD4⁺ Foxp3⁺ T cells was significantly down-regulated in the combination group of OBP-301 and IL-2 treatment in comparison to that of the IL-2 treated group (Fig. 4b). The decrease in the percentage of peripheral CD4⁺ Foxp3⁺ T cells was observed only in the combination and IL-2 groups.

Adenoviral E1A protein expression and tumor degeneration in an orthotopic tumor and metastatic lung tumors

In order to investigate the effect of the OBP-301 action, the mice and tumors were histologically examined on day 14 after each treatment. An immunohistochemical analysis confirmed the expression of the adenoviral E1A protein in both the orthotopic RENCA tumor and metastatic lung tumors with OBP-301 treatment (Fig. 5a), indicating that intravenously administered OBP-301 spread via the blood circulation and distributed into the orthotopic and lung tumors. The E1A protein expression was not observed in the normal organs including the liver. A histopathological analysis revealed massive tissue degeneration in the orthotopic tumor and metastatic lung tumors of the OBP-301 and IL-2 combination group (Fig. 5b). Such tissue degeneration was not observed in the tumors of the PBS treated group. These findings provided consistent evidence for tumor volume reduction or growth inhibition observed after the combined treatment. In addition, the analysis of liver and other body tissue specimens indicated no histological damage in each treatment group (data not shown).
Discussion

Oncolytic adenoviruses have been developed as a novel anti-tumor therapeutic strategy and their effectiveness is being currently tested in clinical trials.\textsuperscript{9} In order to improve the therapeutic index of anti-cancer agents, it is necessary that they selectively target cancer cells and spares the normal cells. Investigators have been focusing on developing such cancer-specific biochemical drugs.\textsuperscript{8,13} A replication-selective tumor-specific adenoviral agent, OBP-301 was constructed for the development of a cancer-specific therapeutic modality. OBP-301 has potent anti-cancer activities against many types of human malignancies such as lung, colon and prostate cancer.\textsuperscript{6,9}

The \textit{in vitro} assay with mouse renal cell carcinoma cells demonstrated the cytotoxic effect of OBP-301. On the other hand, no cytotoxic effect was induced by the IL-2 treatment alone. It is reasonable because IL-2 will not be able to exhibit it's anti-cancer effects in the absence of the immune cells. In the \textit{in vivo} experiment, intravenously administrated OBP-301 had significant cancer-killing effects in the both orthotopic renal tumor and metastatic lung tumors. There was no definite histological damage in the examined normal organs. In addition, the immunohistochemical examination disclosed the definite cancer selectivity because adenoviral E1A expression was clearly observed in the tumor tissue but not in the normal tissue. These results indicate that intravenously injected OBP-301 can specifically target the cancer tissue in the kidney and lung through the blood vessel and can replicate in the tumor.

The current study revealed that the combined administration of intravenous OBP-301 plus intraperitoneal IL-2 induced robust therapeutic effects on both the orthotopic renal tumor and metastatic lung tumors. To examine the anti-cancer immunomodulation in each mouse, the ratio of peripheral CD4\textsuperscript{+} Foxp3\textsuperscript{+} regulatory T cells (Tregs) was
measured by FACS analysis. Although there was no inhibitory effect of the Tregs population in the OBP-301 alone, the population of Tregs was significantly down-regulated after the OBP-301 and IL-2 combined therapy in comparison to the IL-2 alone group. Therefore, the *in vivo* synergistic effect of OBP-301 treatment on the IL-2 induced down-regulation of peripheral Tregs was observed in the current RCC model. This immunological synergistic effect in the suppression of Tregs may explain the robust anti-tumor therapeutic effects by the OBP-301 and IL-2 combination therapy.

IL-2 is a T cell growth factor that is thought to play a critical role in the regulation of T cell-dependent immune responses. High-affinity IL-2 receptor-mediated cell signaling is critical for the regulation of Tregs *in vivo*. Therefore, it appears that the therapeutic effect of IL-2 is at least partially due to the immunological activation by the down-regulated Tregs lymphocytes. In addition, a recent study showed that OBP-301 virus-mediated oncolysis might be an effective stimulus for immature dendritic cells to induce specific response against cancer cells in humans. These findings may provide the clue to clarify the mechanism of synergistic anti-tumor immunological response elicited by the combination of OBP-301 and IL-2.

In conclusion, the combined administration of OBP-301 and IL-2 exhibited a potent therapeutic effect in the model of RCC without any definite toxicities. These findings are noteworthy in terms of the clinical applications of this combination therapy for patients with both primary and metastatic renal cell carcinoma. The outcomes of this study also provide important implications regarding replication-selective oncolytic immunovirotherapy in human cancers.
References


Figure legends

Figure 1

*In vitro* cell-killing assay of OBP-301 and recombinant IL-2 protein in RENCA renal cancer cells.

(a,b)

OBP-301 and IL-2 were added to the culture medium at the indicated concentration and then the cells were incubated for the indicated days. The relative cell number (%) is shown in comparison to the control group (no treatment). The number of experiments was ten. *: A significant difference was observed in comparison to the group with no treatment.

Figure 2

Anti-tumor effect of OBP-301 and/or IL-2 treatment on the growth of the orthotopic and lung RENCA tumors in BALB/c mice.

(a)

Bioluminescence images of five mice from each treatment group were shown on the indicated day. The animals with the tumors derived from luciferase- stably transfected RENCA cells were imaged after luciferin injection via an IVIS instrument.

(b)

The tumor volume was analyzed by IVIS after the indicated treatment. Tumor volume of each mouse was determined by region-of-interest analysis of total photons per second. Five mice were analyzed in each group. *: A significant difference was observed in comparison to the group with IL-2 alone and OBP-301 alone.
Figure 3
Anti-tumor effect of OBP-301 and/or IL-2 treatment on the growth of the orthotopic and lung RENCA tumors in BALB/c mice.

(a) Representative macroscopic view of the resected orthotopic tumors is shown in the indicated treatment groups (upper panel). The tumor tissue weight (including normal kidney) was measured and analyzed between the groups. Twelve to fourteen mice were analyzed in the treatment groups. *: A significant difference was observed in comparison to the group of IL-2 alone and OBP-301 alone.

(b) Representative macroscopic view of the resected lungs is shown in the indicated treatment groups (upper panel). The tumor tissue weight (including normal lung) was measured and analyzed between the groups. Twelve to fourteen mice were analyzed in the treatment groups. *: A significant difference was observed in comparison to the group of IL-2 alone and OBP-301 alone.

Figure 4
The percentage of peripheral CD4+ Foxp3+ regulatory T cells (Tregs) in the total lymphocytes was analyzed by FACS in each mouse.

(a) The representative FACS data were shown. The blood sample was collected prior to initiating the treatment (day 0) and on day 14 after the treatment.

(b)
The percentage of CD4^+ Foxp3^+ T cells in the total lymphocytes was quantified by FACS analysis and was shown in the indicated groups and treatment days. Five mice were analyzed in the treatment groups. *: A significant difference was observed in comparison to the group of IL-2 alone.

Figure 5
Both histological and immunohistochemical analyses were performed on orthotopic and lung RENCA tumors.
(a)
The expression of adenovirus E1A protein in the orthotopic and lung RENCA tumors was examined by immunohistochemistry on day 14 after the indicated treatment.
(b)
Hematoxylin and eosin staining of the orthotopic and lung RENCA tumors on day 14 after the indicated treatment.
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