

## Irradiation Stimulates Human Lung Fibroblasts to Release Inflammatory Cell Chemotactic Activity

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**Background** : Radiation therapy plays an important role in the treatment of lung cancer. However, it also radiation therapy often induces serious complications such as radiation-induced pneumonitis and its underlying mechanism remains to be elucidated.

**Objectives** : The present study examined whether radiation might stimulate human lung fibroblasts (HLF) to release neutrophil and monocyte chemotactic activity (NCA and MCA).

**Methods** : HLF were exposed to varying doses of radiation (3-12 Gy) at varying incubation times (12-72 hr). The chemotaxis assays of NCA and MCA were performed by a 48-well microchemotaxis chamber method. The effect of polyclonal antibodies of various cytokines on NCA and MCA was evaluated. Furthermore, the protein and mRNA expression of these effective cytokines was assessed by ELISA and RT-PCR.

**Results** : HLF released NCA and MCA in response to irradiation in a dose- and time- dependent manner. NCA was significantly inhibited by anti-interleukin (IL)-8 antibody and MCA was significantly attenuated by anti-monocyte chemoattractant protein (MCP)-1 antibody. The protein secretion of IL-8 and MCP-1 was significantly increased by irradiation, and mRNA expression of IL-8 and MCP-1 was upregulated by irradiation.

**Conclusion** : These findings suggest that HLF may, at least partly, participate in the development of radiation-induced pneumonitis. *Shinshu Med J 58 : 57-68, 2010*

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**Key words** : irradiation, human lung fibroblast, chemotaxis, IL-8, MCP-1

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### I Introduction

Radiation therapy plays an important role in the treatment of localized lung cancer<sup>1)-3)</sup>. However, it often induces serious complications, including radiation-induced pneumonitis and fibrosis. In particular, radiation-induced pneumonitis occurs in 5 to 20 % of patients irradiated for lung cancer and is sometimes a limiting factor in radiation therapy<sup>4)-8)</sup>. An early acute pneumonitis, characterized by non-pro-

ductive cough, fever and dyspnea on effort, develops 2 to 12 weeks after completion of a course of radiotherapy<sup>9)-11)</sup>. It is thought that the early onset of symptoms is indicative of a more serious and protracted clinical course<sup>12)</sup>. However, the underlying molecular and cellular mechanisms of radiation-induced pneumonitis remain to be elucidated.

Pathological characteristics of radiation-induced pneumonitis are interstitial edema, interstitial and alveolar cellular inflammation, and an increase in the number of type II pneumocytes<sup>13)</sup>. An increase in inflammatory cells including neutrophils and monocytes is often found in the lumen of airways, the interstitium and the alveolar spaces in rat fol-

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lowing thoracic irradiation<sup>14</sup>). Therefore, it is thought that inflammatory cells may play an important role in radiation-induced pneumonitis. Sequestration of peripheral blood neutrophils and monocytes within the lung is characteristic of a number of acute and chronic pulmonary diseases<sup>15)16)</sup>. The presence of neutrophils is determined by the local generation of chemotactic agents, which direct neutrophil migration from the vascular compartment to the alveolar space along chemotactic gradients. The alveolar macrophage is also derived predominantly from differentiated peripheral blood monocyte migration and to a limited extent from local macrophage replication<sup>17)</sup>. Although elicited neutrophils and macrophages serve a vital role in host defense against a number of organisms, the presence of increased numbers of activated inflammatory cells can lead to tissue injury and fibrosis-promoting activities via the excessive elaboration of inflammatory cytokines, proteolytic enzymes, and oxygen radicals<sup>16)18)</sup>. Substantial investigation has focused on the alveolar macrophages as a primary source of the chemotactic factor<sup>19)</sup>. However, neutrophil chemotactic activity (NCA) and monocyte chemotactic activity (MCA) have been found to be produced by endothelial cells<sup>20)</sup>, lung fibroblasts<sup>21)</sup>, and pulmonary epithelial cells<sup>22)</sup>.

The fibroblast is the principal cell of most connective tissues and is involved in constituting collagenous and noncollagenous components of the extracellular matrix. This synthetic activity serves an important structural function by providing a framework for organ integrity. In addition to this traditionally accepted function, recent studies<sup>23)–26)</sup> have demonstrated that fibroblasts not only serve to maintain the connective tissue but are important participants in the orchestration of acute and chronic inflammation. In this context, fibroblasts released monocyte chemoattractant protein (MCP)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF)- $\beta$  in response to interleukin (IL)-1  $\beta$  and tumor necrosis factor (TNF)- $\alpha$ , and platelet-derived

growth factor, suggesting their contribution to certain disease states<sup>23)–26)</sup>. Recently, it was reported that bleomycin and cyclophosphamide stimulate human lung fibroblast (HLF) to release NCA and MCA, including IL-8, granulocyte colony-stimulating factor (G-CSF), MCP-1, and GM-CSF<sup>27)28)</sup>. Therefore the fibroblast, because of its anatomic location, is in a pivotal position to participate in and establish direct communication between interstitial and vascular events in pulmonary inflammation and fibrosis.

Although airway epithelial cells and alveolar macrophages may play a role in inflammatory cell migration from the interstitium to the alveolar and bronchial spaces in response to irradiation, the role of HLF in inflammatory cell recruitment in response to irradiation is uncertain. The purpose of the present study was to determine whether HLF could participate in the recruitment of inflammatory cells into the lung. Specifically, the possibility of lung fibroblasts releasing NCA and MCA in response to irradiation was evaluated. The results demonstrated that HLF released NCA and MCA in response to irradiation, including IL-8 and MCP-1, suggesting that HLF may participate in the development of radiation-induced pneumonitis.

## II Materials and Methods

### A Culture of HLF

We used fetal HLF (lung, diploid, human, passage 29) which are an established cell line and commercially available (American Type Tissue Culture Collection, Manassas, VA). The HLF were suspended at  $1.0 \times 10^6$  cells/ml in Ham's F-12 medium supplemented with penicillin (50 U/ml, GIBCO BRL life technologies, Grand Island, NY), streptomycin (50  $\mu$ g/ml, GIBCO BRL), and fungizone (2  $\mu$ g/ml, GIBCO BRL) and 10 % heat-inactivated fetal bovine serum (GIBCO BRL). After the cells reached confluence, they were harvested with 0.25 % trypsin and 0.1 % ethylenediamine tetraacetic acid in phosphate buffered solution (Sigma, St. Louis, MO), centrifuged at low speed (250 x g, 5 min), and were re-suspended in fresh medium at  $1 \times 10^6$  cells/ml in

30 mm tissue culture dishes. The cells reached confluence after 4-6 days incubation, and were then used for the experiments.

### **B Exposure of HLF to irradiation**

The medium was removed from cells by washing twice with serum-free Ham's F-12, and cells were incubated in the presence and absence of irradiation. To determine the dose- and time-dependent release of NCA and MCA the cells were exposed to varying doses of radiation (3, 6, 9, 12 Gy, by high X-ray source, 1.5 MeV, linear accelerator, Mitsubishi, Tokyo, Japan), and were incubated for 12, 24, 48, and 72 hr at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere. Irradiation did not cause injury (no deformity of cell shape, no detachment from tissue culture dish, and greater than 98 % of cells were viable by trypan blue exclusion) after 48 hr incubation at 6 Gy. The supernatant fluids were then harvested and stored at -80 °C until assayed. At least six separate HLF supernatant fluids were harvested for each experimental condition.

### **C Measurement of NCA and MCA**

Polymorphonuclear leukocytes were purified from heparinized normal human blood by the method of Boyum<sup>29</sup>. The resulting cell pellet, as determined by trypan blue and erythrosine exclusion, consisted of >96 % neutrophils and >98 % viable cells. The cells were suspended in Gey's balanced salt solution (GIBCO BRL) containing 2 % bovine serum albumin (BSA, Sigma) at pH 7.2 to give a final concentration of 3.0x10<sup>6</sup> cells/ml. This suspension was used for the neutrophil chemotaxis assay.

Mononuclear cells for the chemotaxis assay were obtained from normal human volunteers by Ficoll-Hypaque density centrifugation to separate red blood cells and neutrophils from mononuclear cells. The preparation routinely consisted of 30 % large monocytes and 70 % small lymphocytes determined by morphology and alpha-naphthyl acetate esterase staining (Sigma) with >98 % viability as assessed by trypan blue and erythrosin exclusion. The cells were suspended in Gey's balanced salt solution containing 2 % BSA (Sigma) at pH 7.2 to give a final

concentration of 5.0X10<sup>6</sup> cells/ml. This suspension was used for the monocyte chemotaxis assay.

The chemotaxis assay was performed by a 48-well microchemotaxis chamber (NeuroProbe Inc., Cabin John, MD), as described previously<sup>30</sup>. The bottom wells of the chamber were filled with 25 µl of fluid containing the chemotactic stimulus or media in duplicate. A 10 µm thick polyvinylpyrrolidone-free polycarbonate filter (Nucleopore, Pleasanton, CA), with a pore size of 3 µm for neutrophil chemotaxis and 5 µm for monocyte chemotaxis, was placed over the bottom wells. The silicon gasket and upper pieces of the chamber were attached, and 50 µl of the cell suspension was placed into the upper wells above the filter. The chambers were incubated in humidified air in 5 % CO<sub>2</sub> at 37 °C for 30 min for neutrophil chemotaxis and 90 min for monocyte chemotaxis. After incubation the chamber was disassembled and non-migrated cells were wiped away from the filter. The filter was then immersed in methanol for 5 min, stained with Diff-Quik (American Scientific Product, McGaw Park, IL), and mounted on a glass slide. Cells completely migrated through the filter were counted by using light microscopy in ten random high power fields (HPF, ×1000) per well.

To ensure that monocytes, but not lymphocytes, were the primary cells that migrated in the monocyte chemotaxis assay, some membranes were stained with alpha-naphthyl acetate esterase according to the manufacturer's directions (Sigma).

All volunteers provided written informed consent in advance for the use of their bloods. The study was performed in compliance with the Declaration of Helsinki.

### **D Effects of LTB<sub>4</sub> receptor antagonist on NCA and MCA released from HLF in response to irradiation**

LTB<sub>4</sub> receptor antagonist (ONO 4057, Ono Pharmaceutical Co., Tokyo, Japan) at a concentration of 10<sup>-5</sup> M was used to evaluate the involvement of LTB<sub>4</sub> as NCA and MCA in the supernatant fluids incubated with irradiation at 6 Gy for 48 hr.

### **E Effects of polyclonal antibodies to IL-8, G-CSF, GM-CSF, MCP-1, M-CSF, or RANTES on NCA and MCA released from HLF in response to irradiation**

The neutralizing antibodies to IL-8, G-CSF, MCP-1, GM-CSF, M-CSF, and RANTES were purchased from Genzyme (Cambridge, MA). These antibodies were obtained from mouse and added to HLF supernatant fluids, which were harvested at 48 hr incubation in response to 6 Gy irradiation at the suggested concentrations to inhibit the migratory potential of these cytokines. Then these samples were used for chemotactic assay. The antibodies inhibited each chemokine-induced neutrophil and monocyte chemotactic activity, and each antibody did not influence the neutrophil and monocyte chemotaxis induced by activated serum or fMLP (formyl methionyl leucyl phenylalanine), as we have previously reported<sup>27)28)</sup>. We used non-immune IgG as negative control.

### **F Measurement of IL-8 and MCP-1 proteins in HLF supernatant fluids**

Because IL-8 and MCP-1 antibodies significantly inhibited NCA and MCA in the supernatant fluids, the concentrations of IL-8 and MCP-1 in HLF supernatant fluids cultured for 48 hr after 6 Gy irradiation were measured by enzyme linked immunosorbent assay (ELISA) according to the manufacturers' direction. IL-8 and MCP-1 kits were purchased from R&D systems (Minneapolis, MN), and the minimum detectable concentration was 10.0 pg/ml for IL-8 and 31.3 pg/ml for MCP-1.

### **G Evaluation of IL-8 and MCP-1 mRNA expressions**

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to evaluate mRNA expression of IL-8 and MCP-1 in HLF cultured for 48 hr after 6 Gy irradiation. Total cellular RNA was extracted from adherent cells using a modification of the methods of Chomczynski and Sacchi<sup>31)</sup>. One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) using cDNA synthesis kit (MuLV Transcriptase, Rnase Inhibitor in PERKIN ELMER, Dnase I in TAKARA Co. Tokyo,

Japan) and then amplified for 26 cycles in PERKIN ELMER Gene Amp PCR System 9600-R (denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 30 sec, and primer extension at 72 °C for 30 sec). The sequences of IL-8 and MCP-1 used in the present study were as follows :

IL-8 sense, 5'-AAC ATG ACT TCC AAG CTG GC-3', and anti-sense, 5'-ACT GGC ATC TTC ACT GAT TC-3'; MCP-1 sense, 5'-CAGCCAGATG-CAATCAATGC-3' and anti-sense, 5'-GTG GTC CAT GGA ATC CTG AA-3';  $\beta$ -actin sense, 5'-TGA CCC AGA TCA TGT TTG AG-3' and anti-sense, 5'-TCA TGA GGT AGT CAG TCA GG-3'

Preliminary studies indicated that more than 26 cycles were subsaturating for the mRNA tested, and thus was appropriate for comparison of relative levels of mRNA between groups. PCR products were separated by electrophoresis on 3% agarose gel.  $\beta$ -actin mRNA, which has been shown not to change by stimulation, was measured in both normal and stimulated RNA samples at each point, using the same cDNA that was analyzed for cytokines. The intensity of the bands was quantified by densitometry. The results were expressed as the ratio of intensity to the  $\beta$ -actin.

### **H Statistics**

In experiments where multiple experiments were made, differences between groups were tested for significance using one-way analysis of variance with Fisher's multiple range test applied to data at specific time and dose points. In experiments where a single measurement was made, the differences between groups were tested for significance using Student's paired t-test. In all cases, a p value less than 0.05 was considered significant. Data in figures and tables were expressed as means  $\pm$  S.E.

## **III Results**

### **A Release of NCA and MCA from HLF**

HLF released NCA and MCA in a dose-dependent manner in response to irradiation (Fig. 1A, B). The lowest dose of irradiation to stimulate HLF significantly was 3 Gy for NCA and MCA. Increasing doses of irradiation progressively increased the

release of NCA and MCA up to 12 Gy. Less than 3 Gy irradiation did not significantly release NCA and MCA from human lung fibroblast.

Although HLF released NCA and MCA constitutively, HLF further released NCA and MCA in

response to irradiation in a time-dependent manner (Fig. 2A, B). The release of NCA and MCA was significantly increased after 12 hr exposure to irradiation (Fig. 2A, B). The release of chemotactic activity reached a plateau after 48 hr.

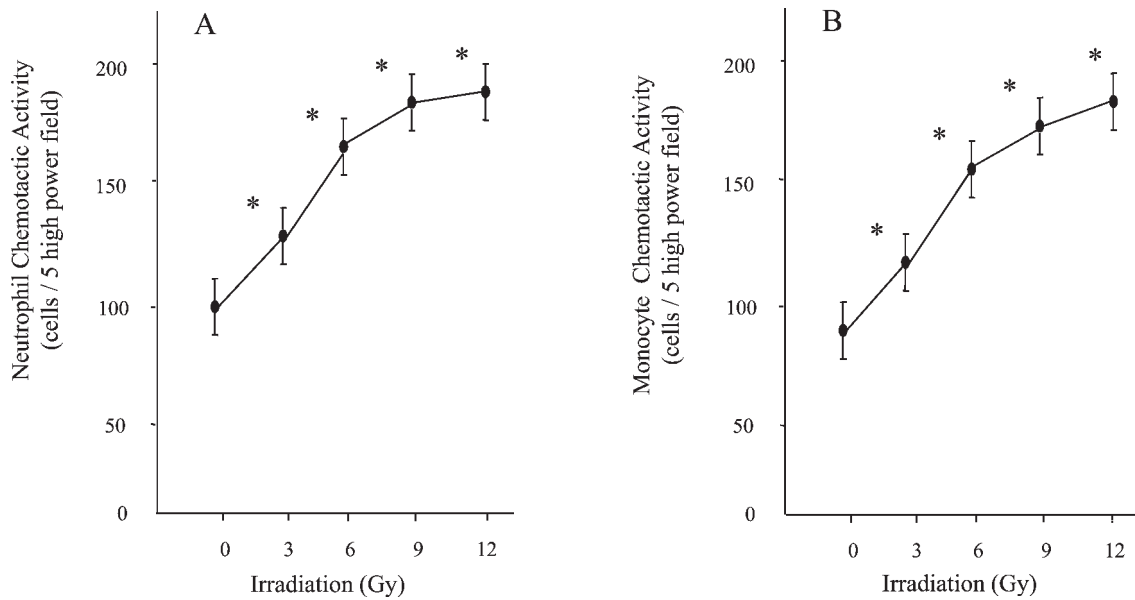


Fig.1 Dose-dependent release of neutrophil (panel A) and monocyte chemotactic activity (panel B) in response to irradiation from HLF monolayers after 48 hr incubation (n = 12 monolayers). Values are expressed as means±S.E.

\*p<0.05 compared with supernatant fluids without irradiation.

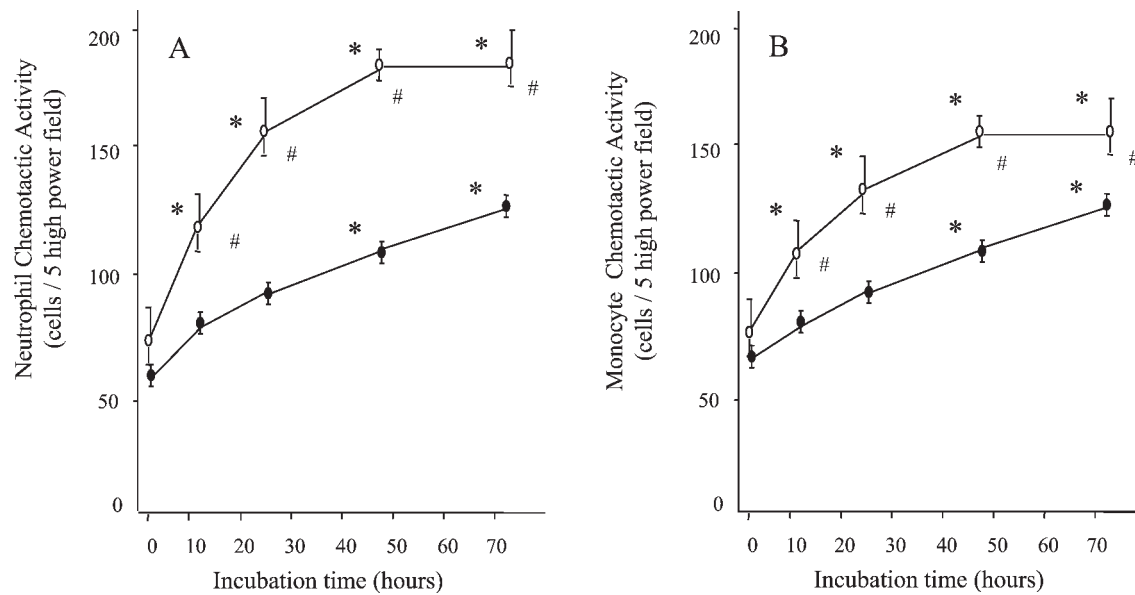


Fig.2 Time-related release of neutrophil (panel A) and monocyte chemotactic activity (panel B) in response to 6 Gy irradiation (open circles) and baseline release (closed circles) from HLF monolayers (n = 12 monolayers).

Values are expressed as means±S.E.

\* : p<0.05 compared with medium alone.

# : p<0.05 compared with supernatant fluid without irradiation.

Confirmation that the migrated cells were monocytes was provided by the following lines of evidence: 1) >90 % of the migrated cells appeared to be monocytes morphologically by light microscopy; 2) >90 % of the migrated cells were esterase positive; and 3) lymphocytes purified by allowing the monocytes to attach to plastic and tested in the chemotaxis assay yielded 0–20 % of the chemotactic activity of the monocyte preparation.

### B Inhibition of NCA and MCA by polyclonal antibodies to IL-8, G-CSF, MCP-1, GM-CSF, M-CSF, and RANTES, and by LTB4 receptor antagonist

Because HLF had the potential to release chemokines, and the chemokines released from HLF might be responsible for NCA and MCA, we used polyclonal blocking antibodies to IL-8, G-CSF, MCP-1, GM-CSF, M-CSF and RANTES. Among these antibodies, anti IL-8 antibodies inhibited NCA ( $p < 0.05$ , Fig. 3A). Anti G-CSF antibody attenuated

NCA, but not significantly. NCA in the crude samples was slightly attenuated by the addition of LTB4 receptor antagonist, but this too was not significant (Fig. 3A). The combination of anti-IL-8 and G-CSF antibodies, and LTB4 receptor antagonist inhibited NCA completely ( $p < 0.05$ , Fig. 3A).

Anti-MCP-1 antibodies inhibited MCA ( $p < 0.05$ , Fig. 3B). In contrast, Anti-GM-CSF, M-CSF and RANTES antibodies did not inhibit MCA (Fig. 3B). MCA in the crude samples was not inhibited by the addition of LTB4 receptor antagonist (Fig. 3B). The combination of anti-MCP-1, GM-CSF, M-CSF and RANTES antibodies, and LTB4 receptor antagonist also similarly inhibited MCA completely ( $p < 0.05$ , Fig. 3B).

Non-immune IgG was used to evaluate the effect of nonspecific antibody. Non-immune IgG did not attenuate the NCA and MCA in the same irradiation-conditioned medium.

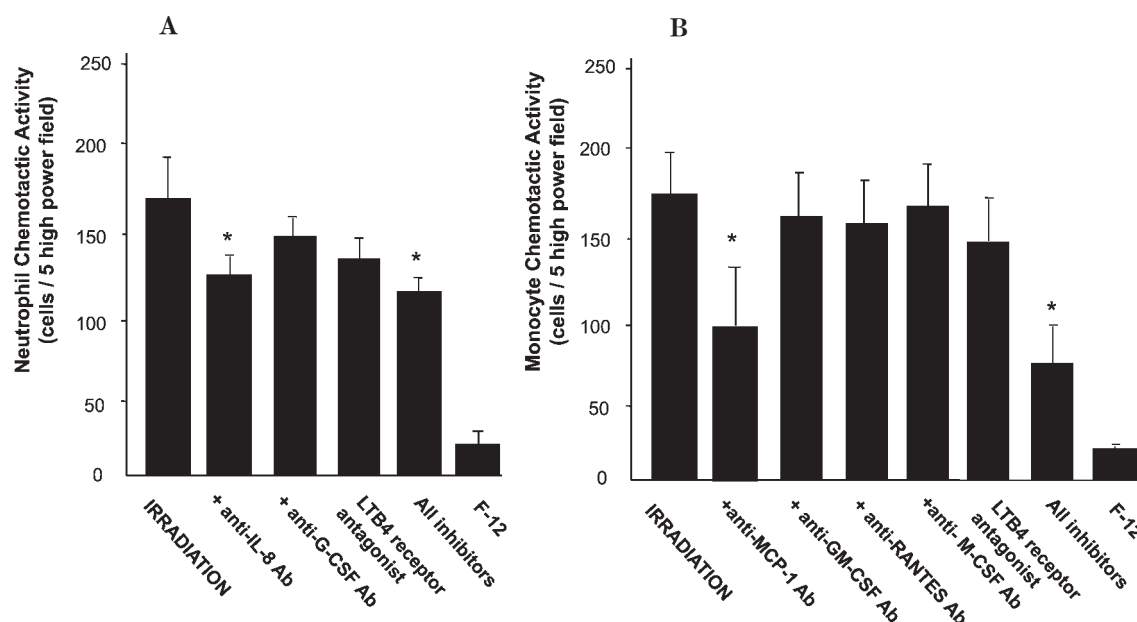


Fig. 3 Panel A: Inhibition of NCA in HLF supernatant fluids harvested at 48 hr in response to 6 Gy of irradiation combined with anti-IL-8 antibody, anti-G-CSF antibody, or LTB4 receptor antagonist ( $n = 12$  monolayers).

Panel B: Inhibition of MCA in HLF supernatant fluids harvested at 48 hr in response to 6 Gy of irradiation combined with anti-MCP-1 antibody, anti-GM-CSF antibody, anti-M-CSF antibody, anti-RANTES antibody, or LTB4 receptor antagonist ( $n = 12$  monolayers).

Chemotactic activity is on the ordinate, and the experimental groups are on the abscissa.

Values are expressed as means  $\pm$  S.E.

\* :  $p < 0.05$  compared with untreated supernatant fluids.

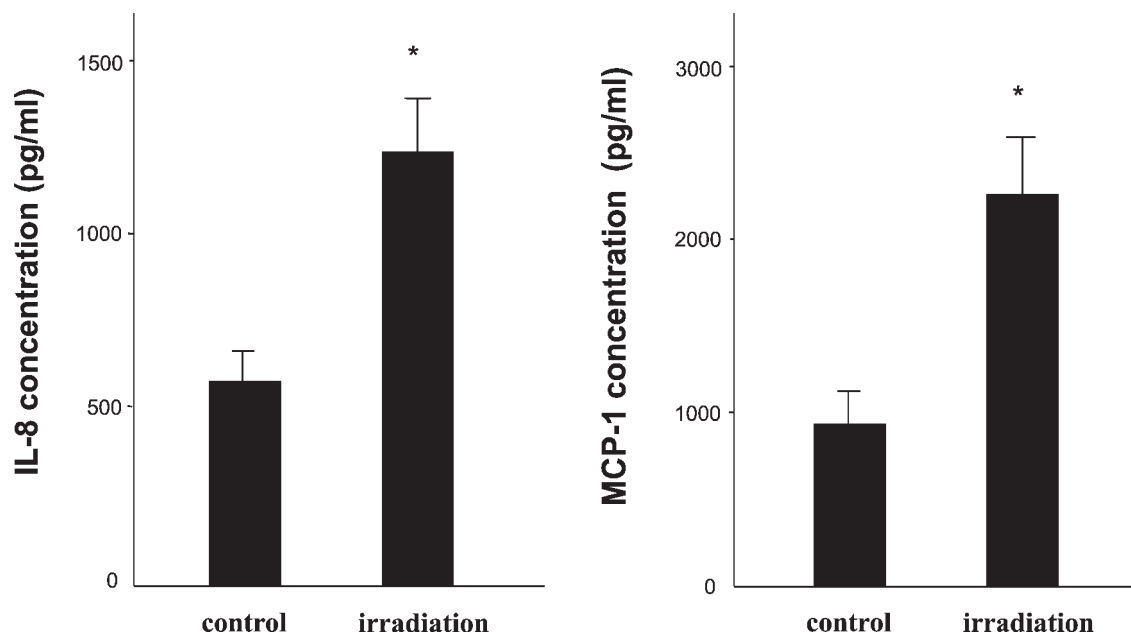


Fig. 4 The release of cytokines from HLF in response to irradiation. Panel A shows the release of IL-8 from HLF in response to irradiation at 6 Gy for 48hr. Panel B shows the release of MCP-1 in response to irradiation from HLF.

Values are expressed as means  $\pm$  S.E.

\* $p < 0.05$  compared with supernatant fluids without irradiation.

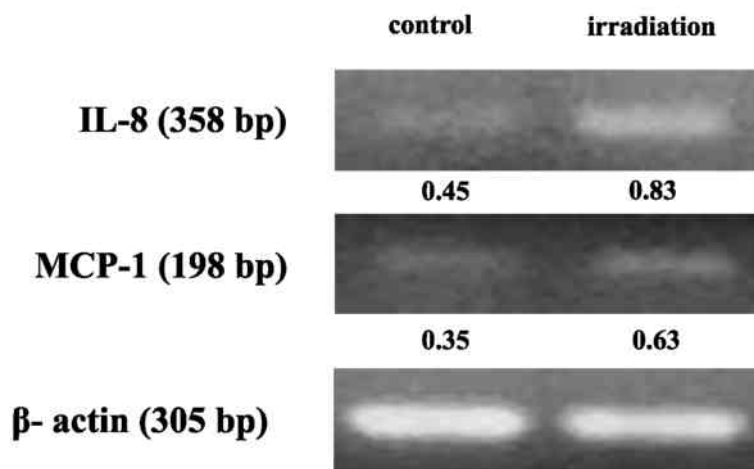


Fig. 5 Augmentation of IL-8 and MCP-1 mRNA expression by 6 Gy irradiation after 48 hr incubation in HLF monolayers. Data presented is representative of three experiments. The densitometry data with IL-8 is expressed as a ratio of IL-8 mRNA to  $\beta$ -actin mRNA (IL-8 mRNA/  $\beta$ -actin mRNA). The densitometry data with MCP-1 is expressed as a ratio of MCP-1 mRNA to  $\beta$ -actin mRNA (MCP-1 mRNA/  $\beta$ -actin mRNA).

### C Release of IL-8 and MCP-1 from HLF

HLF was exposed to 6 Gy radiation and incubated for 48 hr after irradiation. The concentration of IL-8 and MCP-1 in the culture supernatant fluid significantly increased ( $p < 0.05$ , Fig. 4A, B).

### D Upregulation of IL-8 and MCP-1 mRNA expression from HLF in response to irradiation

HLF was exposed to 6 Gy radiation and incubated for 48 hr after irradiation. The expression of IL-8 and MCP-1 mRNA was upregulated (Fig. 5).

#### IV Discussion

In the present study, HLF released NCA and MCA by irradiation in a dose- and time-dependent manner. Pretreatment by anti-IL-8 antibody inhibited NCA released from HLF. Pretreatment by anti-MCP-1 antibody inhibited MCA released from HLF. In response to irradiation, the release of IL-8 and MCP-1 was significantly increased. Furthermore, the mRNA expression of IL-8 and MCP-1 was upregulated by irradiation. These data suggest that an interaction between HLF and irradiation may modulate inflammatory cell recruitment from the vasculature to the interstitium after irradiation through the generation of chemotactic cytokines.

Although the pathogenesis of radiation-induced pneumonitis is complex and not yet fully elucidated, it is thought that part of the pathogenesis of radiation pneumonitis is concerned with free radicals<sup>12)</sup>. The ionizing radiation collides with and excites electrons, which in turn generate free radicals<sup>32)</sup>. Free radicals are highly reactive molecules capable of causing the scission of covalent bonds, resulting in molecular changes that can lead to significant biochemical, structural, and functional abnormalities. Damage to structural molecules can lead to immediate effects, such as leaky cell membranes or faulty transport of intracellular material, and then resulting in rapid cell death. Moreover, free radicals are also capable of causing direct damage to DNA, which would lead to cell death. Although there is no doubt that direct damage to cells by irradiation is one etiology of radiation-induced pneumonitis, radiation-induced pneumonitis sometimes extends beyond the irradiated lung field and tends to follow a rapid course which is severe and fatal<sup>33)</sup>. In such a case, radiation-induced lung injury cannot be explained solely by the direct action of radiation. It is reported that regional increases of lymphocytes in irradiated lung have been revealed in bronchoalveolar lavage fluid (BALF)<sup>9)34)35)</sup> and histological examinations<sup>33)</sup>. However, it is also reported that the findings were similar between the irradiated lung and the "out of field" lung<sup>9)35)</sup>. In

addition, one of the pathological findings of radiation-induced pneumonitis in rat following thoracic irradiation was infiltration of inflammatory cells, including neutrophils and monocytes, into the interstitium and alveolar space<sup>13)14)</sup>. Based on these reports, hypersensitive immune reaction mediated by leukocytes is thought to be closely involved in the radiation-induced lung injury<sup>9)35)</sup>. Thus hypersensitive immune reaction that recruits and activates large numbers of leukocytes often involves specific chemotactic mediators<sup>36)</sup>. In this context, previous studies have shown that dermal and synovial fibroblasts can release soluble chemotactic factors that direct the migration of neutrophils and monocytes in response to TNF and IL-1<sup>22)23)</sup>. The present study demonstrated that HLF released NCA and MCA constitutively and further in response to irradiation. Thus, lung fibroblasts may modulate their local immunologic environment by releasing chemotactic activity for both neutrophils and monocytes, and may contribute to the lung inflammation in addition to other uncertain reasons.

In animals, a number of authors have described a threshold dose level before any biochemical or histological changes begin to occur<sup>37)38)</sup>, which is followed by a steep sigmoid dose-response curve with increasing lethality with increasing dose. This has been documented by Travis and Tucker in a review of a number of experiments of single-dose lung irradiation in animals<sup>38)</sup>. The study by Van Dyk of humans treated by single-dose whole-lung irradiation shows a similar threshold level and steep sigmoid dose-response curve with increase in pulmonary symptoms and lethality with increasing dose<sup>39)</sup>. The probability of pneumonitis increased from zero at single fraction of less than 7.5 Gy, to 5 % at 8.2 Gy, to 50 % at 9.3 Gy, and 95 % at 10.6 Gy<sup>39)</sup>. This has also been documented by Mah from the same institution in a re-evaluation of fractionated irradiation converted to estimated single dose, again showing a steep sigmoid dose-response curve<sup>40)</sup>. Thus, lung damage from radiation can occur immediately after the start of treatment and is dose-dependent. These studies document a dose-



related pulmonary toxicity. The study by Johnson et al shows that the level of mRNA of TGF- $\beta$  increased from 5 Gy in C57BL/6 mice<sup>41</sup>. Rube et al show that after exposure to a single radiation dose of 6 Gy in C57BL/6 mice, the lung tissue revealed only a minor radiation-mediated TGF-beta mRNA response<sup>42</sup>. But only a minor histopathologic change is seen after thoracic irradiation with 6 Gy. Histological change therefore seemed to be seen over 10 Gy, but an increase in cytokines and their mRNA levels seemed to be seen from 5 Gy in the animal model. Furthermore, Brach et al document that the level of NF KB binding activity to DNA is increased from 2 Gy in human KG-1 myeloid leukemia cell lines<sup>43</sup>. Our study shows that the release of NCA and MCA from HLF is increased from a single 3 Gy irradiation. Based on the above and present reports, a very low dose of single irradiation could cause cellular response without histologic changes.

Recent research has supported the view that a variety of cytokines play important roles during radiation-induced lung injury. Some reports suggest that cytokines such as IL-6, IL-1, TNF- $\alpha$ , and TGF- $\beta$  have the potential to be a predictor of radiation pneumonitis<sup>44-46</sup>, while Hart et al have reported that blood IL-8 level has a predictive value for symptomatic radiation-induced pneumonitis in patients receiving thoracic radiation<sup>47</sup>. Although resident tissue macrophages are recognized as the major local cellular source of chemokines, some reports have suggested that lung fibroblasts have the potential to release NCA, such as IL-8 and G-CSF activities constitutively and further in response to LPS and bleomycin<sup>28</sup>, cyclophosphamide<sup>27</sup>, TNF, and IL-1<sup>48</sup>. Furthermore Daniele Brouty-Boye et al reported that the transcription for IL-8 cytokine was detected in cultured fibroblasts isolated from a variety of human tissues and pathogenic states, including radiation fibrosis<sup>49</sup>. Our study shows that the blocking antibody to IL-8 attenuated NCA from HLF significantly and moreover IL-8 was detected. These findings suggest that IL-8 may play a key role in the pathogenesis of pulmonary disorders by

recruiting neutrophils at the local microenvironment in response to irradiation. Furthermore, although the blocking antibody to anti-G-CSF antibody or LTB4 receptor antagonist alone did not attenuate NCA significantly, the combination of G-CSF antibody and LTB4 receptor antagonist with anti-IL-8 antibody further inhibited NCA more strongly than anti-IL-8 antibody alone. Therefore, G-CSF and LTB4 may have the potential to be involved in NCA by irradiation.

It has been reported that MCP-1 was detected in lung tissue or BALF after irradiation in the animal model<sup>41,50,51</sup>. Although there were no reports that lung fibroblasts released MCA in response to irradiation, some have documented that lung fibroblasts have the potential to release MCA, such as MCP-1, GM-CSF, RANTES, etc. in response to LPS and bleomycin<sup>28</sup>, cyclophosphamide<sup>27</sup>, TNF, and IL-1<sup>48</sup>. Our study shows that the blocking antibody to MCP-1 significantly attenuated MCA from HLF and confirmed the release and production of MCP-1 from HLF. Thus HLF released MCP-1, at least partly, as the responsible MCA.

Our results demonstrated that irradiation stimulated HLF to induce mRNA expression of IL-8 and MCP-1. However, the exact mechanism for irradiation to stimulate fibroblasts resulting in the expression of IL-8 and MCP-1 mRNA and the release of cytokines is uncertain. There is now evidence that irradiation may induce synthesis of inflammatory gene products<sup>37</sup>. Gene transcription, an important prerequisite of protein synthesis, is under the control of a group of proteins known as promoters or transcriptional factors, such as AP-1 response elements and NFkB. These factors are important inducers of transcription of many genes, especially cytokine genes. Irradiation stimulates the synthesis and/or binding to DNA of these factors<sup>43</sup>. If the synthesis and activation of these transcription factors is indeed directly induced by irradiation, it is quite likely that transcription of a wide variety of AP-1, and NFkB responsive genes will result. These findings might be involved in the expression of mRNA of IL-8 and MCP-1 by irradiation.

Because many stimuli, including IL-1 beta, TNF alpha, and LPS caused similar nuclear reactions, the lung infection observed in clinical settings may augment these responses.

In conclusion, irradiation stimulated HLF to release NCA and MCA. The released activity was blocked by anti-IL-8 and MCP-1 antibodies. The

release of IL-8 and MCP-1 from HLF was increased by irradiation. The mRNA expression of IL-8 and MCP-1 in HLF was upregulated by irradiation. These data suggest that HLF might participate in the development of radiation-induced pneumonitis by the release of NCA and MCA in response to radiation exposure.

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