Lipopolysaccharide-activated Alveolar Macrophages Having Cytotoxicity toward Lung Tumor Cells through Cell-to-Cell Binding-dependent Mechanism

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Abstract. *Background: In lung tumor biotherapy, local macrophages such as alveolar macrophages and tumor-associated macrophages (TAMs) which normally exist in contact with tumor cells are thought to be hopeful target. It is advantageous to clarify the potential for and mechanism by which lung tumor cells are killed by the neighboring macrophages in order to establish new lung cancer therapy using a drug delivery system to lung tissue. Materials and Methods: A549, a human lung adenocarcinoma cell line, and Lewis lung carcinoma LLC1, a mouse lung cancer cell line, were co-cultured with NR8383, a rat alveolar macrophage cell line, and AMJ2-C11, a mouse alveolar macrophage cell line, at a ratio of 1:10 and 1:5, respectively. Macrophages were activated with lipopolysaccharide (LPS) and cytotoxicity toward tumor cells was evaluated by a dye-uptake method and ^{3}H-thymidine release assay, respectively. Nitric oxide (NO) production was estimated by Griess assay, and tumor necrosis factor (TNF)-α and interleukin (IL)-1β were measured by ELISA. Results: Significant macrophage cell aggregation and cytotoxicity against A549 cells and LLC1 cells was observed with NR8383 cells and AMJ2-C11 cells in the presence of LPS. A high concentration of NO and TNF-α were detected in the supernatant of co-culture medium with LPS. Inhibition of cell-to-cell contact restored A549 cell growth. Conclusion: The LPS-activated alveolar macrophages demonstrated an increased cell-to-cell contact with lung tumor cells and inducing cytotoxicity with production of NO and cytokines TNF-α and IL-1β. These results suggest that moderate activation of local macrophages in lung (alveolar macrophages and TAMs) is thought to be a hopeful means of establishing new immunotherapy for lung cancer.*

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Lung cancer is the leading cause of cancer death among men and women (1). Despite better surgical techniques and developments in radiotherapy and chemotherapy, it is still the case that fewer than 15% of patients with this disease survive for five years (2). Surgery cannot be performed in many cases because of the progressive state of lung cancer, and non-small cell lung cancer, which makes up 80% of the lung cancer cases in Japan, is resistant to chemotherapy (3). Therefore, new innovative therapies are still required.

Macrophages exist in all animals and are well known for performing pleiotropic functions essential for tissue remodeling, inflammation and immunity, including phagocytosis, cytotoxicity and secretion of a wide array of humoral factors (4). Recently, macrophages have been broadly divided into two phenotypes, classically activated macrophages (also called M1) and alternatively activated macrophages (M2) (5). M1-activated macrophages are potent effector cells that kill microorganisms and tumor cells, produce inflammatory cytokines, and express high levels of major histocompatibility complex (MHC) molecules. In contrast, M2-activated macrophages induce inflammatory responses and adaptive Th1 immunity, scavenge debris, and promote angiogenesis, tissue remodeling and repair (6). Moreover, tumor cells can induce M2 macrophage phenotype, which promotes tumor growth (6, 7). It has been reported that an antitumor effect was observed in tumor

tissue after re-education of tumor-associated macrophages (TAMs) to the activated M1 type (8-10). Based on these facts, if tissue macrophages are activated to the M1 type by some materials such as lipopolysaccharide (LPS) (11), it is thought that the tumors might be effectively eliminated.

In the lung, there are many alveolar macrophages which eliminate foreign substances (12). Due to their large population and close relationship with tumor regression, alveolar macrophages would become attractive targets of lung cancer immunotherapy if M1-type macrophage-activating drugs can be delivered to the lungs. Recently, a dry powder inhaler has been developed as an apparatus to deliver some agents to the lungs (13). An anti-tuberculosis agent for delivery to alveolar macrophages in tuberculosis-infected animals by dry powder inhaler has been already developed (14, 15). Thus, in the near future, alveolar macrophage activation may be effective to overcome these diseases.

However, the knowledge of how alveolar macrophages kill lung cancer cells is not fully understood. The anti-tumor functions of alveolar macrophages need investigation in order to enable lung tumor to be treated with delivering macrophage-activating drugs. In the present study, in order to establish the basis for therapy for lung cancer through pulmonary administration of macrophage-activating drugs, we examined whether cytotoxic activities toward lung carcinoma cells could be induced by activating alveolar macrophages possessing lung tissue-specific characteristics.

Materials and Methods

Cell lines. All cell lines were purchased from the American Type Culture Collection (NR8383, CRL-2192; AMJ2-C11, CRL-2456; A549, CCL-185; LLC1, CRL-1642). The rat alveolar macrophage cell line NR8383 was maintained in F-12 Kaighn's Nutrient Mixture (F12-K) medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS) (Invitrogen) and 60 μg/ml ampicillin (Meiji Seika Kaisha, Tokyo, Japan). AMJ2-C11, a mouse alveolar macrophage cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% FBS and 60 μg/ml ampicillin. The human lung adeno-carcinoma cell line A549 was maintained in F-12K medium supplemented with 10% FBS and 60 μg/ml ampicillin. A mouse lung cancer cell line LLC1 was maintained in DMEM supplemented with 10% FBS and 60 μg/ml ampicillin. All cells were incubated at 37˚C in a 5% CO_2 atmosphere.

Measurement of macrophage and lung tumor cell contact. A549 cells were placed in Culture Slides (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) at a density of 1.2×10^4 cells/well and were incubated in F-12K medium with 5% FBS for 24 hours at 37˚C. After that, NR8383 cells were added to each well at a ratio of 10:1 (NR8383:A549 cells), with or without 1 μg/ml of *Escherichia coli* LPS (Calbiochem, Darmstadt, Germany), and were incubated for a further 6 hours at 37˚C. The number of A549 cells which were bound by NR8383 cells was counted by microscopic observation after staining with crystal violet.

Cytostatic activity by LPS-stimulated macrophages under co-culture conditions. A549 cells were placed in 96-well plates at a density of 5×10^3 cells/well and incubated in F-12K medium with 5% FBS for 4-6 hours at 37˚C. After the A549 cells had adhered to the plate, NR8383 cells were added to each well at a ratio of 10:1 (NR8383:A549 cells), with or without 1 μg/ml LPS and were then incubated for 1, 2, 3, 5, and 7 days at 37˚C. After co-culture, each well was washed with saline to remove the macrophages and dead A549 cells. The surviving A549 cells were stained with 0.1% crystal violet/MeOH (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at room temperature for 10 minutes. The plates were then read on a microplate reader at an absorbance of 570 nm (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). The optical absorbance due to surviving A549 cells in the absence of macrophages (control absorbance) was set at 100%, and the experimental absorbance was divided by the control absorbance to obtain cytostasis as a percentage.

Cytocidal activity by LPS-stimulated macrophages under co-culture conditions. Cytocidal activity of NR8383 cells toward A549 cells, and AMJ2-C11 cells against LLC1 cells, was measured using ^3H release assay. A549 cells placed in 24-well plates at a density of 4×10^5 cell/well and incubated in F-12K medium with 10% FBS for 24 hours at 37˚C in the presence of 185 kBq [methyl-^3H] thymidine (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). After washing two times with culture medium, ^3H-labeled A549 cells were placed in 96-well plates at a density of 5×10^3 cells/well and incubated in F-12K medium with 5% FBS for 4-6 hours at 37˚C. After the A549 cells had adhered to the plate, NR8383 cells were added to each well at a ratio of 10:1 (NR8383:A549 cells), with or without 1 μg/ml LPS, and were then incubated for 1, 2, 3, 5 and 7 days at 37˚C. Supernatant was then collected from each well and radioactivity was measured by a liquid scintillation spectrometer (LSC-3600, Aloka Co., Ltd., Tokyo, Japan). The viability of A549 cells was calculated according to the equation shown below.

Viability (%)=(1−([experimental ^3H release]−[spontaneous ^3H release]) / ([total ^3H incorporated]−[spontaneous ^3H release])) ×100

Supernatant of A549 cells incubated with and without NR8383 cells was collected to obtain experimental and spontaneous release of ^3H, respectively, and the cell suspension was mixed with hydrochloric acid (Wako Pure Chemical Industries, Ltd.) to determine the total amount of ^3H that had been incorporated into A549 cells. LLC1 cells labeled with ^3H were also prepared by a similar method to that of A549 cells using DMEM. In the case of AMJ2-C11 cells, interferon (IFN)-γ was added besides LPS. The ratio of LLC1 cells and AMJ2-C11 cells was 1:5.

Cytostatic activity by LPS-stimulated macrophages under non-contact culture conditions. A549 cells were placed in 24-well plates at a density of 3×10^4 cells/well and incubated in F-12K medium with 5% FBS, for 4-6 hours at 37˚C. After the A549 cells had adhered to the plate, a cell culture insert of 0.4 μm pore size (Becton, Dickinson & Co.) was set in a well of 24-well plate. NR8383 cells were then added to the inside of the insert at a ratio of 10:1 (NR8383:A549 cells), in the presence or absence of 1 μg/ml LPS, and were incubated for 1, 2 and 3 days at 37˚C. Cytostatic effect was assayed by crystal violet method.

Cytostatic activity of culture medium after culture of LPS-stimulated macrophages. NR8383 cells (3×10^5 cell/well) and A549 cells (3×10^4 cell/well) were cultured separately in the presence or

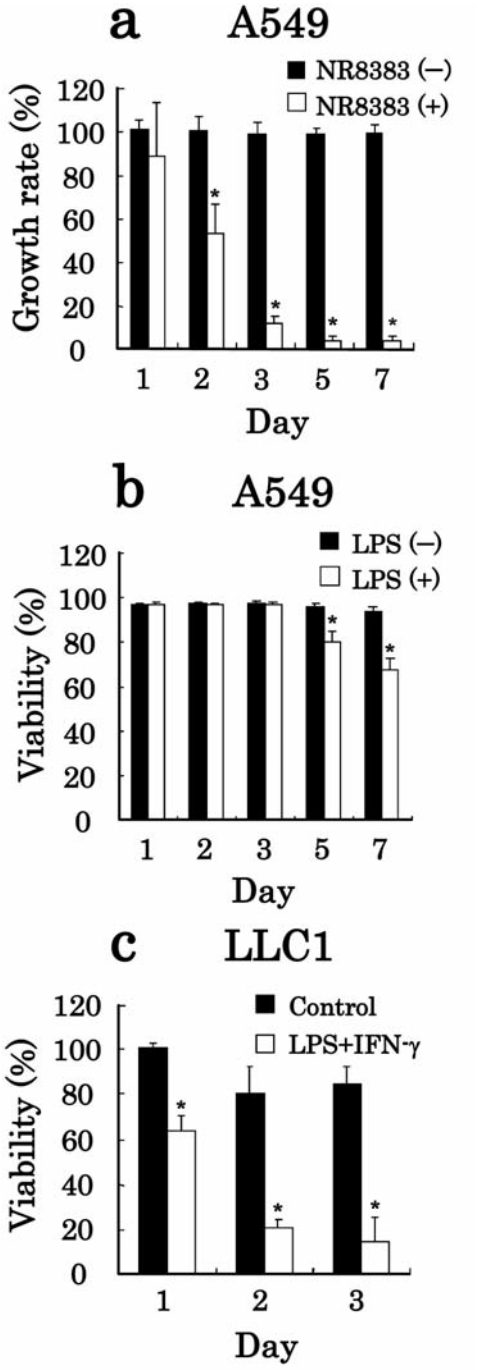

Figure 1. *Cytotoxic effects of activated alveolar macrophages on lung carcinoma cells. Cytostatic effects were evaluated by growth rate (a), and cell-killing effects were evaluated by viability (b, c). A549 cells were cultured with and without NR8383 cells (1:10) in the presence of LPS for 1, 2, 3, 5 and 7 days, and the growth rate of A549 cells compared with those in the absence of LPS for each day was determined (a). Cytotoxicity toward A549 cells after co-culture with NR8383 cells (1:10), represented by viability, in the presence and absence (control) of LPS (b), and that of LLC1 cells after co-culture with AMJ2-C11 cells (1:5) in the presence of LPS and IFN-γ (c) were determined. The results without LPS and IFN-γ were taken as control (c). The data are indicated as the mean±S.D. of 9-18 experiments (*p<0.05 vs. control).*

absence of 1 μg/ml LPS. The culture was maintained in F-12K with 5% FBS at 37˚C for 3 days. At the end of culture, supernatants were collected and A549 cells were cultured at a density of 5×10^3 cell/well in each supernatant. Cytostatic effect was assayed by crystal violet method as described above.

Measurement of TNF-α, IL-1β, and nitric oxide (NO). TNF-α and IL-1β were determined using enzyme-linked immunosorbent assay (ELISA) kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The NO concentration was measured by microplate assay method with Griess reagent (16, 17).

Statistical analysis. Data were analyzed with Student's *t*-test. Differences between treatment and control groups were considered statistically significant at $p<0.05$.

Results

Anti-tumor effect of activated macrophage against lung cancer cells under co-culture conditions. To determine whether alveolar macrophages could induce anti-tumor effects on lung cancer cells, A549 cells and LLC1 cells were co-cultured with alveolar macrophages (NR8383 cells and AMJ2-C11 cells, respectively) in the presence and absence of LPS. Cytostatic effects were evaluated by the growth rate, and cell-killing effects were evaluated by the viability. As shown in Figure 1, NR8383 cells exhibited cytostatic and cell-killing effects toward A549 cells when co-cultured at a ratio of 10:1 (NR8383:A549) in the presence of LPS. A cytostatic effect was observed on the second day or later and the growth rate of A549 cells was reduced to about 4% by day 7 (Figure 1a). A cell-killing effect as expressed by viability was verified at day five and later, and the viability of A549 cells was decreased to 67% at day 7 (Figure 1b). These effects did not occur without incubation with NR8383 cells. AMJ2-C11 cells also exhibited cell-killing effect toward LLC1 cells when incubated at a ratio of 5:1 (AMJ2-C11:LLC1) but only in the presence of LPS and IFN-γ. The viability of LLC1 cells was reduced to about 14% at day 3. These results suggested that activated alveolar macrophages exhibited cellular cytotoxicity against lung cancer cells.

Increase in the contact of macrophages with lung tumor cells by activation of macrophages with LPS. As clearly shown in Figure 1, tumor cell death occurred as a result of the co-culture of lung cancer cells with alveolar macrophages in the presence of LPS. LPS did not induce cell death of lung cancer cells directly (Figure 1a). To observe the behavior of LPS-activated NR8383 cells against A549 cells by cytological staining, A549 cells and NR8383 cells were cultured separately (Figure 2a and 2b, respectively), and together in the absence and presence of LPS (Figure 2c and 2d, respectively). NR8383 cells co-cultured with A549 cells in the presence of LPS migrated and adhered to A549 cells, but not in the absence of LPS. This adhesion was quantified

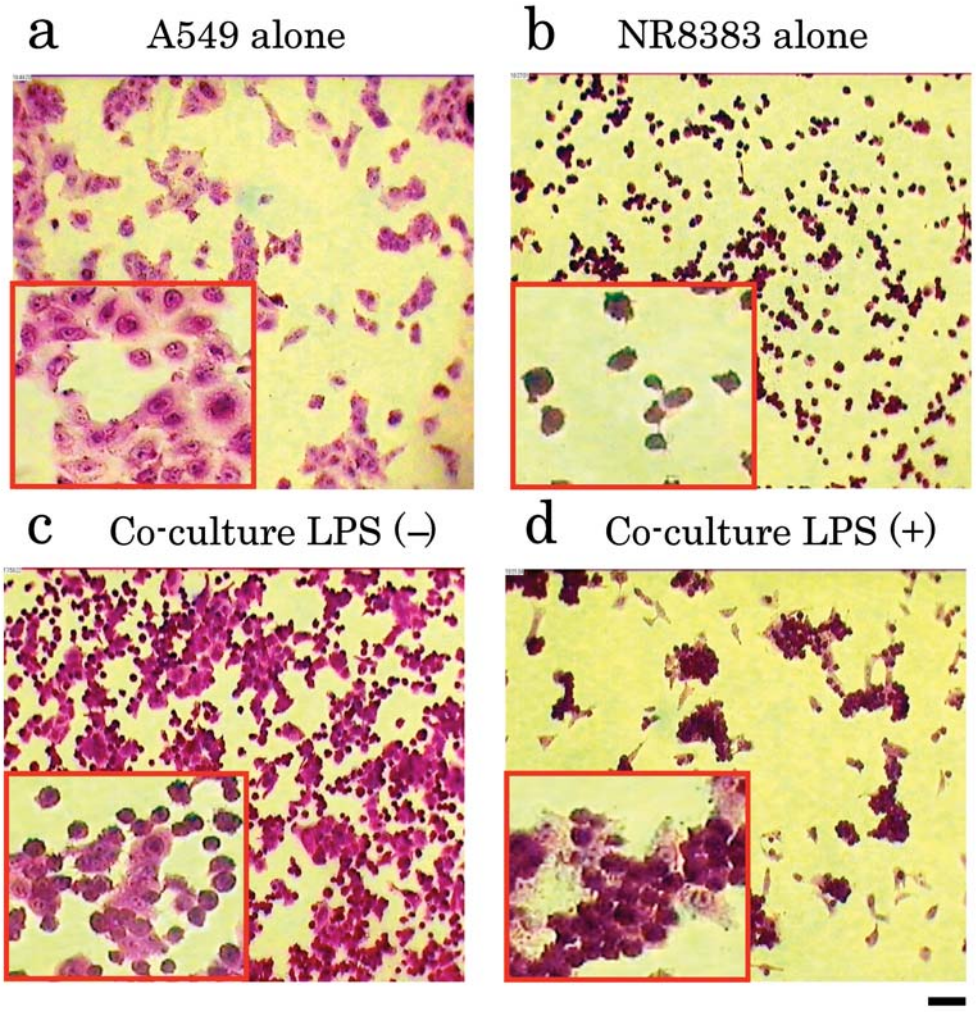

Figure 2. *Microscopic observation of A549 and NR8383 cell cultures at day 1. Each micrograph shows culture of A549 cells alone (a), NR8383 cells alone (b), and A549 cells co-cultured with NR8383 cells in the absence and presence of LPS (c and d, respectively). The cells were stained with crystal violet. Magnification, main image ×100, inset ×200. The bar corresponds to 100 μm.*

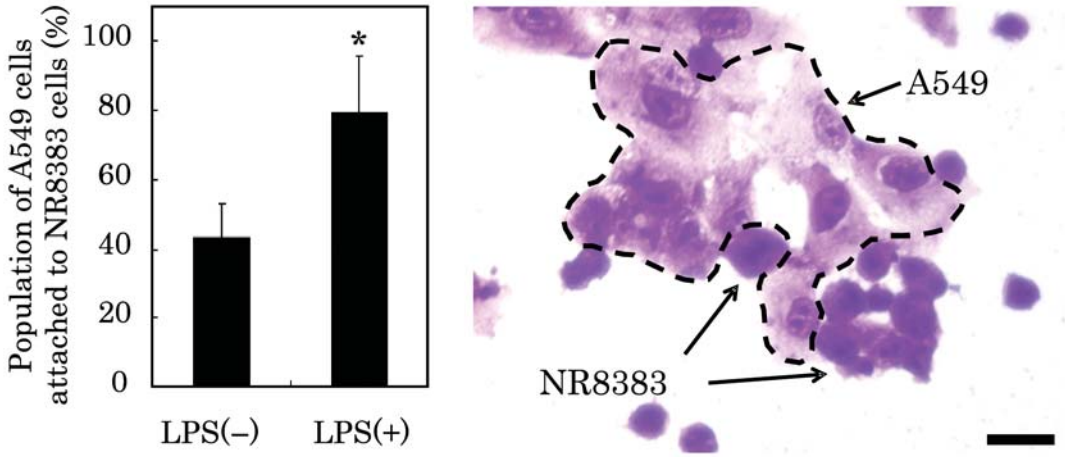

Figure 3. *Quantification of cell-to-cell contact. a: A549 cells were co-cultured with NR8383 cells (1:10) in the presence and absence (control) of LPS for 6 hours, and the population of A549 cells attached to NR8383 cells was counted. b: Micrograph of co-cultured cells after 6 hours. The data are indicated as the mean±S.D. of 10 experiments (*p<0.05 vs. control). Magnification, ×400. The bar corresponds to 20 μm.*

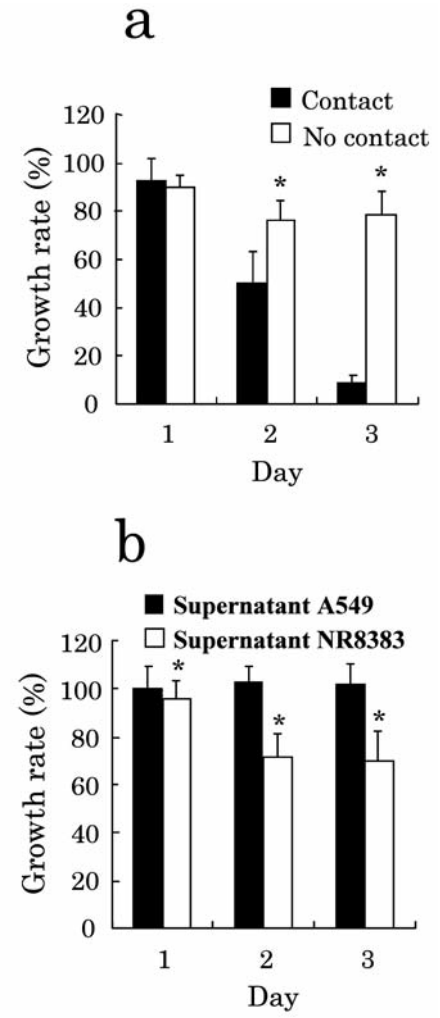

Figure 4. *The influence of cell contact on cytotoxic effects. a: A549 cells were co-cultured with NR8383 cells at a ratio of 10:1 under contact conditions and under noncontact conditions in the presence of LPS for 1, 2 and 3 days, and the growth rate of A549 cells was determined. The data are indicated as the mean±S.D. of 6-14 experiments (*p<0.05 vs. control). b: A549 cells were incubated for 1-3 days in the supernatant of culture medium after culture of NR8383 cells, and of A549 cells for 3 days in the presence of LPS. The growth rate of A549 cells (absorbance in the absence of LPS for each day was set at 100%) was then determined. The data are indicated as the mean±S.D. of 30 experiments (*p<0.05 vs. control).*

in terms of the population of A549 cells attached to NR8383 cells relative to that of the total A549 cells. As a result, LPS increased the adhesion of NR8383 cells to A549 cells almost two-fold in six hours (Figure 3).

Influence of cell-to-cell contact on cytotoxic effect. To evaluate the influence of cell-to-cell contact of NR8383 cells on the growth rate of A549 cells, NR8383 cells were cultured separately from A549 cells by using a cell culture insert tool in the presence and absence (control) of LPS, and the growth

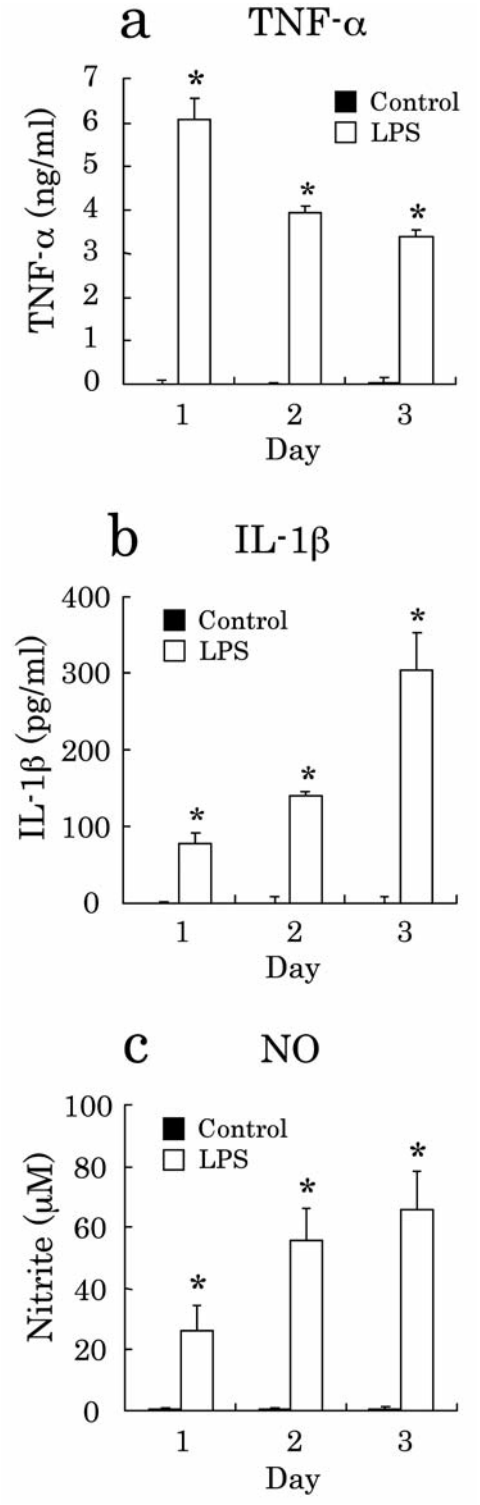

Figure 5. *Influence of humoral factors produced by LPS-activated NR8383 cells. NR8383 cells were cultured with A549 cells (10:1) in the presence and absence (control) of LPS for 1, 2 and 3 days, and production of TNF-α (a), IL-1β (b), and NO (c) from NR8383 cells was determined in the supernatants of the culture medium. The data are indicated as the mean±S.D. of 4 (a and b) and 17 experiments (c) (*p<0.05 vs. control).*

rate of A549 cells was determined. The growth rate under non-contact conditions was compared with that of A549 cells co-cultured with LPS-activated NR8383 cells. As a result, the growth rate of A549 cells cultured separately from NR8383 cells was only reduced to 78%, whereas that of A549 cells co-cultured with NR8383 cells in the presence of LPS significantly decreased to about 8% at day 3 (Figure 4a). The influence of culture supernatant on cytotoxic effects was then examined under co-culture conditions without the insert tool (Figure 4b). The cytotoxic effect of the supernatants of the conditioned medium obtained after culture of LPS-activated NR8383 cells for 3 days reduced the growth rate of A549 cells to 70% after incubation for 2 and 3 days, this level being almost the same as that observed when A549 cells were cultured separately from NR8383 cells (see Figure 4a). In contrast, the growth rate of A549 cells was not changed when A549 cells were cultured in the supernatant of the culture medium after culture of A549 cells in the presence of LPS for 3 days. These results indicate that cell contact of alveolar macrophages with tumor cells is important in suppressing the growth of lung tumor cells.

Next, we investigated the amounts of the humoral factors TNF-α and IL-1β, and NO that were produced from LPS-activated NR8383 cells (Figure 5). All of these were significantly induced by stimulation of NR8383 cells with LPS. Hence, the cytotoxic effect of LPS-activated alveolar macrophages on tumor cells could be due to the action of any single combinations of these humoral factors.

Discussion

Although alveolar macrophages of the activated M1 phenotype can kill lung cancer cells, the anti-tumor function of alveolar macrophages is less defined. To analyze the killing and cytostatic mechanism of alveolar macrophages against lung tumor cells, we demonstrated in this study that alveolar macrophages activated by LPS are able to kill lung cancer cells under conditions of cell-to-cell contact. This means that the binding of macrophages to tumor cells is an important event in causing the tumor cell-killing effect.

In this study, LPS was used to induce M1-like activated alveolar macrophages. The results showed a high level of cytotoxicity when A549 cells were co-cultured with NR8383 cells in the presence of LPS. This cytotoxicity was not observed without LPS. Similar cytotoxic effects were observed for the co-culture of LLC1 cells with AMJ2-C11 cells in the presence of LPS and IFN-γ (Figure 1). Thus, activation of alveolar macrophages by LPS strongly enhances the cytotoxic activity against lung cancer cells in the allogeneic (LLC1 and AMJ2-C11) and xenogeneic (A549 and NR8383) combinations.

As macrophages have pleiotropic functions, they modulate tumor growth, both in terms of progression and regression (4). Reduction of tumor growth by macrophages can be mediated by direct contact with tumor cells and by indirect killing by effector molecules released from the activated macrophages. In the microscopic observation, LPS-activated NR8383 cells aggregated and contacted A549 cells (Figures 2 and 3). It is reported that membrane receptors of macrophages recognize tumor antigenic molecules enabling macrophages to intimately bind to tumor cells (18, 19). Prevention of the cell-to-cell contact between NR8383 cells and A549 cells almost restored the growth rate of A549 cells to normal levels (Figure 4). On the other hand, the supernatant of LPS-activated macrophage culture medium showed some growth inhibition of tumor cells, but the effect was limited. Thus, the major mechanism of anti-tumor effects of alveolar macrophages may be due to cell-to-cell contact.

Moreover, we found significant quantities of effector molecules, such as TNF-α, IL-1β and NO, in the supernatant of LPS-stimulated NR8383 cells. It is reported that TNF-α inhibits the growth of tumor cells, and this indicates the possible effectiveness of TNF-α in cancer therapy (20). IL-1, which plays an important role in various immunity and inflammatory reactions, is also an anti-tumor factor produced by macrophages (21). NO is a low molecular mediator that has a range of functions and is closely related to tissue injury, inflammation, and shock. It is also known that apoptosis of tumor cells is induced by large amounts of NO (22, 23). Additionally, NO generates peroxynitrite by reacting with superoxide radicals (24) and peroxynitrite is known to have strong cytotoxic activity (25). Further study on the actions of these effector molecules is important to understand fully the cytotoxic effect of LPS-stimulated alveolar macrophages on tumor cells under cell-to-cell contact.

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