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MDP-Lys (L18), a Synthetic Muramyl Dipeptide Derivative, Enhances Antitumor Activity of an Inactivated Tumor Vaccine

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Abstract The adjuvant effect of a muramyl dipeptide (MDP) derivative, MDP-Lys (L18), on enhancing of antitumor immunity induced by X-irradiated tumor cells against highly metastatic B16-BL6 melanoma cells was examined in mice. Mice immunized intradermally (i.d.) with a mixture of X-irradiated B16-BL6 cells and MDP-Lys (L18) [Vac+MDP-Lys (L18)] followed by an intravenous (i.v.) inoculation of 10^4 viable tumor cells 7 days after immunization, showed a significant inhibition of experimental lung metastasis of B16-BL6 melanoma cells. The most effective immunization for the prophylactic inhibition of tumor metastasis was obtained from the mixture of 100 μg of MDP-Lys (L18) and 10^4 X-irradiatied tumor vaccine. Furthermore, immunization of mice with Vac+MDP-Lys (L18), 3 days after tumor challenge, resulted in a significant inhibition of lung metastasis of B16-BL6 melanoma cells in an experimental lung metastasis model. Similarly, the administration of Vac+MDP-Lys (L18), 1 or 7 days after tumor removal, markedly inhibited tumor metastasis of B16-BL6 in a spontaneous lung metastasis model. When Vac+MDP-Lys (L18) was i.d. administered 3 days after subcutaneous (s.c.) inoculation of tumor cells (5×10^5/site) on the back, mice treated with Vac+MDP-Lys (L18) showed inhibition of significantly tumor growth on day 20. These results suggest that MDP-Lys (L18) is able to enhance antitumor activity induced by X-irradiated tumor vaccine to reduce lung metastasis of tumor cells, and is a potent immunomodulating agent which may be applied prophylactically as well as therapeutically to treatment of cancer metastasis.

Key words: Bacteria cell wall, MDP, adjuvant, tumor vaccine, tumor metastasis

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A variety of natural and synthetic agents such as lymphokines, lipopolysaccharide (LPS), and *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP), which is the minimal structural unit of the peptidoglycan of bacterial cell wall, are known to have diverse biological functions like immunomodulatory activities [1, 6, 27] and to stimulate host resistance against cancer [9] and infections [11, 17, 19, 21]. Among these, MDP has received much attention as a candidate immunomodulator compound due to its wide spectrum of biological activities [2, 4, 30]. Many derivatives of MDP have also been synthesized and their immunological and immunotherapeutic properties have been reported [7,8,13,16,22].

In a series of studies on immunomodulating and antitumor activities of MDP and its synthetic derivatives, we previously demonstrated that B30-MDP, a lipophilic derivative of MDP, increased specific tumor immunity by enhancing cytotoxicity mediated by T lymphocytes, when it was co-immunized into mice with X-irradiated tumor cells [33]. In addition, MDP-Lys (L18) [N^{α}-acetylmuramyl-L-alanyl-D-isoglutaminyl-N^{ϵ}-stearoyl-L-lysine], which is another derivative of MDP, has been found to be a potent adjuvant to activate immune responses [3] and to stimulate nonspecific resistance against bacterial and viral infections [14, 15, 23, 24]. MDP-Lys (L18) has also been shown to induce interleukin-1 (IL-1) [26], which has various biological activities including the growth inhibition of some tumorigenic cells [20], and colony stimulating factors (CSFs) [26, 31, 32] which function as regulatory molecules related to the proliferation and differentiation of myeloid stem cells [25]. Furthermore, the restorative effect of MDP-Lys (L18) by stimulating hematopoiesis on leukopenia associated with anticancer chemotherapy and/or radiation therapy in cancer patients has recently been demonstrated [5, 10, 29].

Previously, we showed that MDP-Lys (L18) has adjuvant activity to enhance immunogenicity of viral vaccines such as a recombinant hepatitis B surface protein and a hantavirus inactivated vaccine [28, 37]. However, there is no report available on the immunoadjuvant effect of MDP-Lys (L18) on enhancement of specific immunity induced by tumor vaccines against cancers.

In the present study, we investigated whether MDP-Lys (L18) is effective in enhancing antitumor activity against highly metastatic B16-BL6 melanoma cells in experimental and spontaneous murine models by immunization with X-irradiated tumor cells.

Materials and Methods

Animals and Reagents

Specific-pathogen-free C57BL/6 mice, 7–8 weeks old, were purchased from the Sizuoka Laboratory Animal Center, Hamamatsu, Japan. Mice were maintained in the Laboratory of Animal Experiment at the Institute of Immunological Science, Hokkaido University, Japan, or at the College of Medicine, Konynag University, Korea, under lamina air-flow conditions. MDP-Lys (L18) (Fig. 1) was obtained from Dai-ichi Seiyaku Co. Ltd, Tokyo, Japan. An appropriate amount of each compound was suspended in 0.1 M phosphate-buffered saline (PBS, pH 7.4).

Cell Culture

Lung metastatic B16-BL6 melanoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, non-essential amino acids, and L-glutamine.

Preparation of X-ray Treated Tumor Cells

B16-BL6 melanoma cells were suspended in EMEM at a density of 5×10^6/ml and irradiated with 100 Gy (X-ray generator, MBR-1520R, Hitachi, Japan) [33]. Irradiated tumor cells were resuspended in PBS in an appropriate cell density and admixed with MDP-Lys (L18) before immunization. For monitoring growth of the X-irradiated B16-BL6 (5×10^3 per well) cells resuspended in a complete culture medium, they were plated in flat-bottom 96-well plates and incubated at 37°C for the indicated days. The cells were pulsed with 0.5 mCi [^{3}H]thymidine (specific activity 23 Ci/mmol, Amersham International, Buckinghamshire, U.K.) for 5 h before harvest. The cultures were then adsorbed to glass filters using a Filtermate 196 (Packard Instrument, Mercian, U.S.A.). The amount of radioactivity in each well was measured using a Matrix 96™ Direct Beta Counter (Packard).

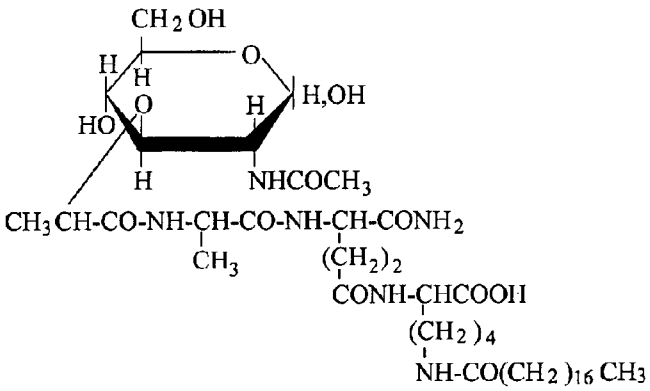

Fig. 1. Chemical structure of MDP-Lys (L18).

Assays for Experimental Lung Metastasis

C57BL/6 mice were given i.d. inoculation of X-irradiated B16-BL6 tumor cells with or without MDP-Lys (L18) before or after challenging with viable B16-BL6 melanoma cells (4×10^4). For the pre-immunization experiment, mice were inoculated i.v. with B16-BL6 cells 7 days after the immunization, and killed 14 days after tumor inoculation [34]. Lung-tumor colonies were counted by a dissecting microscope after fixing the lung samples in a Bouin's solution. For the post-immunization experiment, mice were treated with X-irradiated tumor cells which were mixed with or without MDP-Lys (L18), 3 days after tumor challenge, and killed 14 days after tumor challenge.

Assay for Spontaneous Lung Metastasis

C57BL/6 mice were inoculated s.c. with B16-BL6 cells (5×10^5) into the right hind footpads, and the primary tumors were surgically removed 21 days after tumor inoculation as described [36]. Mice were immunized i.d. with X-irradiated tumor cells (10^4) admixed with or without MDP-Lys (L18) (100 μg), and tumors were excised 1 or 7 days after immunization. Mice were killed 35 days after tumor inoculaton and lung tumor colonies were counted under a dissecting microscope.

Inhibition Assay of Tumor Growth *In Vivo*

The assay of growth inhibition of B16-BL6 cells were conducted by modification of the method previously described [36]. Three C57BL/6 mice per group were inoculated s.c. with B16-BL6 (5×10^5/site) melanoma cells at two sites of the back. Three days after tumor inoculation, mice were immunized i.d. with X-irradiated tumor vaccine (10^4) admixed with or without 100 μg of MDP-Lys (L18). The diameter of the tumor mass was measured 20 days after tumor inoculation.

Statistical Analysis

To analyze statistical significance, differences between groups were determined by applying the Student's two-tailed t-test.

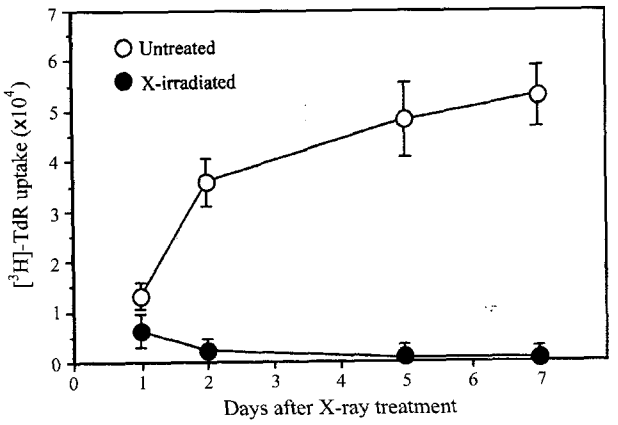

Fig. 2. Effect of X-irradiation on the growth of B16-BL6 melanoma cells.
B16-BL6 melanoma cells inactivated by X-ray irradiation (100 Gy) were plated into each well (5×10^3/100 μl/well) in 96-well microtiter plates and incubated for the indicated days. The cells were incorporated with [^{3}H]-TdR for 5 h before the termination of each incubation. Cell growth was determined by measuring the activity of [^{3}H]-TdR of each well.

Table 1. Effect of MDP-Lys (L18) on enhancement of antitumor activity induced by X-irradiated tumor cells.

Immunized with		No. of lung metastasis	
Tumor vaccine	MDP-Lys (L18)	Mean±SD (inhibition %)	Range
-	-	130±38	(96-184)
-	10 μg	138±32	(102-76)
10^4	-	92±14 (29.2)[a]	(74-104)
10^4	100 μg	33±8 (74.6)[b,c]	(24-44)
10^5	-	76±22 (41.5)[b,c]	(56-96)
10^5	100 μg	32±11 (75.4)[b,c]	(21-46)
10^6	-	74±25 (43.1)[b,c]	(48-101)
10^6	100 μg	30±12 (76.9)[b,c]	(23-44)

Groups of five C57BL/6 mice were immunized i.d. with the indicated number of X-irradiated tumor cells admixed with or without 100 μg of MDP-Lys (L18). One week after the immunization, mice were inoculated i.v. with 4×10^4 viable melanoma cells and killed 14 days after tumor inoculation for evaluation.
[a]$p<0.01$; [b]$p<0.001$, compared with untreated control; [c]$p<0.001$, compared with tumor vaccine alone (by Student's two-tailed t-test).

RESULTS

Effect of MDP-Lys (L18) on Induction of Antitumor Immunity by X-irradiated Tumor Vaccine

In order to prepare tumor vaccine for induction of specific immunity against B16-BL6 melanoma, the cells were inactivated by X-irradiation (100 Gy). As shown in Fig. 2, X-irradiated B16-BL6 cells displayed no DNA synthesis within 3 days after the treatment. Using the X-irradiated B16-BL6 cells as a tumor vaccine, we first examined whether MDP-Lys (L18) could enhance induction of antitumor activity, when it was co-immunized with X-irradiated tumor vaccine prior to tumor challenge. As shown in Table 1, immunization of X-irradiated tumor vaccine 7 days before tumor challenge induced antitumor immunity to inhibit experimental lung metastasis of B16-BL6 cells, indicating that X-irradiated B16-BL6 cells were highly immunogenic. However, no significant difference in antitumor activity was observed between the different numbers of X-irradiated tumor cells, ranging from 10^4 to 10^6 cells. In addition, MDP-Lys (L18) (100 μg), when co-immunized with X-irradiated cells, significantly enhanced the antimetastatic activity raised by inactivated tumor vaccine. We, next, investigated the dose-dependent activity of MDP-Lys (L18) to enhancing antitumor activity of X-irradiated tumor cells. Table 2 shows that the most effective dose for MDP-Lys (L18) to enhance antitumor activity by X-irradiated tumor vaccine was iobserved to be 100 μg per mouse. These results clearly indicate that MDP-Lys (L18) is able to enhance the prophylactic effect induced by immunization of X-irradiated tumor vaccine on experimental lung metastasis of B16-BL6 melanoma cells.

Table 2. Dose-dependent effect of MDP-Lys (L18) on inhibition of experimental lung metastasis induced by X-irradiated tumor cells.

Immunized with		No. of lung metastasis	
Tumor vaccine	MDP-Lys (L18)	Mean±SD (inhibition %)	Range
-	-	112±38	(78-151)
-	20 μg	108±21	(84-137)
-	100 μg	114±13	(98-126)
-	300 μg	103±8	(92-114)
10^4	-	82±12 (16.8)[a]	(58-92)
10^4	20 μg	43±11 (61.6)[b,d]	(34-52)
10^4	100 μg	32±14 (71.4)[b,e]	(19-47)
10^4	300 μg	52±9 (53.6)[b,c]	(44-60)

Groups of five C57BL/6 mice were immunized i.d. with X-irradiated tumor vaccine (10^4) admixed with or without the indicated doses of MDP-Lys (L18). One week after the immunization, mice were inoculated i.v. with 4×10^4 B16-BL6 melanoma cells and killed 14 days after tumor inoculation for evaluation.
[a]$p<0.05$; [b]$p<0.001$, compared with untreated control; [c]$p<0.05$, [d]$p<0.01$; [e]$p<0.001$, compared with tumor vaccine alone (by Student's two-tailed t-test).

Application of MDP-Lys (L18) to Tumor Immunotherapy

To investigate the beneficial effect of MDP-Lys (L18) on active immunization with X-irradiated tumor vaccine after tumor inoculation, C57BL/6 mice were given i.d. injection with X-irradiated tumor cells (10^4) admixed with or without MDP-Lys (L18) (100 μg) [Vac+MDP-Lys (L18)], 3 days after tumor challenge. As shown in Table 3, i.d. immunization of Vac+MDP-Lys (L18), 3 days after tumor challenge, exhibited significant inhibition of experimental lung metastasis of B16-BL6 melanoma cells, as compared to the X-irradiated tumor vaccine alone. We thereafter examined the therapeutic effect of Vac+MDP-Lys (L18) on spontaneous lung metastasis of B16-BL6 cells in a murine

Table 3. Therapeutic effect of immunization with X-irradiated tumor cells admixed with MDP-Lys (L18) on the inhibition of experimental lung metastasis.

Immunized with		No. of lung metastasis	
Tumor vaccine	MDP-Lys (L18)	Mean±SD (inhibition %)	Range
-	-	89±17	(83-110)
10^4	-	55±8 (38.2)[a]	(48-62)
10^4	100 μg	37±6 (58.4)[b,c]	(29-44)

Groups of five C57BL/6 mice were inoculated i.v. with 4×10^4 B16-BL6 melanoma cells. Mice were immunized i.d. with X-irradiated tumor vaccine (10^4) admixed with or without 100 μg of MDP-Lys (L18) on day 3, and killed 14 days after tumor inoculation for evaluation.
[a]$p<0.05$; [b]$p<0.01$, compared with untreated control; [c]$p<0.05$, compared with tumor vaccine alone (by Student's two-tailed *t*-test).

Table 4. Therapeutic effect of immunization with X-irradiated tumor cells admixed with MDP-Lys (L18) on inhibition of spontaneous lung metastasis.

Immunized with			No. of lung metastasis	
On day	Tumor vaccine	MDP-Lys (L18)	Mean±SD (inhibition %)	Range
Tumor control (untreated)			102±13	(77-112)
1	10^4	-	73±26 (28.4)[a]	(51-110)
	10^4		49±31 (52)[b]	(20-82)
7	10^4	-	87±35	(59-126)
	10^4	100 μg	60±29 (41.2)[b]	(40-103)

Groups of six C57BL/6 mice were inoculated s.c. with 5×10^5 viable B16-BL6 melanoma cells. The primary tumors were surgically amputated 21 days after tumor inoculation. Mice were immunized i.d. with X-irradiated tumor vaccine (10^4) admixed with or without 100 μg of MDP-Lys (L18) on the indicated days after amputation. Mice were killed 14 days after tumor amputation.
[a]$p<0.05$; [b]$p<0.001$, compared with untreated control (by Student's two-tailed *t*-test).

spontaneous metastasis model. Single i.d. administration of Vac+MDP-Lys (L18), 1 day after tumor removal, led to inhibition of spontaneous lung metastasis significantly, showing higher inhibitory effect compared to that of the X-irradiated tumor vaccine alone (Table 4). Furthermore, Vac+MDP-Lys (L18) administered on the late period (on day 7) after tumor amputation was still effective to inhibit spontaneous lung metastasis, whereas X-irradiated tumor vaccine alone had no effect. These results suggest that MDP-Lys (L18) serves as a potential immunoadjuvant to induce therapeutic inhibition, not only for experimental, but also for spontaneous lung metastasis of B16-BL6 cells, by enhancing antitumor immunity raised by X-irradiated tumor vaccine.

Effect of Vac+MDP-Lys (L18) on Tumor Growth *In Vivo*

Since tumor growth is one of the most important processes for metastasis, neovascularization, and acquisition of

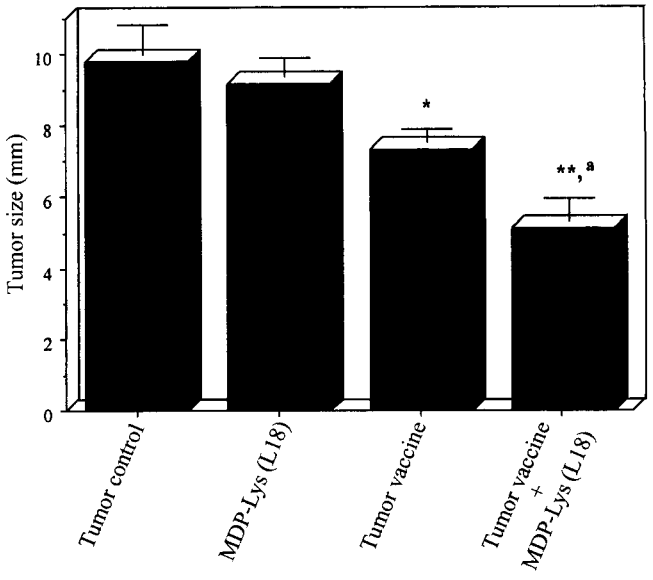

Fig. 3. Inhibitory effect of administration of X-ray irradiated tumor cells admixed with MDP-Lys (L18) on the growth of B16-BL6 melanoma cells *in vivo*.
Three C57BL/6 mice per group were inoculated s.c. with B16-BL6 melanoma cells (5×10^5/site) at two sites of the back, and injected i.d. with tumor vaccine (10^4) admixed with or without MDP-Lys (L18) (100 μg) 3 days after tumor inoculation. Twenty days after tumor inoculation, tumor size was measured for evaluation. *$p<0.05$; **$p<0.01$, compared with untreated control (tumor control); [a]$p<0.01$, compared with tumor vaccine alone (by Student's two-tailed *t*-test).

malignancy of tumor cells, the control of tumor growth is directly associated with the inhibition of tumor metastasis. Here, we examined whether the antimetastatic activity of Vac+ MDP-Lys (L18) is related to inhibition of tumor growth using tumor-bearing C57BL/6 mice. Mice inoculated s.c. with B16-BL6 cells followed by i.d. administration of Vac+MDP-Lys (L18), 3 days after tumor inoculation, showed a significantly reduced tumor growth on day 20, as compared with those treated with X-irradiated tumor vaccine alone (Fig. 3). These results indicate that the antitumor activity of Vac+MDP-Lys (L18) to inhibit lung metastasis of B16-BL6 cells may be related to the suppression of tumor growth.

Discussion

For the past decades, many investigators have demonstrated that the components derived from bacterial cell wall and their synthetic analogues have various biological functions such as immunostimulating [1, 4, 18] and anti-cancer activities [4-6]. Furthermore, a recent report revealed that administration of a bacterium (*Bifidobacterium* spp.) isolated from fecal samples prevented experimental colon carcinogenesis induced by 1,2-dimethylhydrazine in mice [12].

A successful development of tumor vaccines is dependent on their capacity to induce humoral and cell-mediated

immune responses against tumors. In order to obtain a successful immune responses against tumors, a variety of biological response modifiers (BRM) have been applied to cancer therapy. It has long been recognized that MDP is the minimal structural unit of bacterial cell-wall components responsible for a variety of biological activities [2, 4]. The effects of MDP include immunopotentiating (adjuvant) activities in delayed-type hypersensitivity (DTH) and antibody responses, immunotherapeutic activity against cancer and infection, and production of various cytokines [30].

In a series of trials to develop anticancer reagents using synthetic MDP derivatives, we demonstrated that B30-MDP served as an adjuvant to enhance immunogenicity against various antigens such as inactivated tumor cells and viral vaccines [28, 33, 37]. Furthermore, we exhibited that MDP-Lys (L18), a lipophilic derivative of MDP, nonspecifically inhibited tumor metastasis [34], and it enhanced specific immune responses against hantavirus when co-injected with a hantavirus-inactivated vaccine [37]. In nonspecific inhibition of tumor metastasis by MDP-Lys (L18), single administration of MDP-Lys (L18) (100 μg) 2 days before tumor inoculation inhibited prophylactically tumor metastasis [34], however, in this study, MDP-Lys (L18) administered at the same dose 7 days before tumor inoculation had no effect (Table 1 and Table 2). As described in the previous report [34], the prophylactic effect of MDP-Lys (L18) on tumor metastasis was paralleled with enhancement of macrophage-mediated tumoricidal activity by MDP-Lys (L18). The increase of macrophage-mediated cytolytic activity by MDP-Lys (L18) against tumor cells was also found during 4 days after MDP-Lys (L18) treatment, and its activity reduced thereafter to the normal state. Consistent with the effect of MDP-Lys (L18) on macrophage activation, it enhanced the number of leukocytes in peripheral blood (hematopoiesis) during 4 days after the treatment to mice [35]. Taken together, MDP-Lys (L18) plays a role in potentiating nonspecific as well as specific immune responses, and the expression of its biological functions may vary depending on the timing of administration or the presence of antigens. Therefore, the failure to inhibit tumor metastasis by administration of MDP-Lys (L18) 7 days before tumor inoculation was due to inappropriate timing of MDP-Lys (L18) administration and tumor inoculation.

In addition to the prophylactic effect on tumor metastasis, the capability of MDP-Lys (L18) as an immunoadjuvant for therapeutic trials for tumor vaccination was proven in experimental and spontaneous lung metastasis models (Table 3 and 4), and in *in vivo* experiments to inhibit tumor growth (Fig. 3). Collectively, it is likely that MDP-Lys (L18) has the activity not only to enhance nonspecific defense systems of the host, but also to augment specific immune response raised by immunogens, and that its ability to enhance the nonspecific host, defense system depends on the timing of administration. In conclusion, the present study demonstrated that MDP-Lys (L18) is a potent immunoadjuvant enhancing the ability of X-irradiated tumor vaccine to elicit immune responses against tumors, and it is suggested that MDP-Lys (L18) is a promising reagent to apply as a tumor vaccination in the clinic.

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