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Production of interleukin-10 by alveolar macrophages from lung cancer patients

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Interleukin (IL)-10 is known to be an autoregulatory factor of functions of monocyte macrophages. The purpose of this study was to determine whether IL-10 production by alveolar macrophages (AMs) is altered in patients with lung cancer. AMs were obtained by bronchoalveolar lavage from 25 patients with lung cancer and 14 control patients. The production of IL-10 by AMs was quantitated by enzyme immunoassay with or without stimulation with lipopolysaccharide (LPS). No significant difference in spontaneous and LPS-stimulated IL-10 production by AMs was observed between lung cancer patients and control patients (mean ± SEM; 288·0 ± 56·7 *vs.* 249·6 ± 58·4 pg ml^{-1}). IL-10 production of LPS-stimulated AMs was not impaired even in lung cancer patients with systemic metastasis. IL-4 failed to suppress LPS-induced production of IL-10 by AMs both in control patients and in lung cancer patients. In eight patients with lung cancer, IL-10 production by AMs was estimated before and after systemic chemotherapy and IL-10 production by LPS-stimulated AMs tended to increase after systemic chemotherapy from 152·3 ± 51·9 to 278·0 ± 112·8 pg ml^{-1}. As IL-10 is a potent inhibitor of tumour angiogenesis, an important process of tumour progression, these results suggest that, even in advanced cancer patients, macrophages can produce potent angiogenesis inhibitor and systemic chemotherapy may augment this inhibitory activity in the lung.

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Introduction

Angiogenesis is a multistep process involving degradation of extracellular matrix proteins and activation, proliferation and migration of endothelial cells. Although normal angiogenesis is strictly regulated and self-limiting, recent investigations have revealed that pathological angiogenesis contributes to various disease status, including tumour progression (1,2). Several soluble factors have been identified that have angiogenic activity and in several tumours, macrophages, as well as tumour cells, are considered to be a source of these angiogenic molecules (3–5).

It is well known that functions of monocyte macrophages are regulated through various mechanisms and, among those factors, interleukin (IL)-10 is known as an autoregulatory factor which is produced by monocyte macrophages (6). As IL-10 suppresses production of angiogenic molecules and, moreover, augments production of angiogenesis inhibitor by activated macrophages (7,8), it is of interest to examine the ability of monocyte macrophages to produce endogenous IL-10 in the tumour-bearing state. Although various previous studies have revealed that various functions of monocyte macrophages, such as chemotactic activity, antibody-dependent cell-mediated cytotoxicity and cytostatic activity, are impaired in cancer patients (9–11), no information is yet available on the ability of monocyte macrophages to produce IL-10 in cancer patients.

In the present study, we examined production of IL-10 by alveolar macrophages (AMs), which are major effector cells in inflammatory and/or immune responses in the lung (9–12), in lung cancer patients in comparison with those in control patients and the influence of systemic chemotherapy was also examined.

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Materials and Methods

PATIENTS WITH LUNG CANCER

Twenty-five patients (22 smokers and three non-smokers) with primary lung cancer were studied after obtaining informed consent. Their mean age was 69·3 years (range 57–79 years). They had not received any conventional anticancer therapy before the study. Lung cancer was diagnosed by either histological examination of tissue specimens or cytological examination of sputum, or specimens obtained by bronchial brushing, lymph-node biopsy or lung aspiration. On histological examination, the tumours were identified as five small-cell lung cancers

(SCLCs) (mean age 68·2 years, range 57–74 years) and 20 non-small-cell lung cancers (NSCLC) (mean age 69·6 years, range 59–79 years) (adenocarcinoma in 10 cases, squamous cell carcinoma in eight cases, large-cell carcinoma in one case and adenosquamous cell carcinoma in one case). The stage of lung cancer was graded according to the tumour–node–metastasis classification system (Union Internationale Contre le Cancer, 1987). Clinical staging was determined according to the criteria of the Japanese Lung Cancer Association. The stage classification was as follows: SCLC (stage IIIB, 2; stage IV, 3); adenocarcinoma (stage I, 1; stage II, 1; stage IV, 8); squamous cell carcinoma (stage IIIA, 2; stage IIIB, 4; stage IV, 2); large cell carcinoma (Stage IV, 1) and adenosquamous carcinoma (Stage IV, 1). Eight of the patients (three with SCLC, three with adenocarcinoma, two with squamous cell carcinoma) received a second examination with a bronchofibrescope about 10 weeks later, after two cycles of chemotherapy. Two patients received cisplatin-based chemotherapy and the other six patients received carboplatin-based chemotherapy. The therapeutic responses were evaluated at the same time: partial response (SCLC, 3; squamous cell carcinoma, 2; adenocarcinoma, 1), no change (adenocarcinoma, 1) and progressive disease (adenocarcinoma, 1). No complication of atopic diseases was observed in these lung-cancer patients.

CONTROL PATIENTS

Fourteen control subjects (seven smokers and seven non-smokers) were studied: nine men and five women aged 25–73 (mean 52·8 years). The objective of bronchofibrescopy was further examination of chronic cough in one patient, of recent small haemoptysis in six patients and of chest radiographical opacities in seven patients. In each case, no malignant lesion was found on examination with the bronchofibrescope. No complication of atopic diseases was observed in these control patients.

REAGENTS

RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with glutamine (1 mM) and gentamicin (50 μg ml^{-1}) was used in all experiments. Recombinant human IL-4 (lot 801; specific activity, $1{\cdot}0 \times 10^6$ U mg $protein^{-1}$) was a kind gift from Ono Pharmaceutical Co. (Osaka, Japan). None of these reagents contained endotoxins as judged by *Limulus amoebocyte* assay (Seikagaku Kogyo Co., Tokyo, Japan) in which the minimum detection level is 0·1 ng ml^{-1}. Lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5 strain was purchased from Difco Laboratory (Detroit, MI, U.S.A.).

PREPARATION OF HUMAN ALVEOLAR MACROPHAGES

AMs were obtained by bronchoalveolar lavage (BAL) after obtaining informed consent as described in detail previously (13). For BAL, the oral cavity and the upper airway were anaesthetized with lidocaine spray, and the tip of an Olympus fibre-optic bronchoscope (Model BF-1T20; Olympus Co., Tokyo, Japan) was wedged into one segment of the lung. Endobronchial findings in all control patients were normal and in this group the right middle lobe or lingular segment was lavaged. In the cancer group, BAL was performed in the non-tumour-bearing subsegment of the middle lobe or lingular segment at the diagnostic fibre-optic bronchoscopy. The lung was washed with 50 ml of sterilized saline (0·9%) prewarmed to 37°C and the fluid was gently sucked out with a 50 ml syringe. This process was repeated three times. A total of 150 ml of saline was instilled, of which about 40–70% was recovered. Immediately after collection, an aliquot was reserved for cytocentrifugation and staining (Diff-Quick method, American Scientific products, McGaw Park, IL, U.S.A.) for cell differential analysis. The remainder of the cells were counted, washed twice, and suspended in serum-free RPMI 1640 medium. There was no difference in total BAL cell number or percentage of AMs in BAL cells between lung cancer patients and control patients ($1{\cdot}15 \pm 0{\cdot}12$ vs $1{\cdot}28 \pm 0{\cdot}18 \times 10^7$ cells; $96{\cdot}5 \pm 0{\cdot}34$ *vs.* $95{\cdot}6 \pm 0{\cdot}43$ %). The viability of AMs was over 90%, as assessed by the trypan blue dye exclusion test. Cells were plated in each well of 96-well, flat-bottomed culture plates (Falcon 3072, Becton Dickinson, Lincoln Park, NJ, U.S.A.) at a concentration of 5×10^4 AMs per well, incubated for 1 h at 37°C under 5% CO_2 in air and then washed twice with warm medium to remove non-adherent cells. At this point, over 99% of the adherent cells in monolayers were macrophages, as judged by morphological examination and non-specific esterase staining.

IN VITRO ACTIVATION OF AMs

AMs were incubated in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Grand Island, NY, U.S.A.) with or without LPS (1 μg ml^{-1}) in the presence or absence of IL-4 (100 U ml^{-1}) for 18 h at 37°C under 5% CO_2 in air. LPS at 1μg ml^{-1} was chosen to examine the potency of AMs to produce cytokines, and IL-4 at 100 U ml^{-1} was chosen as a optimal concentration for suppression of cytokine production by monocyte macrophages (14,15). The cell-free supernatants were harvested and kept in a freezer for measurement of cytokine secretion.

MEASUREMENTS OF IL-10 AND IL-1β BY ENZYME IMMUNOASSAY

The supernatants of the AMs culture were pooled and protein concentrations of IL-10 and IL-1β were measured quantitatively by enzyme immunoassay (EIA). EIAs for human IL-10 and IL-1β were performed essentially as described previously (13–15). The sensitivity limits of the EIAs for IL-10 and IL-1β were 20 pg ml^{-1}.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. The statistical significance of differences between test groups was analysed by the Mann–Whitney *U*-test. Probability values of less than 0·05 were considered statistically significant.

Results

IL-10 PRODUCTION BY AMs FROM LUNG-CANCER PATIENTS AND CONTROL PATIENTS

We compared IL-10 production by AMs from lung-cancer patients with that from control patients. Samples of AMs from 14 control patients and 25 lung cancer patients were incubated with or without LPS (1 μg ml^{-1}) for 18 h. Then, IL-10 in the culture supernatants was quantitated by EIA. AMs from two out of 14 control patients produced low levels of IL-10 (39 pg ml^{-1} and 63 pg ml^{-1}) spontaneously. AMs from other control patients and from none of the lung-cancer patients produced IL-10 spontaneously. There was no difference between LPS-induced production of IL-10 by AMs from lung-cancer patients and from control patients (Fig. 1) (288·0±56·7 *vs.* 249·6±58·4 pg ml^{-1}). LPS-induced production of IL-10 by AMs from smokers with lung cancer (294·0±61·7 pg/ml^{-1}) was similar to that from control smokers (229·0 ±86·1 pg ml^{-1}). LPS-induced production of IL-10 by AMs from non-smokers with lung cancer (241·0±165·0 pg ml^{-1}) was also similar to that from control non-smokers (270·0±84·8 pg ml^{-1}).

IL-10 PRODUCTION BY AMs FROM LUNG-CANCER PATIENTS WITH DIFFERENT HISTOLOGICAL FEATURES AND AT DIFFERENT CLINICAL STAGES

We compared IL-10 production by AMs from patients with different histological types of lung cancer. As SCLC is reported to impair immune functions (16,17), the patients were divided into two groups; SCLC and NSCLC. As shown in Table 1, there was no difference in the production of IL-10 by AMs between the SCLC group and the NSCLC group.

Next, we compared the IL-10 production by AMs from lung-cancer patients with or without clinical distant metastasis. Results are also shown in Table 1. There was no difference between the production of IL-10 by AMs in patients without clinical distant metastasis (Stage I–IIIB) and in patients with clinical distant metastasis (Stage IV) .

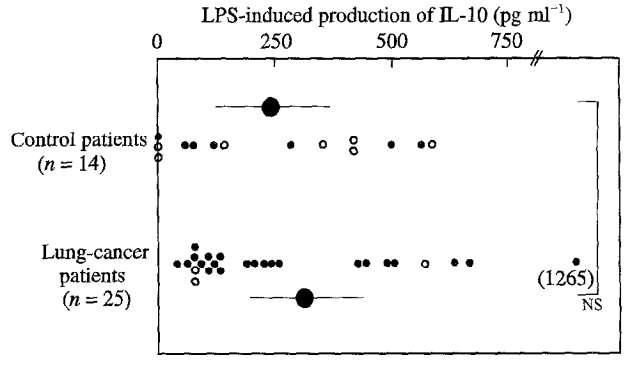

FIG. 1. IL-10 production by alveolar macrophages from lung-cancer patients and control patients. Large symbols and bars show means with SEMs for each group. ●, Smokers; ○, non-smokers; NS, not significant.

EFFECT OF IL-4 ON IL-10 PRODUCTION OF AMs FROM LUNG-CANCER PATIENTS

We have already reported that IL-4 suppresses pro-inflammatory cytokine production by human AMs (14,15). In the present study, we compared the effect of IL-4 on IL-10 production by AMs with its effect on IL-1β production by AMs from lung-cancer patients and from control patients. For this, samples of AMs from six control patients and eight lung cancer patients were incubated with or without LPS (1 μg ml^{-1}) for 18 h in the presence or absence of 100 U ml^{-1} of IL-4. Then, IL-10 levels in the culture supernatants were quantitated by EIA. Among the control and lung cancer patients, AMs produced low levels of IL-10 only in one case, and IL-4 alone had only a marginal effect on the spontaneous production of IL-10 (increase from 39 pg ml to 45 pg ml^{-1}). LPS-induced production of IL-1β by AMs was suppressed by IL-4 both in the control patients and lung-cancer patients and the difference was statistically significant in lung-cancer patients ($P=0·027$) (Fig. 2). In these experimental conditions, IL-4 had failed to suppress LPS-induced production of IL-10 by AMs from lung-cancer patients or from control patients (Fig. 2).

INFLUENCE OF SYSTEMIC CHEMOTHERAPY ON IL-10 PRODUCTION OF AMs FROM LUNG-CANCER PATIENTS

Finally, the production of IL-10 by AMs from eight lung cancer patients before and after platinum-containing chemotherapy were compared as follows. Samples of AMs were incubated in medium with or without LPS (1 μg ml^{-1}) for 18 h and then the culture supernatants were harvested and IL-10 was quantitated by EIA. Results are shown in Fig. 3. IL-10 was undetectable in unstimulated AMs supernatants in the group before or after chemotherapy. After stimulation with LPS, mean IL-10 secretion from AMs in the group after chemotherapy (278·0±112·8 pg ml^{-1}) was higher than that in the group before chemotherapy (152·3±51·9 pg ml^{-1}), but the range of values was large and the difference was not statistically significant. Within the limit of the six patients who had responded to systemic chemotherapy, the mean IL-10 level excreted by LPS-stimulated AMs after chemotherapy (299·5±145·0 pg ml^{-1}) was also higher than that before chemotherapy (177·7±67·2 pg ml^{-1}), but the difference did

TABLE 1. LPS-induced production of IL-10 by AMs from lung-cancer patients with different histological features and at different clinical stages

	LPS-induced production of IL-10 (pg ml^{-1})	
Histology		
Small-cell lung cancer (n=5)	300·2±113·7	NS
Non-small-cell lung cancer (n=20)	284·9±66·4	
Clinical stage		
Stage I–IIIB(n=10)	258·3±70·6	NS
Stage IV(n=15)	307·7±83·6	

LPS, lipopolysaccharide; NS, not significant.

not reach statistical significance. In the SCLC responder group, on *in vitro* treatment with LPS, IL-10 production by AMs obtained after chemotherapy (550·0±205·6 pg ml^{-1}) was also higher than that by AMs obtained before chemotherapy (208·3±133.9 pg ml^{-1}). In non-responders to systemic chemotherapy, LPS-induced IL-10 production by AMs decreased (from 74 pg ml to 25 pg ml^{-1}) in one patient and increased (from 78 to 402 pg ml^{-1}) in another upon systemic chemotherapy.

Discussion

In the present study, we observed that AMs from lung-cancer patients produce similar amounts of IL-10 upon stimulation with LPS to those produced by control patients and this is the first demonstration of the ability of monocyte macrophages to produce IL-10 in cancer patients. Moreover, LPS-induced production of IL-10 by AMs tended to increase upon systemic chemotherapy.

There are various reports on the functional changes of AMs in patients with lung cancer, such as a decrease in chemotactic activity, antibody-dependent cell-mediated cytotoxicity and cytostatic activity (9–11). On the other hand, we have already reported that AMs in lung-cancer patients have a similar ability to produce cytokines, such as tumour necrosis factor-α, IL-1β and the IL-1 receptor antagonist, to that of control subjects (18,19). To extend these previous findings, AMs from lung-cancer patients were found to have a similar ability to produce IL-10 to those of control patients (Fig. 1).

Various studies have revealed that tumour-associated macrophages play important roles in tumour progression through the production of various soluble factors (3–5) and recent attention has been focused on the angiogenesis-related molecules produced by macrophages. Macrophages produce various angiogenic cytokines, such as IL-1, IL-8, granulocyte-macrophage colony-stimulating factor, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), and proteases which degrade the extracellular matrix, such as metalloproteinases and serine proteases (20,21). In addition, macrophages produce various angiogenic inhibitors. Tissue inhibitor of metalloproteinases (TIMPs) is a natural inhibitor produced by macrophages (22,23). In addition, IL-10 is known to inhibit angiogenic cytokine synthesis by human macrophages (6,24). Moreover, as IL-10 augments the production of TIMPs by macrophages (7), it can be

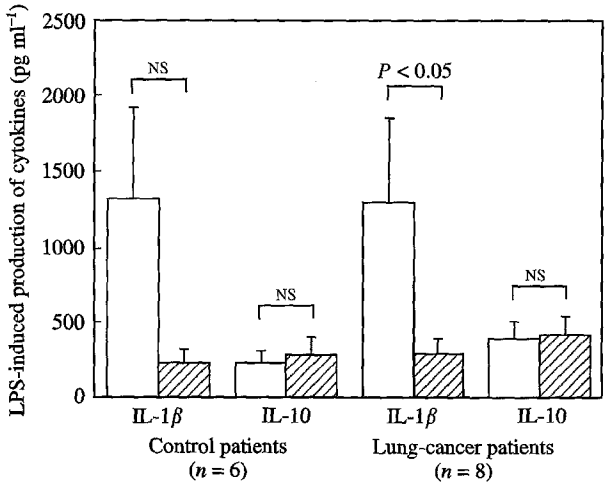

FIG. 2. Effect of IL-4 on the production of IL-1β and IL-10 by AMs treated with lipopolysaccharide (□,LPS; ▨, LPS+IL-4). Data are given as mean+SEM.

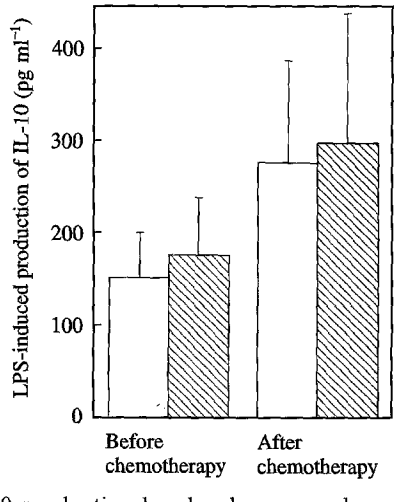

FIG. 3. IL-10 production by alveolar macrophages from lung-cancer patients before and after systemic chemotherapy. Data are given as mean+SEM.□, All patients; ▨, responders to chemotherapy (n=6).

considered an autoregulatory factor of macrophage-mediated angiogenesis. In a nude mouse system, Huang *et al.* (8) reported that the transfection of IL-10 to melanoma cells resulted in significantly slower subcutaneous tumour growing and fewer lung metastasis than control cells and considered suppression of macrophage-derived angiogenic factors by IL-10 as a possible mechanism of anti-tumour action. The present study revealed that, in lung-cancer patients, the ability of AMs to produce IL-10 is not impaired, even in advanced stage or SCLC patients (Table 1). Therefore, augmentation of IL-10 production by macrophages in the tumour site could be a strategy to control tumour progression through the inhibition of angiogenesis.

We have previously reported that IL-4 suppresses pro-inflammatory cytokine production by human AMs in normal subjects (14,15). To confirm and extend these previous findings, IL-4 suppressed IL-1β production by AMs not only from control patients, but also from lung cancer patients (Fig. 2). On the other hand, we have shown for the first time that IL-4 failed to suppress LPS-induced production of IL-10 by AMs from control or lung-cancer patients (Fig. 2). The reason for the difference in action of IL-4 on the production of IL-1β and IL-10 by AMs must be clarified in future studies, but it is of interest to speculate that IL-10 production is important as an autoregulatory mechanism of monocyte macrophage function and is resistant to modulation by other factors.

Systemic chemotherapy is known to affect various functions of competent cells of the host immune system (17,25,26). The phagocytic oxidizing activity of AMs in SCLC patients was found to be suppressed during cytotoxic treatment (27). On the other hand, restoration of impaired functions of monocyte macrophages upon systemic chemotherapy has been reported (28,29) and a direct effect of chemotherapeutic agents on monocyte macrophages and decreased tumour burden upon chemotherapy are considered to be possible mechanisms for the restoration. In the present study, IL-10 production by AMs tended to increase upon systemic chemotherapy (Fig. 3), which suggests that the important role of IL-10 as an endogenous inhibitor of angiogenesis is still maintained after systemic chemotherapy.

Besides its role as an autoregulatory factor of monocyte macrophage function IL-10 is known to be a member of the Type 2 cytokines which suppress cellular immune responses (30,31) and a shift of the Type 1/Type 2 balance towards Type 2 predominance has been reported in cancer patients (32–34). Previous studies have revealed that cancer cells produce IL-10 (35–37), and an increased expression of IL-10 by tumour-infiltrating lymphocytes has also been reported (38,39). Although Huang *et al.* (40,41) have reported that tumour-derived soluble factors induce IL-10 production by macrophages *in vitro*, and considered macrophages, in addition to tumour cells and lymphocytes, to contribute to the predominant Type 2 cytokine pattern, the present study did not confirm the overexpression of IL-10 by AMs in lung-cancer patients.

In conclusion, we have demonstrated that macrophages have a potent ability to produce IL-10 in lung-cancer patients. Establishment of the protocol to apply this potent anti-angiogenic activity in cancer treatment is warranted. At the same time, further investigation is necessary to clarify whether macrophage-derived IL-10 has a role in the imbalance of Type 1/Type 2 cytokines in cancer patients.

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