Antitumor Effect of Inhalatory Lipopolysaccharide and Synergetic Effect in Combination with Cyclophosphamide

KEIJI HIROTA[1,2], YUSUKE OISHI[1], HIROAKI TANIGUCHI[1], KEIICHI SAWACHI1, HIROYUKI INAGAWA[2,3,4,5], CHIE KOHCHI[2,4,5], GEN-ICHIRO SOMA[2,4,5] and HIROSHI TERADA[1,2]

[1]Faculty of Pharmaceutical Sciences, [2]Center for Drug Delivery Research, Tokyo University of Science, Noda, Chiba 278-8510, Japan;
[3]National Fisheries University, Shimonoseki, Yamaguchi 759-6595, Japan;
[4]Institute for Health Science, Tokushima Bunri University, Nishihama Bouji, Yamashiro-cho, Tokushima 770-8514, Japan;
[5]Faculty of Medicine, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

Abstract. *Background: Macrophage cells differentiate into killer macrophages, named M1 macrophages, that effectively eliminate cancer cells by generating cytokines. We examined the feasibility of a lung cancer therapy using lipopolysaccharide (LPS), which triggers this differentiation. It is expected that the delivery via inhalation of LPS directly into the lungs, where alveolar macrophages reside abundantly, would be effective at minimizing the possible toxic effect of LPS. Materials and Methods: We determined the effects of intratracheal insufflation of LPS on (i) the exudation of lactate dehydrogenase, (ii) generation of tumor necrosis factor-α and interleukin-12, and (iii) tumor metastases in Lewis lung carcinoma-bearing C57BL/6 mice. Results: Pulmonary insufflation of LPS resulted in a consistent accumulation of tumor necrosis factor-α, and transient increase in interleukin-12 without significant release of lactate dehydrogenase from the lung cells. In addition, a significant antitumor effect of LPS was observed; and this antitumor effect was potentiated by combination of LPS with the antitumor agent cyclophosphamide. Conclusion: Pulmonary inhalation of LPS combined with a chemotherapeutic agent is a promising approach to lung cancer therapy.*

Lung cancer is the leading cause of death from malignant neoplasms (1, 2). In addition, resistance to chemotherapeutic agents is frequently observed in non-small cell lung cancer (NSCLC) and becomes a clinical problem, with the 5-year survival rate of NSCLC patients being less than 15% (3, 4).

Recently, it was reported that macrophage cells can be classified into two subsets: killer macrophages (M1 macrophages) and healer macrophages (M2 macrophages) (5, 6). M1 macrophages enhance cellular immunity by secreting inflammatory cytokines and nitric oxide (7, 8). This characteristic response is observed in the presence of a classical macrophage-activating agent, such as lipopolysaccharide (LPS). In contrast, M2 macrophages suppress inflammatory responses, scavenge debris, and promote angiogenesis. It is reported that a substantial number of M2 macrophages are present in malignant tumors and play a role in aiding tumor growth and metastasis (9-12), and that these cells are converted into antitumor M1 macrophages by modulation of nuclear factor-kappa B (13).

We hypothesized that pulmonary inhalation of LPS should activate alveolar macrophages without systemic undesirable effects, exerting antitumor effect as a result of an increase in the population of M1 macrophages. In addition, combination of it and the chemotherapeutic agent cyclophosphamide (CPA), which also possesses immunostimulatory activity, would be beneficial to this immunotherapy (14). In this study, we examined the feasibility of cancer therapy by pulmonary inhalation of the immunostimulatory agent LPS.

Materials and Methods

Mice. C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Male mice of 6 weeks of age were used. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science.

Reagents. LPS derived from *Escherichia coli* O111:B4 was purchased from Calbiochem (Darmstadt, Germany); pentobarbital sodium, from Dainippon Sumitomo Pharma Co., Ltd. (Osaka,

Correspondence to: Hiroshi Terada, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki Noda, Chiba 278-8510, Japan, Tel: +81 471213663, Fax: +81 471213782, e-mail: teradah@rs.noda.tus.ac.jp

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Japan); and CPA, from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available.

Pulmonary insufflation of LPS. Mice were anesthetized by an intraperitoneal (*i.p.*) injection of pentobarbital sodium at 40 mg/kg and then subjected to intratracheal (*i.t.*) insufflation with 150 μl of LPS suspended in phosphate-buffered saline using a MicroSprayer® Model IA-1C for mice (Penn-Century, Inc., Wyndmoor, PA, USA). The dose of LPS delivered by insufflation was 450 μg/kg for determination of lactate dehydrogenase (LDH) activity in the normal mice, and 50 μg/kg for examination of cytokine production and the antitumor effect in the tumor-bearing mice. The animals were sacrificed at appropriate time periods after insufflation for obtaining lung, serum, and bronchoalveolar lavage fluid (BALF) samples. Fluorescein isothiocyanate (FITC)-labeled LPS at a dose of 450 μg/kg was used for observing the distribution of the LPS in the lungs after 6 h of insufflation and was detected with a fluorescence stereomicroscope (VB-G05; Keyence, Osaka, Japan).

Serum and bronchoalveolar lavage fluid. After *i.t.* insufflation of LPS, the chest cavities were opened under anesthesia with pentobarbital sodium, and a blood sample was collected from the heart. Serum was obtained by centrifugation of the coagulated blood at 750×g. The trachea was exposed through a midline incision and cannulated with a polyfluorocarbon tube. Bronchoalveolar lavage was performed on the chest-open animals by using 1 ml of saline. Serum and BALF thus obtained were stored at –80˚C for subsequent analysis.

Determination of LDH, tumor necrosis factor (TNF)-α, and interleukin (IL)-12. LDH, used as an indicator of tissue damage, was determined in the BALF samples with a cytotoxicity detection kit for LDH (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions, using LDH from rabbit muscles as a standard. The concentration of TNF-α in the serum and BALF samples was determined by performing the L929 cell bioassay (15). Briefly, L929 cells were placed in the wells of a 96-well plate at 8×10^4 cells/100 μl/well and incubated for 4 h. Subsequently, 50 μl of serum and BALF samples serially diluted with saline were added to each well followed by addition of 50 μl of actinomycin D solution to give a final concentration of 1 μg/ml. After incubation for another 18 h, L929 cells attached to the well were stained with 0.1% (w/v) crystal violet solution. The absorbance of each well at 570 nm was measured after extraction of the dye with 0.5% (w/v) sodium dodecyl sulfate solution. Cytotoxic activity of TNF-α at a dose that kills 50% of the L929 cells corresponds to 1 U/ml. The concentration of IL-12 in the BALF samples was determined with an appropriate ELISA kit (Mabtech AB, Nacka Strand, Sweden), as recommended by the manufacturer.

Determination of antitumor effect. Lewis lung carcinoma (LLC) cells derived from the C57BL mouse were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 60 μg/ml ampicillin. C57BL/6 mice were inoculated with 4×10^5 LLC cells by *i.v.* injection into a tail vein, and then treated with either 100 mg/kg CPA by *i.p.* injection on day 7, or 50 μg/kg LPS by *i.t.* insufflation on day 14, or their combination (100 mg/kg CPA on day 7 and 50 μg/kg LPS on day 14) according to a protocol shown in Figure 1. On day 21, the lungs were excised and their weight measured. The number of metastases on the lung surface was counted.

Results

Stereomicroscopic observation of FITC-labeled LPS insufflated into mouse lungs. Our strategy to overcome lung cancer by induction of the cytotoxic effect was based on the association of LPS with the biochemical function of alveolar macrophages. For this, the homogeneous distribution of LPS in the lung tissue would be necessary. To assess the distribution of LPS in the lungs, we determined the amount of FITC-labeled LPS in the mouse lungs 6 h after insufflation at a dose of 450 μg/kg by observation using a fluorescence stereomicroscope. To avoid the contamination of LPS with trachea and primary bronchi, they were excised from the lung tissue. As shown in Figure 2, the green fluorescence derived from FITC-LPS was observed in all five lobes of the lungs. The fluorescence intensity in each of the right lobes appeared to be higher than that in the left lobe. In particular, the deposition of FITC-LPS in the right lower and right accessory lobes was greater than that in the other lobes. Although a uniform distribution of LPS in the lungs was not observed, LPS was concluded to be delivered efficiently to the alveoli by insufflation using the MicroSprayer®. Almost no fluorescence of lobes due to the insufflated saline was observed.

Toxicity test of intratracheal insufflation of LPS into normal mice. As LPS is a cause of sepsis (16), it was necessary to examine the effect of LPS insufflation on the degree of damage to the lungs. LDH has been used as an indicator of lung tissue damage because it is released from alveolar cells upon their destruction. We thus examined the toxic effect of LPS on the lungs in terms of the amount of LDH leaked from the alveoli. LDH activity in the BALF on day 7 after insufflation of LPS at a dose of 450 μg/kg into the lungs was 4.3±1.8 mU/ml. This level was as low as that induced by *i.t.* instillation of sterilized distilled water to rats in our preliminary experiments (data not shown). In contrast, the insufflation of 450 μg/kg SiO_2 as a crystalline quartz powder with a diameter of 170 nm, which is known to lead to chronic inflammation and tissue destruction in the lungs, in the form of suspension resulted in the production of LDH activity of approx. 90 mU/ml on day 7. These results suggest that *i.t.* insufflation of LPS at a dose of less than 450 μg/kg was not toxic to lung tissue.

Determination of Th1 cytokines after insufflation of LPS into tumor-bearing mice. To ascertain the effect of LPS delivered to the lung alveoli on alveolar macrophages, we determined the amounts of Th1 cytokines, such as TNF-α and IL-12,

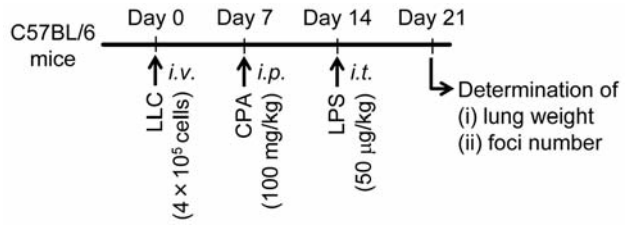

Figure 1. *Protocol using LPS for lung cancer treatment of LLC-bearing C57BL/6 mice. C57BL/6 mice were inoculated with 4×10^5 LLC cells by i.v. injection, and then treated with either 100 mg/kg CPA by i.p. injection on day 7, or 50 μg/kg LPS by i.t. insufflation on day 14, or their combination (100 mg/kg CPA on day 7 and 50 μg/kg LPS on day 14). The lung weight and number of metastasized foci were determined on day 21 for evaluation of the antitumor effects of treatments.*

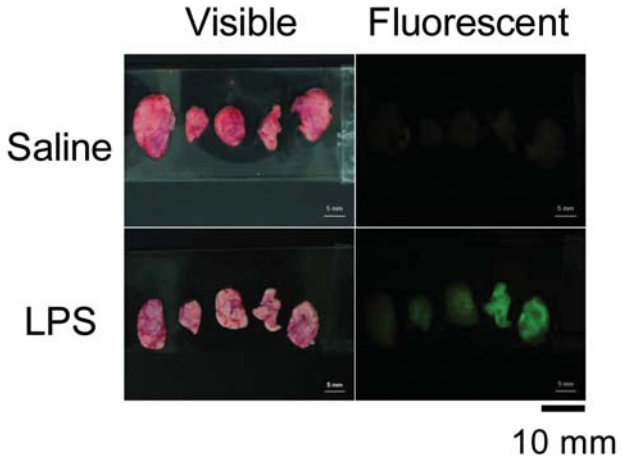

Figure 2. *Pulmonary distribution of LPS by i.t. insufflation. Lobes shown, from left to right, are the left, right upper, right middle, right accessory, and right lower lobe after 6 h of i.t. insufflation of saline (upper images) and FITC-labeled LPS at a dose of 450 μg/kg (lower images). Images on the left are those observed under visible light and those on the right, under excitation light passed through a blue-light excitation filter.*

produced by the macrophages in the BALF of tumor-bearing mice at various time periods after pulmonary insufflation of LPS at the amount of 50 μg/kg. As summarized in Table I, the *i.t.* insufflation of LPS generated TNF-α to the level of approx. 100 U/ml 2 h after insufflation. The production elicited by LPS increased with time up to 12 h, at which time more than 600 U/ml of TNF-α was generated in the alveoli. It is interesting to note that TNF-α generation was not observed at all in the serum by *i.t.* insufflation of LPS. In contrast, the *i.v.* administration of LPS did not stimulate TNF-α production in the alveoli, but did cause transient production of 4 U/ml of TNF-α in the serum. Significant generation of TNF-α in the BALF of tumor-bearing mice was not observed following *i.t.* insufflation of saline. These results suggest that pulmonary

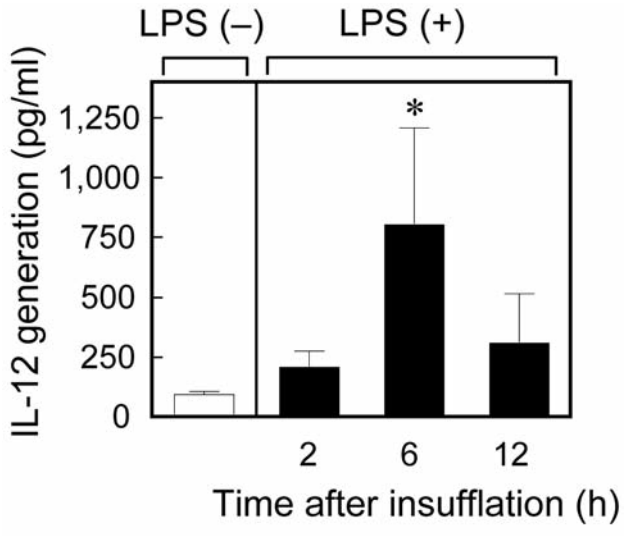

Figure 3. *IL-12 generation in the mouse lungs after i.t. insufflation of LPS. IL-12 generation in BALF samples from tumor-bearing mice treated with i.t. insufflation of 50 μg/kg LPS at various time periods was examined, and the results are shown as closed columns. The open column indicates the amount of IL-12 of tumor-bearing mice without any treatment, used as a reference. The data are expressed as the mean±S.D. of 3 experiments. The asterisk indicates a significant difference from the reference at $p<0.05$, as assessed by Dunnett's multiple comparison test.*

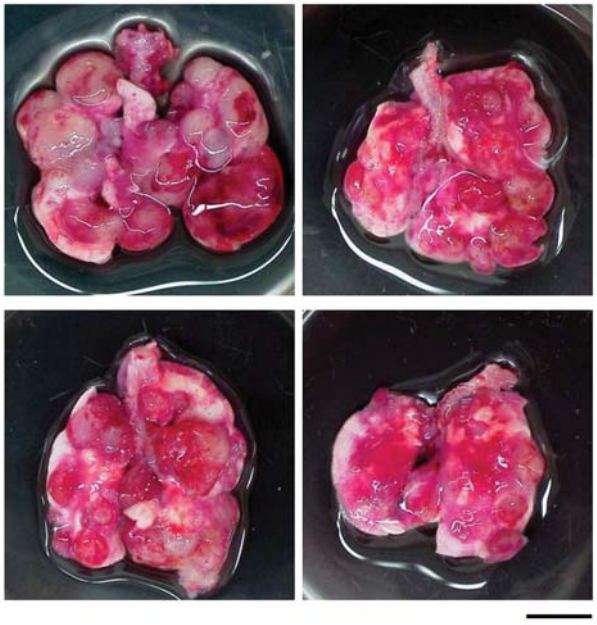

Figure 4. *Lung images of tumor-bearing mice after immunological treatments. C57BL/6 mice were inoculated with 4×10^5 LLC and then treated with saline (A), 100 mg/kg CPA by i.p. injection on day 7 (B), 50 μg/kg LPS by i.t. insufflation on day 14 (C), or their combination (100 mg/kg CPA by i.p. injection on day 7 and 50 μg/kg LPS by i.t. insufflation on day 14) (D) according to the protocol shown in Figure 1. Images were obtained by use of a stereomicroscope. The bar corresponds to 5 mm.*

Table I. *TNF-α generation after i.t. insufflation of LPS. BALF and serum samples were obtained from tumor-bearing mice at various time periods after treatment with i.t. insufflation of saline or 50 μg/kg LPS or i.v. injection of 50 μg/kg LPS. The amount of TNF-α was determined by performing the L929 cell bioassay as described in the Materials and Methods. The data are expressed as the mean±S.D. of 4 experiments.*

	Time after insufflation (h)		
TNF-α generation (U/ml)[a]	2	6	12
BALF			
Saline *i.t.*	N.D.	N.D.	N.D.
LPS *i.t.*	95.9±56.2	219.5±58.5	622.3±320.5
LPS *i.v.*	N.D.	N.D.	N.D.
Serum			
Saline *i.t.*	N.D.	N.D.	N.D.
LPS *i.t.*	N.D.	N.D.	N.D.
LPS *i.v.*	3.9±2.6	N.D.	N.D.

[a]Cytotoxic activity of TNF-α at a dose killing 50% of the L929 cells corresponds to 1 U/ml. N.D., The level of TNF-α was less than 0.5 U/ml and thus not detectable.

Table II. *Antitumor effect of i.t. insufflation of LPS on LLC pulmonary metastases in C57BL/6 mice. C57BL/6 mice were inoculated i.v. with 4×10^5 LLC and treated with either 100 mg/kg CPA by i.p. injection on day 7, 50 μg/kg LPS by i.t. insufflation on day 14, or both in combination (100 mg/kg CPA on day 7 and 50 μg/kg LPS on day 14) according to the protocol shown in Figure 1. After treatment, the lung weight and the focus number were determined. The data represent the mean±S.D. of at least 8 experiments.*

Treatment	Lung weight (g)	Number of foci
Saline	0.20±0.06	10.1±6.0
LPS	0.21±0.06	6.7±3.5
CPA	0.16±0.02	6.4±2.8
LPS+CPA	0.17±0.03	2.8±0.7*

The asterisk indicates a significant difference from the treatment with saline, used as a reference, at $p<0.01$, as assessed by Dunnett's multiple comparison test.

inhalation of an immunostimulatory agent enables local immune potentiation, and this action should be advantageous for immune treatment of lung cancer.

In contrast to TNF-α, IL-12 was significantly generated at approx. 100 pg/ml in the BALF of tumor-bearing mice, as shown in Figure 3. The level of IL-12 generated increased to approx. 800 pg/ml by 6 h after pulmonary insufflation of LPS, being approx. 8 times greater than that generated without LPS treatment. Then the production of IL-12 decreased to approx. 300 pg/ml by 12 h after insufflation. These results show that the immunostimulatory effect of LPS was sustained for at least 12 h after insufflation of the polysaccharide, although the effect on TNF-α endured longer than that on IL-12. Hence, the population of M1 macrophages was likely to have increased by administration of LPS to the lungs.

Possible cancer immunotherapy by pulmonary insufflation of LPS. Mice were inoculated with 4×10^5 LLC cells by *i.v.* injection, and then treated with either the chemotherapeutic agent CPA by *i.p.* injection at a dose of 100 mg/kg on day 7, or LPS by *i.t.* insufflation at a dose of 50 μg/kg on day 14, or their combination (100 mg/kg CPA on day 7 and 50 μg/kg LPS on day 14). Figure 4 shows lung images after these treatments, and Table II summarizes the lung weight and number of metastases in the lungs. Widespread metastases were developed in the mouse lungs by inoculation with LLC cells. The saline-treated lungs, the weight of which was approx. 0.20 g, bore more than 10 foci, and the size of several foci exceeded 3 mm. Treatment with either CPA or LPS reduced the number of metastases to a similar extent, and the foci were smaller than those observed in the lungs treated only with saline. However, the increase in the lung weight was suppressed only when CPA was given. It is noteworthy that metastatic cancer foci significantly decreased in number with the combination therapy of CPA and LPS, resulting in a focus number of less than 3. In addition, the huge metastases exceeding 3 mm in size almost disappeared with the combination therapy. These results reveal that an antitumor effect of LPS was exerted by its *i.t.* insufflation and that this effect was potentiated by the combination with CPA given by *i.p.* injection.

Discussion

LPS derived from gram-negative bacteria, such as *Escherichia coli*, has been regarded to trigger sepsis (16). However, an evolutionary perspective has been recently suggested that the host-microbial interaction is symbiotic and important to maintain host homeostasis (17). Several reports suggest that LPS helps in the treatment of various diseases, such as cancer and allergy, by controlling innate immunity involving macrophages (18, 19). M1 macrophages are developed by a stimulation of immature macrophages with LPS and play a key role in attacking cancer cells (7, 8), whereas M2 macrophages are differentiated by signaling from cancer cells and aid in tumor growth and metastasis (9-12). Thus, we hypothesized that an appropriate activation by LPS would be effective in cancer therapy.

To avoid systemic undesirable effects, such as shock and fever, LPS should be delivered to a local target tissue, where macrophages are abundant. We thus performed pulmonary insufflation of LPS by using a common insufflator, the MicroSprayer®, and examined the distribution of LPS in the

lungs. We found that LPS in suspension form was deposited and retained in the mouse lungs for at least 6 h. In addition, lung destruction, evaluated in terms of LDH, was not induced by pulmonary insufflation of LPS at a dose of 450 μg/kg to the normal mice. Hence, LPS may be regarded as a safe material for the lung tissue when used at less than 450 μg/kg.

The antitumor function of macrophages is modulated by the generation of immunosuppressing cytokines such as transforming growth factor-β (TGF-β) (20, 21). Thus, it is important to understand the immunological profiles of tumor-bearing mice in anticancer therapy using LPS. We found that LPS insufflated into the lung of tumor-bearing mice caused a significant increase in the levels of TNF-α and IL-12 cytokines, which play a key role in tumor destruction (22, 23). In addition, the generation of TNF-α increased steadily for at least 12 h, whereas that of IL-12 was transient, with a peak at 6 h, after insufflation of LPS. These immunological changes were possibly due to an increase in the population of M1 macrophages by *i.t.* insufflation of LPS and suggest that the development of a Th1 cytokine network initiated by LPS may occur as follows: the alveolar macrophages recognize LPS insufflated into the lungs, differentiating into M1 macrophages which then generate TNF-α and IL-12. Naive T-cells are matured into Th1 cells by the IL-12 generated by the M1 macrophages and generation of TNF-α is then accelerated by these Th1 cells. Further investigation is needed for a full understanding of this mechanism.

It is noteworthy that LPS insufflated into the lungs exhibited an antitumor effect on lung cancer, the effect being comparable to that obtained by treatment with CPA in terms of the development of tumor foci. In addition, the antitumor effect exerted by LPS was greatly potentiated when LPS was combined with CPA. Although many chemotherapeutic agents suppress immunological functions due to their severe bone marrow toxicity, CPA treatment was recently reported to enhance the antitumor effect through the induction of the expression of various cytokines (14). CPA treatment is beneficial to cancer immunotherapy by enhancing the effect of the immunostimulatory agent LPS. Likewise, the alkylating agent melphalan exerts antitumor cytotoxicity by causing a decrease in TGF-β generation from tumor cells (24). A combination of chemotherapeutic agents possessing immunomodulatory activities should be required for optimization of cancer immunotherapy using LPS.

In conclusion, LPS insufflated into the lungs exhibited an antitumor effect on the LLC pulmonary metastases without lung destruction. Possibly, the induction of TNF-α and IL-12, likely due to an increase in the population of M1 macrophages, by the action of LPS is at least one reason why LPS administration had such an effective antitumor effect. In addition, we found that the antitumor effect of LPS was potentiated by the chemotherapeutic and immunomodulatory agent CPA. Pulmonary insufflation of LPS combined with such a chemotherapeutic agent is thus a promising approach to lung cancer therapy.

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