Oral Administration of Immunopotentiator from \textit{Pantoea agglomerans} 1 (IP-PA1) Improves the Survival of B16 Melanoma-Inoculated Model Mice

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Abstract: To investigate the usefulness of the immunopotentiator from \textit{Pantoea agglomerans} 1 (IP-PA1) as a supportive drug in melanoma therapy, we analyzed the immunological effects of IP-PA1 on melanoma-inoculated model mice. Oral administration of IP-PA1 increased the serum levels of tumor necrosis factor (TNF)-\textgamma at 2 h after the administration and interferon (IFN)-\textgamma and IL-12 at 12 h after the administration in naïve BALB/cCrSlc mice as evaluated by ELISA. IP-PA1 did not affect the proliferation of melanoma cells directly determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Combinatory treatment of IP-PA1 with doxorubicin for 9 days increased the serum levels of IFN-\textgamma and IL-12 by 71.0 and 15.3\%, respectively, compared to the treatment of doxorubicin alone as evaluated by flow cytometry. The mean survival period of melanoma-bearing, doxorubicin treated mice was prolonged from 31.4 ± 7.1 to 35.3 ± 8.4, 51.1 ± 5.4, and 45.0 ± 8.4 days by combinatory treatment of IP-PA1 at the daily doses of 0.1, 0.5, and 1 mg/kg, respectively. In conclusion, the results of the present study suggest the usefulness of IP-PA1 as a supportive drug in melanoma therapy.

Key words: chemotherapy, immunomodulator, immunosuppression, IP-PA1, melanoma

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Introduction

Malignant melanoma (MM) is the most aggressive form of skin cancer, and its incidence is increasing faster than that of any other type of cancer [6, 19, 22, 26]. The predominant treatment for primary melanoma is wide local excision; however, even patients treated with surgical resection are at a high risk of nodal and distant metastases [11, 37]. In addition, MM is generally resistant to conventional therapies such as systemic chemotherapy and radiotherapy [5]; therefore, MM carries a poor prognosis [10].

One of the prognostic factors for cancer patients is the function of anti-tumor immunity, i.e., the defense mechanisms against the occurrence and progression of cancer [29, 43]. It has been reported that the tumor-lytic function of tumor-infiltrating lymphocytes is associated with the clinical response in melanoma patients [2]. The majority of tumor cells can potentially inhibit host immunity through various mechanisms such as the production of immunosuppressive IL-10 [9]. In addition, conventional chemotherapy [44], radiotherapy [21], and surgery [4, 24] frequently suppress host immunity; therefore, cancer patients are generally, immunologically suppressed.

Immunopotentiator from Pantoea agglomerans 1 (IP-PA1) is a low-molecular-weight (5 kDa) lipopolysaccharide (LPS) derived from the cell walls of a symbiotic gram-negative bacteria commonly found in various food crops, such as cereals, fruits, and vegetables [17, 18, 20, 27]. IP-PA1 has immune-enhancing functions such as protection from bacterial and parasitic infections in mice [8, 20, 32], and recovery from stress-induced immunosuppression in chickens [14]. Thus, IP-PA1 is considered to be a potentially edible immunomodulator.

To investigate the usefulness of IP-PA1 as a supportive drug in melanoma therapy, we evaluated the effects of the oral administration of IP-PA1 in a melanoma-inoculated mouse model. We initially assessed the effects of IP-PA1 on the proliferation of melanoma cells and on the cytotoxicity of doxorubicin in vitro. Then, we evaluated the immunological effects of a combinatory treatment of IP-PA1 with doxorubicin in melanoma-inoculated model mice. Finally, the survival of melanoma-bearing mice treated with IP-PA1 and doxorubicin was examined for 60 days.

Materials and Methods

Mice

Five- to six-week-old female C57BL/6NCrSlc mice and BALB/cCrSlc mice (Japan SLC Inc., Shizuoka, Japan) were maintained at the Tokyo University of Agriculture and Technology, Tokyo, Japan. The mice were kept in an air-conditioned room, and fed standard laboratory food and water ad libitum. All experiments were approved by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

Cell culture

Murine melanoma B16 cells, originating from female C57BL/6 mice [16], were obtained from the RIKEN BioResource Center (Tsukuba, Japan). B16 cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640; GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with l-glutamine, 10% foetal bovine serum (FBS; HyClone; Thermo Scientific, South Logan, UT, USA), and 100 µg/ml each of penicillin and streptomycin (Nacalai Tesque, Kyoto, Japan).

Reagents

IP-PA1 was purified to more than 99% purity by using previously described methods [34]. LPS derived from Escherichia coli and doxorubicin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Affinity-purified rat anti-mouse tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-12 antibodies, biotin-labeled rat anti-mouse TNF-α, IFN-γ, and IL-12 antibodies, avidin-horseradish peroxidise (HRP), and standard recombinant mouse TNF-α, IFN-γ, and IL-12 were obtained from eBioscience Inc. (San Diego, CA, USA). Unless otherwise indicated, all of the other chemicals used were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Oral administration of IP-PA1 to naïve BALB/cCrSlc mice

Six-week-old naïve female BALB/cCrSlc mice were administered 0, 0.5, or 1 mg/kg of IP-PA1 in PBS. Their
blood samples were collected by heart puncture under deep anesthesia with diethyl ether 2 or 12 h after the administration of IP-PA1.

**ELISA**

NUNC MaxiSorp 96-well ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated with primary antibodies (100 µl, 1 µg/ml) in 0.1 M NaHCO₃ (Nacalai Tesque), incubated overnight at 4°C, and washed with PBS containing 0.05% Tween 20 (PBS-T). The plates were blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 1 h at room temperature, then washed with PBS-T. Standard recombinant TNF-α, IFN-γ, or IL-12 (100 µl) and serum sample diluted with 1% BSA in PBS were added to the wells, and the plates were incubated for 1 h at room temperature; subsequently, the plates were washed with PBS-T. A biotin-labeled detection antibody (100 µl, 1 µg/ml) was added to the wells; after which the plates were incubated for 1 h at room temperature and then washed with PBS-T. HRP-conjugated avidin (100 µl, 1 µg/ml) was added to the wells; the plates were incubated for 1 h at room temperature and then washed with PBS-T. Finally, colorimetric signals were generated using TMB One Solution (Promega Corp., Madison, WI, USA). The reaction was stopped by adding 100 µl of 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl. The absorbance was measured at 595 nm by using a model 550 microplate reader (Bio-Rad Laboratories Inc.). The relative number of viable cells was calculated as the ratio of the optical density of the treated cells to that of the cells before the start of treatment.

**In vivo melanoma-inoculation model**

C57BL/6NCrSlc mice were intraperitoneally injected with 2.0 × 10⁵ B16 cells diluted in calcium- and magnesium-free Hank’s balanced salt solution (Sigma-Aldrich). Tumor-inoculated mice were administered 0–1 mg/kg IP-PA1 in PBS orally everyday and 0 or 0.1 mg/kg doxorubicin in saline intraperitoneally on alternative days starting 1 day after the melanoma inoculation. For the evaluation of the immunological aspects, the mice were administered IP-PA1 and doxorubicin for 8 days. Blood samples were collected by heart puncture under deep anesthesia with diethyl ether 24 h after the final administration, and their spleens were isolated for flow cytometry analysis. To evaluate the effects on the survival of melanoma-bearing mice, the mice were administered IP-PA1 and doxorubicin for 60 days, and the survival and body conditions (behavior, abdominal expansion, wasting, and dehydration) of each mouse were examined twice a day. When mice were reached the humane end point, defined by more than 20% body weight loss, they were euthanatized and their survival periods were defined as the number of days until euthanasia.

**Flow cytometry analysis**

Spleens of mice were individually prepared as single-cell suspensions in 3 ml RPMI 1640 supplemented with L-glutamine, 10% FBS, and 100 µg/ml of penicillin and streptomycin. The red blood cells were removed by a lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA₂Na). The cells were washed with complete RPMI, and counted using a trypan blue exclusion method. The cells were then adjusted to 1.0 × 10⁶ cells/100 µl in complete RPMI and incubated for 30 min on ice in the presence of FITC-conjugated anti-mouse CD4 or CD8 monoclonal antibodies (eBioscience Inc.), or FITC-conjugated anti-mouse pan-NK cells (Sigma-Aldrich). The cells
were subsequently washed 3 times with cold PBS, 0.1% BSA, and 0.01% sodium azide, and analyzed on an Epics Elite Flow Cytometer (Coulter, Tokyo, Japan).

Statistical analysis
All data are expressed as the mean ± SEM. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with post hoc analysis followed by Bonferroni’s test. Kaplan-Meier survival curves were analyzed using the log-rank test.

Results

Cytokine production following the oral administration of IP-PA1 in naïve BALB/c mice
Blood samples were collected from naïve BALB/cCrSlc mice at 2 or 12 h after the oral administration of IP-PA1. The serum levels of TNF-α, IFN-γ, and IL-12 were determined using ELISA. The serum levels of TNF-α were increased at 2 h after the administration of IP-PA1 (Fig. 1A) and remained so at 12 h after the administration (Fig. 1B). The serum levels of IL-12 and IFN-γ were increased only at 12 h after the administration of IP-PA1 (Fig. 1C and 1D).

Effect on the proliferation of B16 melanoma cells
B16 cells pretreated with 0–100 mg/ml IP-PA1 in PBS were treated after 1 h with 0–1 µM of doxorubicin in vitro. Cell proliferation was determined using an MTT assay 72 h after treatment with doxorubicin. IP-PA1 did not affect the proliferation of B16 cells when compared to cells that were not treated with doxorubicin (Table 1). Doxorubicin inhibited the proliferation of B16 cells in a dose-dependent manner; however, IP-PA1 did not affect the inhibitory effects of doxorubicin on B16 cells (Table 1).

Serum cytokine levels in melanoma-bearing mice
C57BL/6NCrSlc melanoma-bearing mice were administered doxorubicin and IP-PA1 for 8 days. Blood samples were collected at 24 h after the final administration and the serum levels of TNF-α, IFN-γ, and IL-12 were determined using ELISA. The serum levels of

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<th>Table 1.</th>
<th>Effects of the pre-treatment of IP-PA1 on the cytotoxicity of doxorubicin to B16 melanoma cells in vitro</th>
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<tr>
<td>IP-PA1 (ng/ml)</td>
<td>Doxorubicin (µM)</td>
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<tr>
<td>0</td>
<td>0.1</td>
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<tr>
<td>0</td>
<td>196.7 ± 19.1</td>
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<td>10</td>
<td>197.2 ± 24.7</td>
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The results are expressed as the mean ± SEM of 3 independent experiments.

**P<0.01 compared to doxorubicin-untreated control cells.
EFFECTS OF IP-PA1 ON MELANOMA-INOCULATED MICE

TNF-α and IL-12 did not change, and the levels of IFN-γ were slightly not significantly decreased in melanoma-bearing mice compared to naïve mice (data not shown). In melanoma-bearing mice, the combinatory treatment of IP-PA1 and doxorubicin increased the serum levels of IFN-γ compared to treatment with no drug or doxorubicin alone (Fig. 2A). In melanoma-bearing mice, the combinatory treatment of IP-PA1 and doxorubicin increased the serum levels of IFN-γ compared to treatment with doxorubicin alone (Fig. 2B). No difference was observed in the serum levels of TNF-α among the groups (data not shown).

Proportion of immune cells in the spleen

C57BL/6NcrSlc melanoma-bearing mice were administered doxorubicin and IP-PA1 for 8 days. Spleens were isolated at 24 h after the last treatment and the proportion of NK cells, CD4+ T cells, and CD8+ T cells were determined using flow cytometry. The population of NK cells and the ratio of CD4+ to CD8+ T cells were slightly decreased in melanoma-bearing mice compared to naïve mice; however, these differences were not statistically significant (data not shown). In melanoma-bearing mice, the population of NK cells and the ratio of CD4+ to CD8+ T cells were significantly increased in mice treated with the combinatory treatment of IP-PA1 and doxorubicin compared to mice treated with no drug or doxorubicin alone (Fig. 2C and 2D).

Improvement of survival in melanoma-bearing mice

C57BL/6NcrSlc mice inoculated with B16 melanoma cells died 19.3 ± 1.2 days after the tumor inoculation when they were received no drug. Depressive behavior, severe wasting, dehydration, and abdominal expansion were observed in more than half of the mice at 10 days after the inoculation. The abdominal cavities of each necropsied mouse were occupied with clusters of melanotic tumors, and their intestinal tracts were transparent. Diffused tumor cells were often observed on the surface of the lungs, liver, spleen, and kidneys. Two of eight drug-untreated control mice were euthanatized because of rapid weight loss before natural death. The intraperitoneal administration of doxorubicin prolonged the survival of melanoma-bearing mice to 31.4 ± 7.1 days (Fig. 3). The combinatory treatment of 0.5 mg/kg IP-PA1 with doxorubicin significantly prolonged the survival compared to mice that were only administered doxorubicin, which was statistically significant under the log-rank test (P=0.02; Fig. 3A). The mean survival periods of mice treated with 0.1, 0.5, or 1 mg/kg/day IP-PA1 with doxorubicin were 35.3 ± 8.4, 51.1 ± 5.4, and 45.0 ± 8.4 days, respectively (Fig. 3B). All mice administered no drug or only doxorubicin died before day 60; however, 25.0, 51.4, and 45.1% of mice treated with 0.1, 0.5, or 1 mg/kg/day IP-PA1 with doxorubicin were still alive on day 60, respectively. The combinatory treatment of IP-PA1 with doxorubicin was more effective at the 0.5 mg/kg/day dose than at the other doses. Clusters of melanotic tumors were not observed in the abdominal cavities of most of the surviving mice.
LPS derived from conventional bacteria activates macrophages and dendritic cells (DCs) via the toll-like receptor (TLR)-4, a specific receptor of LPS [3]. IP-PA1 also stimulates macrophages partly through the activation of nuclear factor (NF)-κB, a major target of TLR-4, in vitro [15]. The oral administration of IP-PA1 to naive BALB/cCrSlc mice increased the serum levels of TNF-α (Fig. 1A and 1B), IFN-γ (Fig. 1C), and IL-12 (Fig. 1D) within 2 (TNF-α) or 12 h (IFN-γ and IL-12), possibly due to activation by IP-PA1 of macrophages and DCs in the gut mucosal tissue.

IP-PA1 increased the serum levels of IFN-γ (Fig. 2A) and IL-12 (Fig. 2B) in melanoma-bearing C57BL/6NCrSlc mice, indicating that IP-PA1 enhances host immunity even in tumor-bearing, chemotherapy-treated, immunosuppressed hosts. IP-PA1 increased the proportion of splenic NK cells (Fig. 2C) and improved the ratio of splenic CD4+ to CD8+ T cells, a typical marker of chemotherapy-induced lymphocytic depletion (Fig. 2D) [25]. These results indicate IP-PA1 induced recovery in the immune system of this model. As IP-PA1 did not directly affect the proliferation of B16 melanoma cells (5), we speculate that the significant improvement of survival induced by IP-PA1 treatment in melanoma-bearing mice (Fig. 3) was predominantly due to the enhancement of anti-tumor immunity (Fig. 4). This suggests that the application range of IP-PA1 extends to other tumor-related diseases as well as a melanoma.

Various kinds of immune cells are involved in anti-tumor immunity. Macrophages directly recognize and kill tumor cells [33, 39]; they phagocytose apoptotic tumor cells [30], and process and present tumor-specific antigens to CD8+ cytotoxic T lymphocytes (CTL) that
directly lyse antigen-specific tumor cells [36]. NK cells play a pivotal role in the surveillance and exclusion of developing cancer [12, 13]. Several cytokines produced by activated macrophages improve anti-tumor immunity by affecting other immune cells. TNF-α enhances T cell responses partly by increasing the expression of the IL-2 receptor [31] and mediates the differentiation of monocytes [41]. IFN-γ enhances the anti-tumor functions of CTL [28] and NK cells [1]. IL-12 supports the effective anti-tumor function of CTL partly by increasing the number of macrophages [38]. BALB/c is regarded as a Th2-skewing strain, which means it is a low producer of Th1 cytokines such as IFN-γ and IL-12 [23]. In the present study, the inductions of IFN-γ and IL-12 by IP-PA1 were shown in BALB/cCrSlc mice (Fig. 1), indicating that IP-PA1 is a strong inducer of Th1-type responses.

Host immunity may support the outcome of chemotherapy. For example, the insufficient removal of apoptotic tumor cells by macrophages prevents effective chemotherapy because apoptotic tumor cells are strong suppressors of anti-tumor immunity [40]. However, chemotherapeutic agents including doxorubicin often suppress host immune cells [8]. IP-PA1 exhibits potent protective effects against doxorubicin-induced immunosuppression such as the inhibition of macrophage growth [15], which may be one of the reasons behind the prolongation of survival of the melanoma-inoculated model mice observed in the present study.

Although LPS from pathological gram-negative bacteria such as Escherichia coli have strong immune-enhancing effects in vitro, their clinical application has been limited because they cause severe septic shock when large amounts enter the blood stream directly [7]. However, in contrast to systemic injection, no toxicity of LPS has been reported when it has been orally administered in safety tests: the oral administration of 1,000 mg/kg IP-PA1 for 28 days generated no toxic effects in rats [18, 35]. In addition, IP-PA1 derived from symbiotic gram-negative bacteria found in crops is considered safe because it has been ingested by humans and animals for a long time [20]. Therefore, IP-PA1 is expected to act as an immunomodulator, which is one of major reasons why we assessed immunological effects and the usefulness of orally-administered IP-PA1 in melanoma therapy.

Orally administered IP-PA1 has shown preventive effects against gastric ulcer and parasitic infections; however, LPS from Escherichia coli or other conventional bacteria has shown very small or negligible effects [27, 32]. IP-PA1 forms smaller micelles in solution than LPS from E. coli because it has a short O-chain, is confer advantages for infiltration of mucosal tissues and stimulation of mucosal macrophages, plausible reasons for the demonstrated effects of orally-administered IP-PA1. There is not enough evidence regarding the safety of oral administration of IP-PA1 to cancer patients; however, the doses of IP-PA1 that demonstrated immunological effects in the present study are much lower than the no observed effect level in toxicity tests. Therefore, IP-PA1 may be useful as an edible supportive drug in melanoma therapy.

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References

8. Dominguez-Rodriguez, J.R., Chaparro-Huerta, V.,


