Injection of cell-wall skeleton of *Mycobacterium bovis* BCG draining to a sentinel lymph node eliminates both lymph node metastases and the primary transplanted tumor

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1. Introduction

About 30 years ago, bacteria and polysaccharides were intensively studied as biological response modifiers (BRMs) for cancer therapy (1-6), but limited effects were observed in patients. In those days, little was known about the mechanisms leading to tumor suppression or eradication, and regimens and target diseases were therefore not optimized. Recently, with the development of immunology, innate immunity in particular, the activation of macrophages and dendritic cells (DCs) has been successfully bridged to elicit acquired immunity (7,8). We now know that a draining lymph node plays a central role in the generation of immunity against invading organisms. Furthermore, the molecular aspects of macrophage and DC activation by microbes are becoming clear (9,10) and agonists of toll-like receptors (TLRs), such as imiquimod and CpG oligodeoxynucleotide, have been highlighted (11-13). BCG-CWS, known to be one of the strongest adjuvants (14,15), also stimulates TLRs (16,17) and gene-induction profiles have been demonstrated (18). This prompted us to reevaluate BCG-CWS based on the recent evidence of cancer immunology. We prepared CWS from *M. bovis* BCG Tokyo 172 strain with purity of more than 97% (SMP-105) and investigated the effect on macrophages to identify TLR2/MyD88-dependent activation (19,20). Partial structures were chemically synthesized and the macrophage-activating activities were investigated (21).

Most studies of cancer immunotherapy involve exogenous tumor antigens (22-25), but cancer...
patients have an enormous number of tumor antigens endogenously. We think that when endogenous tumor antigens are properly exploited and presented to T cells by activating innate immunity, tumor immunity will be generated and tumor cells will be eradicated. The potential of mono-therapy with BCG-CWS was therefore studied.

To select a disease model, we focused our attention on spontaneous lymph node metastases, because we expected that tumor cells would be easily exploited as antigens for activating T cells by targeting the lymph nodes; furthermore, this is an important disease model as the prognosis of cancer treatment is almost always determined by the control of lymph node metastases. A transplantable tumor cell line, line 10 hepatocarcinoma, derived from a diethyl-nitrosamine-induced hepatoma in an inbred strain 2 guinea pig (26), establishes metastases in a sentinel lymph node at high frequency (27,28) and, for more than 100 years, guinea pigs have been used as important experimental animals for the study of infectious microbes. We therefore employed strain 2 guinea pigs inoculated with syngeneic line 10 hepatoma.

Many reports studied the antitumor effects of BCG or the cell wall of BCG using WST-8 (DOJINDO Laboratories, Kumamoto, Japan).

In this study, the injection route was improved to exclude the effect of local inflammation on tumor growth. SMP-105 revealed a strong tumor-eliminating effect both on primary tumor and lymph node metastases. Separate injection from tumor draining to a sentinel lymph node is an improved method in classical guinea pig models for investigating immunostimulators.

2. Materials and Methods

2.1. Animals

Male strain 2 guinea pigs, five weeks of age, were obtained from Japan SLC Inc., Shizuoka and used when they were six weeks old. Animals were maintained under specific pathogen-free conditions. Their maintenance and all experiments were conducted with the approval of the DSP Animal Care and Use Committee.

2.2. Cell line

Line 10 hepatocellular carcinoma cells were implanted intraperitoneally and a range of cell stock was prepared in liquid nitrogen. In each experiment, cells were freshly thawed before intradermal inoculation.

2.3. Preparation of SMP-105

SMP-105 is a product of Dainippon Sumitomo Pharma Co., Ltd and chemical analysis data have been reported by Uenishi et al. (39). Briefly, SMP-105 contains less than 3% (w/w) of sugars and amino acids assumed not to constitute CWS. Both DNA and trehalose dimycolate are removed to less than 0.05% (w/w) and lipopolysaccharide is about 0.0015 EU/mg by gel-clot technique. An oil-in-water emulsion of SMP-105 was prepared and lyophilized on the thousand-vial scale. Each vial contained 1.2 mg of SMP-105, 32 mg of squalane, 20 mg of polysorbate 80 and 100 mg of mannitol. Vehicle preparation used the same formulation except for SMP-105. SMP-105 in emulsified form was used for inoculation, and the suspended form in saline was prepared for in vitro use.

2.4. Direct cytotoxic effect on line 10 hepatoma

Line 10 hepatoma cells were incubated with SMP-105 or mitomycin C for 48 h and viability was assayed using WST-8 (DOJINDO Laboratories, Kumamoto, Japan).

2.5. Antitumor effects

Line 10 hepatoma cells stored in liquid nitrogen were rapidly thawed and washed three times with Hanks’ balanced salt solution (HBSS). 4×10^6 cells were injected into strain 2 guinea pigs intraperitoneally and ascites were collected from failing animals after about 10 days. Line 10 hepatoma cells were prepared by washing the ascites cells three times with HBSS and inoculated intradermally at 1×10^6 cells in 0.1 mL into the right thoracic flank region. SMP-105 or vehicle was injected intradermally into sites distal to the site of tumor inoculation on days 0, 7 and 14. In the postoperative model, the primary dermal tumor nodule was excised on day 7 under ketamine and xylasine anesthesia, and SMP-105 at a dose of 60 μg or vehicle was injected into sites dorsal and ventral to the site of tumor excision on days 7 and 14, respectively. In the no-operation group, SMP-105 was injected on days 0, 7 and 14.

The size of the primary skin tumor nodule was calculated as the squared average of the long and short diameter perpendicular to each other.

Animals were sacrificed by anesthesia with a high concentration of carbon dioxide and an axillary lymph node was collected and weighed. For pathological study, the lymph node was fixed with 10% of formaldehyde solution. It was then cut into two equal pieces in the apsis direction and a slice of the section was stained with hematoxylin and eosin. Metastasis...
was scored from 0 to 4 based on the area occupied by tumor cells.

2.6. Challenge of live tumor cells

Line 10 hepatoma cells collected from ascites were treated with mitomycin C (MMC) (Kyowa Hakko Kagyo Co., Ltd, Tokyo, Japan) at 100 µg/mL for 20 min at 36°C in a water bath, washed three times with HBSS and stored in liquid nitrogen. 1×10⁶ inactivated cells were inoculated into the right thoracic flank region, and SMP-105 or vehicle was injected into the same side distal to the site of tumor inoculation. Fourteen days after treatment, live line 10 hepatoma cells were injected into the opposite side. Animals were observed for tumor growth.

2.7. Distribution of SMP-105 in lymph nodes and spleen

SMP-105 was injected intradermally into the thoracic flank region of strain 2 guinea pigs at 60 µg, and the spleen and bilateral axillary lymph nodes were sampled at various time points over 7 days. Lymph nodes and spleen were fixed with 10% of formaldehyde solution, and SMP-105 was analyzed immunohistochemically using rabbit anti-<i>M. bovis</i> BCG antibody (DAKO Japan Co. Ltd., Kyoto, Japan).

2.8. Statistical analysis

Wet weights of lymph nodes and primary tumor sizes of each observation day were compared with the vehicle group using Steel's test, or Wilcoxon's test when comparing two groups. Statistical analysis was performed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Antitumor effect of SMP-105 on lymph node metastasis and primary tumor

In order to use SMP-105 as an immunostimulator, the direct cytotoxic effect was determined <i>in vitro</i>. SMP-105 showed no cytotoxic activity in line 10 hepatoma cells (Figure 1).

Line 10 hepatoma cells were inoculated and an oil-in-water emulsion of SMP-105 or vehicle was injected into sites different from the tumor inoculation site in order to avoid damage to tumor cells by local inflammation.

SMP-105 demonstrated prominent antitumor activity at very low doses such as 3.75 µg for both the primary tumor (Figure 2A) and lymph node metastases (Figure 2B), from which the effect of local inflammation on tumor cells was excluded. Growth of the primary implanted tumor began to decrease in some animals about 2 weeks after the first dose of SMP-105.

When SMP-105 was injected into the opposite side, no effect was observed on the primary tumor or axillary lymph node metastases (Figures 3A and B).

3.2. Time course study of lymph node metastases

Metastases into a lymph node were pathologically investigated and the Metastasis Score was established as follows:

0: Tumor cells are not observed.
1: Tumor cells can be found as small clusters.
2: Clusters composed of a significant number of tumor cells are clearly observed.
3: Clusters composed of a large number of tumor cells, some of which are undergoing mitosis, are observed.
4: Tumor clusters occupy more than half the area of the lymph node.

Line 10 hepatoma cells were inoculated into the flank region and 60 µg of SMP-105 or vehicle was injected into different sites on days 0, 7 and 14. Clusters of line 10 hepatoma cells emerged by day 8 in the axillary lymph node irrespective whether SMP-105 or vehicle was injected; thereafter, the area and number of clusters developed over time in vehicle-injected guinea pigs, whereas in animals treated with SMP-105, the number and area of tumor clusters diminished and tumor cells finally disappeared on day 18 (Table 1). A period of about 2 weeks was required before the effect of SMP-105 was observed in lymph node metastases.

A lymph node from one of three animals, sampled 21 days after SMP-105 injection was scored as 3, indicating the development of metastases (Table 1). Lymph node metastases failed to be eradicated in this animal, demonstrating that metastases are not always eliminated by treatment with SMP-105.
3.3. Elimination of tumor cells of established micrometastases

The primary dermal tumor nodule was excised on day 7, and SMP-105 or vehicle was injected. The elimination rate was demonstrated as the number of animals with Metastasis Score 0 to the total number of animals in the group. All animals treated with tumor excision alone or tumor excision and vehicle developed progressively growing tumors in the axillary lymph node, but tumor excision followed by SMP-105 injection eliminated tumor cells from the lymph node in four of eight guinea pigs (Table 2).

3.4. Distribution of SMP-105 in lymph nodes and spleen

Immunohistochemical investigation was performed using anti-BCG antibody obtained commercially. The anti-BCG antibody, which was raised against whole BCG bacteria, binds to SMP-105 by in vitro binding assay (data not shown). No unspecific staining was observed (Figure 4).

SMP-105 was inoculated intradermally into the right flank region and both right and left axillary lymph nodes and spleen were collected at each sampling time. Three hours post-inoculation, SMP-105 was detected in the marginal sinus of the axillary lymph node from the injection side, and even in the medullary cords of the lymph node 24 h after injection (Figures 4B and C), but not in the opposite lymph node or spleen (Figures 4B-F). Closer observation identified particles...
Table 1. Time course of lymph node metastases

<table>
<thead>
<tr>
<th>Days after implantation</th>
<th>Vehicle</th>
<th>SMP-105</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1, 1, 1</td>
<td>1, 1, 1</td>
</tr>
<tr>
<td>12</td>
<td>1, 2, 3</td>
<td>0, 0, 1</td>
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<td>15</td>
<td>2, 2, 3</td>
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<td>21</td>
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</tr>
<tr>
<td>25</td>
<td>4, 4, 4</td>
<td>0, 0, 0</td>
</tr>
</tbody>
</table>

Metastasis Score of individual animals (n = 3) defined in the text is indicated in the table. Line 10 hepatoma cells were inoculated and 60 μg of SMP-105 or vehicle was injected intradermally into a site distal to tumor inoculation on days 0, 7 and 14. Axillary lymph node was collected from tumor inoculation side on the indicated days and fixed with 10% formaldehyde solution. A slice of the lymph node was stained with hematoxylin and eosin.

Table 2. Effect of SMP-105 on established micrometastases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Elimination rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/8</td>
</tr>
<tr>
<td>SMP-105</td>
<td>5/8</td>
</tr>
<tr>
<td>Excision alone</td>
<td>0/8</td>
</tr>
<tr>
<td>Excision and vehicle</td>
<td>0/8</td>
</tr>
<tr>
<td>Excision and SMP-105</td>
<td>4/8</td>
</tr>
</tbody>
</table>

Line 10 hepatoma cells were inoculated and the primary dermal tumor nodule was excised on day 7. SMP-105 at a dose of 60 μg or vehicle was injected on days 7 and 14. Animals were sacrificed on day 21 and an axillary lymph node was collected. Elimination rate was the number of animals with Metastasis Score 0 to the total number of animals in the group.
with a positive signal at the marginal sinus engulfed by phagocytes with round or oval nucleus (Figure 4B).

3.5. Generation of systemic tumor immunity

MMC-treated line 10 hepatoma cells and SMP-105 or vehicle were inoculated separately. Two weeks after treatment, live line 10 hepatoma cells were injected into the opposite side. Strong erythema and edema, considered to be delayed-type hypersensitivity reactions, were induced at the challenge site, and tumor cells were rejected in four of eight animals pre-treated with tumor cells and SMP-105, whereas tumor cell growth was observed in all animals of other groups (Figure 5).
Figure 4. Distribution of SMP-105 in lymph nodes and spleen (continued).

Figure 5. Generation of systemic tumor immunity. Two weeks after separate inoculation of MMC-treated line 10 hepatoma cells and 60 μg of SMP-105 or vehicle, live line 10 hepatoma cells were challenged on the opposite side. Animals were observed for tumor growth. Tumor incidences are plotted against days after challenge (n = 8).
4. Discussion

We prepared a highly purified BCG-CWS (SMP-105) and evaluated its potential for cancer immunotherapy. We thought that if innate immunity is properly activated, tumor antigens distributed endogenously in cancer patients will be exploited to activate tumor immunity. We investigated strain 2 guinea pigs inoculated with line 10 hepatoma, a classical animal model, improving the administration route to exclude the effect of local inflammation on tumor growth.

We injected SMP-105 into sites different, but not distant from the tumor. Metastases in the axillary lymph node and primary skin tumor were eliminated after some time at very low doses in microgram order (Figures 2A and B). But when SMP-105 was injected into the opposite side from the tumor, neither metastases nor primary tumor was eradicated (Figures 3A and B). SMP-105 was observed in the axillary lymph node from the injection side (Figures 4B and C), but was not detected in the axillary lymph node from the opposite side or the spleen (Figures 4B-F). These data indicated that inoculation into sites draining to a sentinel lymph node is crucial for the antitumor activity of SMP-105.

Phagocytes engulfing SMP-105 were observed in the marginal sinus of the axillary lymph node at the injection side (Figures 4B and C). Since SMP-105 is insoluble and formulated into oil droplets of about 2 μm in diameter, three candidates can be listed as phagocytes: polymorphonuclear leukocytes, macrophages and immature dendritic cells (iDCs). From the simple, round or oval shape of the nucleus observed in the lymph node, it is likely that the phagocytes included macrophages and iDC. SMP-105 is therefore considered to activate cells that can potentially process antigens and present them to T cells in the lymph node. Further analysis of phagocytes is necessary. Activation of lymph node cells is investigated using mice in our next paper.

It is not clear whether SMP-105 flowed into the lymph node or was carried by phagocytes. As SMP-105 was injected as insoluble oil droplets and that a large amount of SMP-105 remained for a long time at the inoculation site (our next paper), it is more likely that macrophages or iDCs ingested SMP-105 at the inoculation site and migrated into the draining lymph nodes (40,41). Investigations as to how SMP-105 reached the lymph node are important to study the formulation for efficient targeting.

Both primary implanted tumors and metastases began to be eradicated about 2 weeks after injection of SMP-105 (Figure 2A, Table 1). When guinea pigs were challenged with live tumor cells 2 weeks after inoculation of tumor cells lacking proliferation activity and SMP-105 separately as antitumor experiments, strong edema and erythema, which was considered to be delayed-type hypersensitivity reactions, developed and the tumor was rejected in the half of the animals (Figure 5). From this evidence it is assumed that systemic tumor immunity was generated during the lag time and eradicated primary and metastasizing tumors.

Taken together, it is considered that SMP-105 activated immune reactions to antigens in the draining lymph node. When a sentinel lymph node was activated, cells capable of processing antigens and presenting them to T cells stimulated by the microbial components would encounter tumor cells and systemic tumor immunity would be generated.

About 30 years ago, when BRMs were intensively studied, lots of animal experiments were tried but systemic administration failed to slow down the growth of the implanted tumor. Then, intratumoral injection and tumor implantation admixed with a BRM were worked out. By these methods, however, antitumor activities are not properly evaluated (37,38). Our method reported in this paper is a more reasonable one, based on recent development of immunology, for evaluating immunostimulators making use of classical a guinea pig model.

After microscopic metastases settled in the axillary lymph node, the primary tumor was excised and SMP-105 was injected into the same side as tumor excision. Treatment with SMP-105 eliminated lymph node metastases in some animals (Table 2). Shu et al. reported that lymph nodes that harbor metastases demonstrate significant suppression in their ability to respond to antigenic stimulation (42), but our data show that lymph nodes with metastases recovered their ability to respond to malignant tumor by SMP-105.

Hayashi showed the excellent efficacy of BCG-CWS on head and neck cancer with lymph node metastases (43) and Kodama et al. reported the long survival of patients with recurrent supraclavicular lymph node metastases of lung cancer by treatment with BCG-CWS (44). This evidence may support our idea of exploiting lymph node metastases to generate tumor immunity by microbial immunostimulators.

In conclusion, we suggested that the potential of mono-therapy with a strong immunostimulator and that SMP-105 is one of the most promising agents for cancer immunotherapy by improving the administration route using classical guinea pig models. Although several papers have reported the inoculation of BCG or BCG-CWS as vaccine adjuvants separate from inactivated line 10 hepatoma cells (45-50), separate inoculation has not been tried for mono-therapy. This is therefore the first report presenting the potential of mono-therapy with BCG-CWS injection into sites different from the tumor. Separate injection from tumor draining to a sentinel lymph node using guinea pigs bearing line 10 hepatoma will be a useful method for investigating immunostimulators.
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