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Cadmium Induces Interleukin-8 in Alveolar Epithelial Type II Cells (A549)

Katsuhiko Fukuda, Yutaka Hitsuda and Tadashi Igishi

Third Department of Internal Medicine, Faculty of Medicine, Tottori University, Yonago 683, Japan

Cadmium (Cd) is a contaminant in cigarette smoke and may play an important role in the pathogenesis of smoking-related emphysema. It is reported that repeated Cd exposure causes emphysema after neutrophil infiltration in the rat lung. Interleukin-8 (IL-8) is a major neutrophil chemotactic factor commonly involved in a variety of pulmonary disorders including emphysema. The aim of this study is to elucidate the effect of Cd on IL-8 production of alveolar epithelial type II cells, using the A549 cell line. We used 1–100 μM of cadmium chloride ($CdCl_2$) to stimulate the cells for a 48 h period. IL-8 production (2957 ± 137 pg/mL) without any cytotoxic effect was observed in the cell supernatants after 24 h exposure to 50 μM $CdCl_2$. The Cd-removed supernatant caused neutrophil chemotaxis and was inhibited by the anti-IL-8 antibody. Cd-induced IL-8 was inhibited by EDTA, and the inhibition was blocked by copper (II) chloride. The addition of anti-interleukin-1β or tumor necrosis factor-α antibodies did not diminish IL-8 release induced by Cd. These results suggest that Cd increases the production of IL-8 without any cytotoxic effect in alveolar epithelial cells, which may be an important factor in the developmental process of cigarette smoking-related emphysema.

key words: A549; alveolar epithelial type II cell; cadmium chloride; emphysema; interleukin-8

Cadmium (Cd) is not only an air pollutant, but is also one component of cigarette smoke. Cd inhalation can cause mainly 2 types of lung damage depending on its concentration. Acute chemical pneumonitis and bronchitis after a high concentration of Cd inhalation are believed to be the result of direct toxic effect of Cd on the lung tissue (Nermery, 1990). On the other hand, a low concentration of Cd inhalation can cause lung emphysema in rats. Since then, Cd within tobacco smoke has been thought to be a causative factor of cigarette-related emphysema in humans. Human lungs with emphysema had a significantly higher Cd content than those from a nonemphysematous age-matched control group and the degree of emphysema was correlated with the Cd content of the lung tissue (Hirst et al., 1973).

It has been elucidated that neutrophil or neutrophil elastase plays a principal role in the establishment of emphysema in smokers. Recently, it is reported that an increase in the neutrophil influx in smokers was regulated by interleukin-8 (IL-8), a potent neutrophil chemotactic cytokine (Waltz and Kunkel, 1989). Although IL-8 is produced by various types of cells in the lung (Bagglioni et al., 1989), the alveolar epithelial type II cell (type II cell) is known to be one of the major sources of this cytokine.

In this study, we investigated the effects of Cd on IL-8 production in type II cells.

Abbreviations: BALF, bronchoalveolar lavage fluids; D-MEM/F-12, Dulbecco's modified Eagle medium: nutrient mixture F-12; FBS, fetal bovine serum; IL-8, interleukin-8; MTS, 3-(4,5-dimethylthiazol-2-yi)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PBS, phosphate-buffered saline; PMS, phenazine methosulfate

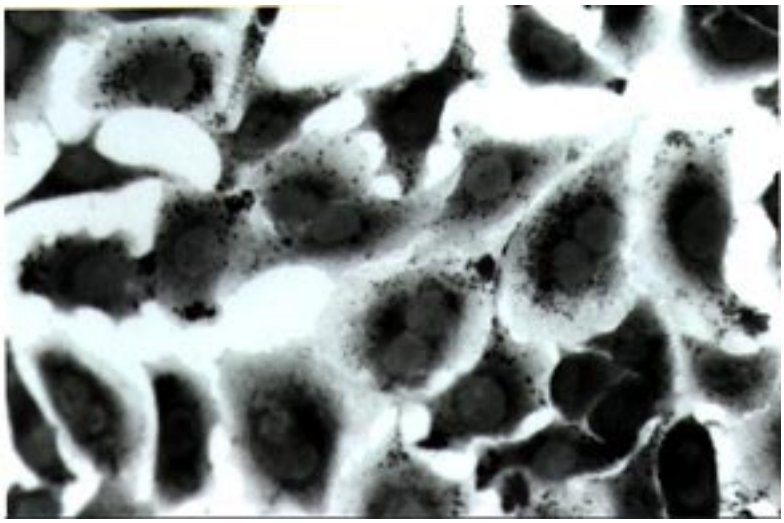


Fig. 1. A confluent monolayer of A549 cells from passage level 82nd was cultured in D-MEM/F-12 media with 10% FBS. The cells were stained with a tannic acid and polyclome stain. The dark cytoplasmic granules are the lamellar bodies. × 400.

Materials and Methods

Cell preparation

The A549 cells (American Type Culture Collection, Rockville, MD) we used are from the epithelial type II cell line derived from a patient with alveolar cell carcinoma (Leiber et al., 1976). The A549 cell line retains the features of type II cells, such as a cuboidal appearance, cytoplasmic multilamellar bodies and the synthesis of surfactants. We used confluent cultures of the A549 cell line at the 82nd to 86th passages (Fig. 1) in the present study. After trypsinization, the cells were plated at a concentration of 3×10^5 cells/well to 24-well microtiter plates (Nunc, Roskilde, Denmark) in 1 mL of Dulbecco's modified Eagle medium: nutrient mixture F-12, 1:1 (D-MEM/F-12) media containing 365 mg/L L-glutamine (Life Technologies, Inc., Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Wako Pure Chemical Industries Ltd., Osaka, Japan). Cultures were incubated in a humidified 95% air and 5% CO_2 atmosphere at 37˚C for 16 h to achieve confluence. A549 monolayers were washed with phosphate-buffered saline (PBS), replaced with serum free media (D-MEM/F-12) and incubated for 2 h. Cadmium chloride ($CdCl_2$; Wako) and other stimulants were suspended in fresh complete media for additional times (8, 16, 24 and 48 h). For blocking studies, A549 cultures were incubated with 100 neutralizing U/mL of monoclonal antibodies to human interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α; Genzyme, Cambridge, MA) 30 min after the addition of either Cd, TNF-α (10 U/mL) or IL-1β (10 U/mL). After each time of incubation with stimulants, the supernatants were filter sterilized, and stored at –30˚C until the IL-8 enzyme-linked immunosorbent assay (ELISA). The cells were lysed with 0.05% triton X-100 in PBS or freeze-thawed 4 times by alcohol/dry ice and a 37˚C water bath according to Gaffar and colleagues (1986) and Rosenthal and co-workers (1994). The cell

lysates were centrifuged and suspended in fresh media to quantify intracellular IL-8. $CdCl_2$, copper (II) chloride ($CuCl_2$; Wako), EDTA, lipopolysaccharide (LPS; W *E. coli* 055: B5; Difco Laboratories, Detroit, MI) and other reagents were dissolved in distilled water and sterilized with a MILLEX-GS 0.22 μm filter (Millipore Ltd., Tokyo, Japan) before use.

Viability and cytotoxic assay

Cell viability was evaluated with the tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yi)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (MTS assay) and trypan blue exclusion to define the cytotoxic threshold dose. The culture media were removed at 8, 16, 24 and 48 h after incubation of the cells with Cd (1, 5, 10, 50 and 100 μM), 20 μL of the MTS/phenazine methosulfate (PMS) solution (Promega Corporation, Woods Hollow Road, WI) were added to the culture wells and agitated cell monolayers in 0.2 mL of D-MEM/F-12 media. After 1 h, a 100 μL sample of each extraction was placed in duplicate in a 96-well plate and the amount of formazan production was assayed by measuring the density at 492 nm using a plate leader (MPR-A4i; Tosoh, Tokyo, Japan).

A549 cell monolayers with 50 μM Cd-treatment or without treatment were detached using trypsin, centrifuged and resuspended in fresh media with tripan blue and inspected under the haemocytometer. Viable cells were expressed as a percentage of the values obtained in the initial incubation.

IL-8 assay

The concentration of IL-8 was measured using a specific ELISA kit (Amersham International plc, Bucks, United Kingdom). This assay employs the quantitative immunometric, "sandwich" enzyme immunoassay technique. Briefly, 100 μL of samples and standards, both in duplicate, were added with assay diluent to a microtiter plate precoated with a monoclonal antibody specific to IL-8. After washing away any unbound sample proteins, these were incubated for 2 h with an enzyme-linked polyclonal antibody specific for IL-8. Following a wash to remove any unbound antibody-enzyme reagent, peroxidase substrate was added and incubated for 20 min. Then the reaction was terminated with sulphuric acid and the intensity of the color was measured at 450 nm in the plate leader (MPR-A4i).

Chemotactic assay

Chemotactic experiments were done using a modified Boyden chamber method (Cates et al., 1978). EDTA at 10^{-4} M was added to the Cd-containing samples after 24 h exposure, and filtrated using Ultrafree-C3 (molecular cut-off 3000; Millipore) to remove the Cd-EDTA complex. Human neutrophils, prepared from normal human peripheral blood, were separated and adjusted by Hanks' Balanced Salt Solution (Life Technologies) at a concentration of 2×10^6 cells/mL. The isolated neutrophils were at least 97% viable by trypan blue exclusion and 95% pure. Cd-removed supernatants (218 μL) were added to the bottom of Blind-Well chemotaxis chambers (Neuro Probe, Inc., Cabin John, MD). The positive control was 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (Wako) and the negative was D-MEM/F-12 alone. The neutrophil suspensions (200 μL) were added to the top chambers after separating from the bottom chambers by 3-μm polycarbonate filters (PVP-free; Portics Corp., Livermore, CA). For neutralizing studies, the test samples were incubated with a 1:100 dilution of monoclonal anti-human IL-8 antibody (Genzyme) at 37˚C for 1 h before adding to the chamber. The chambers were incubated in humidified 95% air and 5% CO_2 at 37˚C for 1 h. The filters were removed, fixed in methanol and stained by May-Giemsa. Neutrophils that migrated to the bottom chambers were counted by using a × 400 magnification in 10 fields. Chemotactic activity was shown as a percentage of the positive control as previously described by Standiford and colleagues (1990).

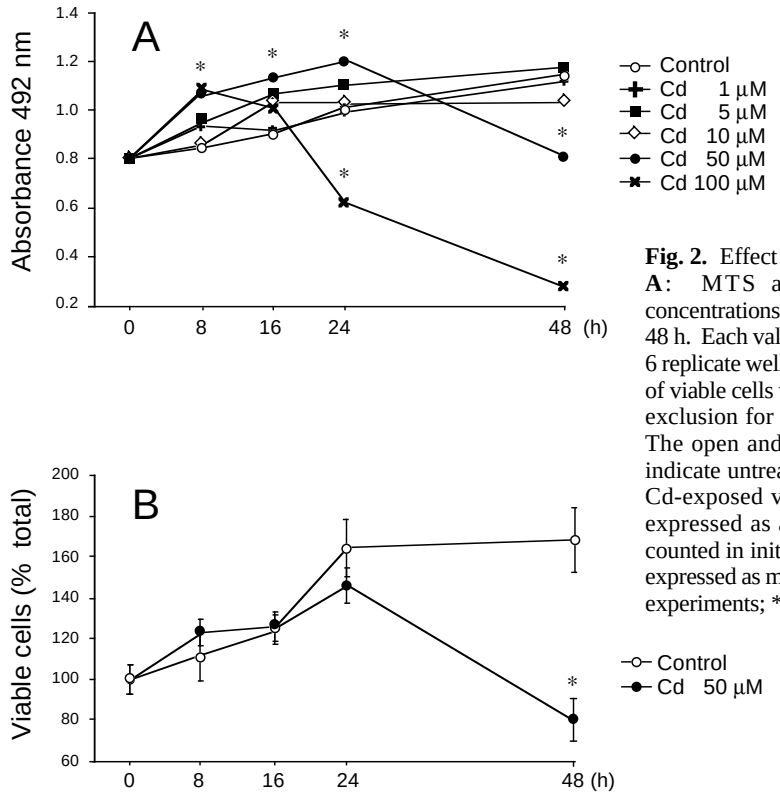


Fig. 2. Effect of Cd on A549 cell viability. **A**: MTS assay for the indicated concentrations of Cd and time intervals over 48 h. Each value represented the mean from 6 replicate wells; **P* < 0.01. **B**: The number of viable cells was monitored by trypan blue exclusion for the indicated time intervals. The open and closed circles respectively indicate untreated control cells and 50 μM Cd-exposed viable cells. The values are expressed as a percentage of viable cells counted in initial incubations. The data are expressed as mean ± SD from 6 independent experiments; **P* < 0.05.

Statistical analysis

Data are expressed as mean ± SD. The Kruskal-Wallis and Mann-Whitney *U* tests were used for statistical comparisons of results between different groups, and were considered significant when *P* values were < 0.05.

Results

MTS assay and trypan blue exclusion

The result of the MTS assay is shown in Fig. 2A. Up to 24 h, the absorbance of 1, 5, 10 and 50 μM Cd-exposed cells did not decrease compared with non-treated control cells (controls). In 50 μM Cd-exposed cells, MTS activities were significantly higher than that of the controls at 8, 16 and 24 h ($P < 0.01$, respectively). In 100 μM exposure of Cd, the absorbance was significantly higher than that of the controls at 8 h ($P < 0.01$), began to decrease at 16 h, and lowered significantly as compared with controls at 24 and 48 h ($P < 0.01$).

Figure 2B shows the result of the trypan blue exclusion for the indicated time intervals. Up to 24 h, there were no significant differences between 50 μM Cd-treated cells and the controls. At 48 h, the number of viable cells with Cd treatment was significantly smaller than that of the controls ($P < 0.05$).

IL-8 contents in supernatants and cell lysates

A549 cells cultured in the presence of Cd at a concentration of 1, 5, 10 and 50 μM induced steep time- and dose-dependent IL-8 release. In every time and dose, Cd-exposed cell cultures showed significantly ($P < 0.01$) high IL-8 levels compared with non-exposed controls. At 24 h postexposure, 50 μM Cd-induced IL-8 releases reached a plateau (2957 ± 137 pg/mL), resulting in a 15-fold increase when compared with untreated controls (190±11pg/mL)(Fig. 3A).

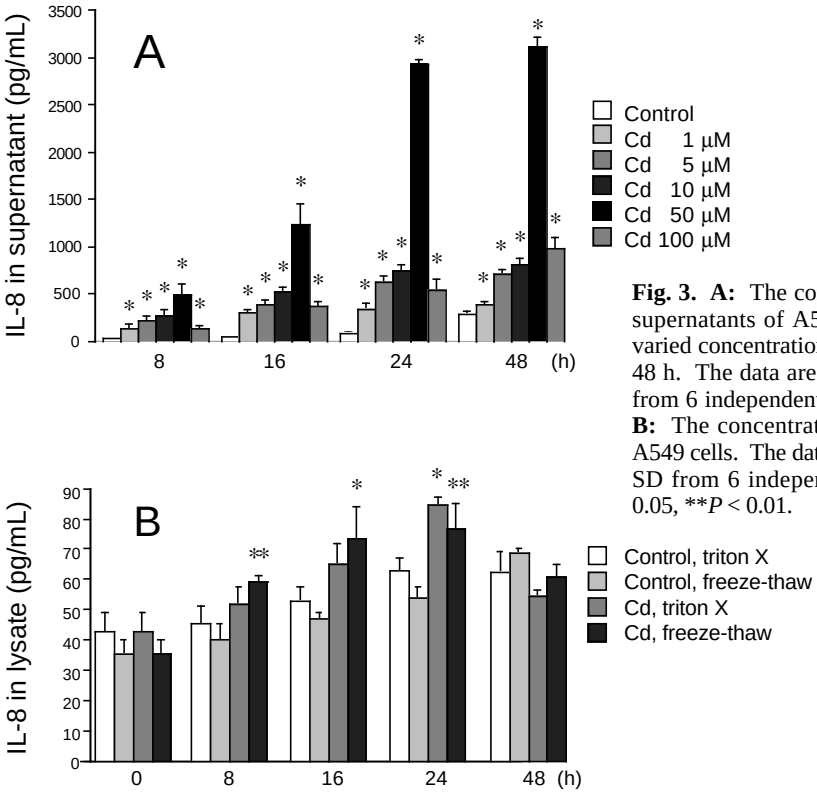


Fig. 3. A: The concentration of IL-8 in the supernatants of A549 cells stimulated with varied concentrations of Cd for 8, 16, 24 and 48 h. The data are expressed as mean ± SD from 6 independent experiments; **P* < 0.01. **B:** The concentration of IL-8 in lysates of A549 cells. The data are expressed as mean ± SD from 6 independent experiments; **P* < 0.05, ***P* < 0.01.

Intracellular IL-8 concentrations were quantified after the cells were lysed with 0.05% triton X-100 or repeated freeze-thawing. At 8 h and 16 h, 50 μM Cd-exposed cell lysates from the freeze-thaw treatment showed significantly (8 h; $P < 0.01$, 16 h; $P < 0.05$) high IL-8 levels compared with the controls. At 24 h, the IL-8 contents in the Cd-exposed cell lysates were significantly greater than those of the non-exposed controls (triton X-100 treated; $P < 0.05$, freeze-thaw; $P < 0.01$) (Fig. 3B). But these IL-8 levels were much lower than those of supernatants derived from Cd-exposed cell cultures.

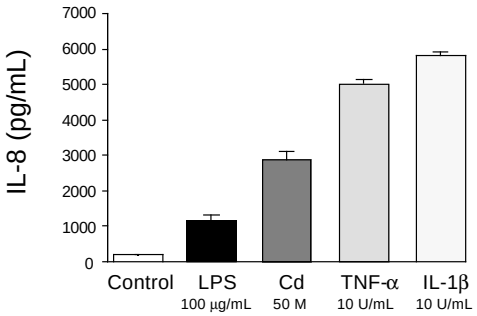


Fig. 4. The concentration of IL-8 in the supernatants of A549 cells stimulated by pretoxic doses of LPS, Cd, TNF-α and IL-1β for 24 h. The data are expressed as mean ± SD from 6 independent experiments.

IL-8 release induced by different stimulants

As shown in Fig. 4, peak levels of IL-8 release were observed after 24 h exposure. The IL-8 levels induced by non-cytotoxic doses of LPS, Cd, TNF-α- and IL-1β-treated cell cultures were 1169 ± 159 pg/mL, 2957 ± 137 pg/mL, 5032 ± 113 pg/mL and 5809 ± 121 pg/mL, respectively. All of them were significantly higher than that of controls ($P < 0.01$). Cd-induced IL-8 production was about 2.5 times potent compared with LPS.

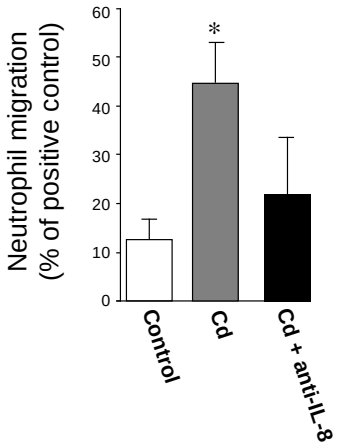
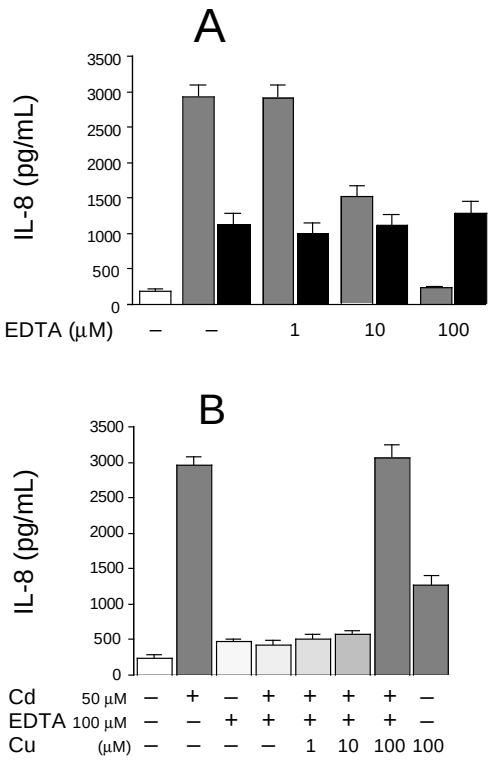


Fig. 5. Chemotactic activity of the supernatants of A549 cells. The activity was represented as neutrophil migration and was expressed as a percentage of the positive control (10^{-7} M formylmethionyleucylphenylalanine) from 6 independent experiments; *P < 0.05.



Chemotactic activity of cell culture supernatants

As shown in Fig. 5, culture supernatants from Cd-treated A549 cell suspensions induced significant chemotactic responses compared with the controls ($P < 0.01$). This activity was inhibited by 52% in the presence of the anti-IL-8 antibody.

Effect of EDTA and $CuCl_2$ on IL-8 release induced by Cd

EDTA at 100 μM inhibited 50 μM Cd-induced IL-8 production to about 86%, but 100 μg/mL LPS-induced IL-8 production was not inhibited by 1–100 μM EDTA (Fig. 6A). The inhibition was blocked by the presence of $CuCl_2$ at 100 μM. The cultured cells exposed to $CuCl_2$ solely produced a significantly large amount of IL-8 (1270 ± 129 pg/mL) compared with the controls ($P < 0.01$). These IL-8 levels were significantly lower than those produced by the cells which were solely exposed to 50 μM Cd (Fig. 6B).

Effects of anti-TNF-α and anti-IL-1β antibodies

Anti-TNF-α or anti-IL-1β monoclonal antibodies failed to block IL-8 release from Cd-treated and non-treated cells. The addition of anti-TNF-α or anti-IL-1β monoclonal antibodies completely blocked the IL-8 release induced by exogenous TNF-α or IL-1β, respectively (Fig. 7).

Discussion

Emphysema in humans takes several different forms: centrilobular, panlobular, paracicatrical and localized types (Foster et al., 1993). The varying morphologic features of these forms of emphysema suggest that they differ in patho-

Fig. 6. A: The effect of EDTA on Cd- and LPS-induced IL-8 in A549 cells. Cells were incubated with either 50 μM Cd (stripe columns) and 100 μg/mL LPS (solid columns), respectively. The data are expressed as mean ± SD from 6 independent experiments. **B:** The effect of EDTA and $CuCl_2$ on Cd-induced IL-8 in A549 cells. The data are expressed as mean ± SD from 6 independent experiments.

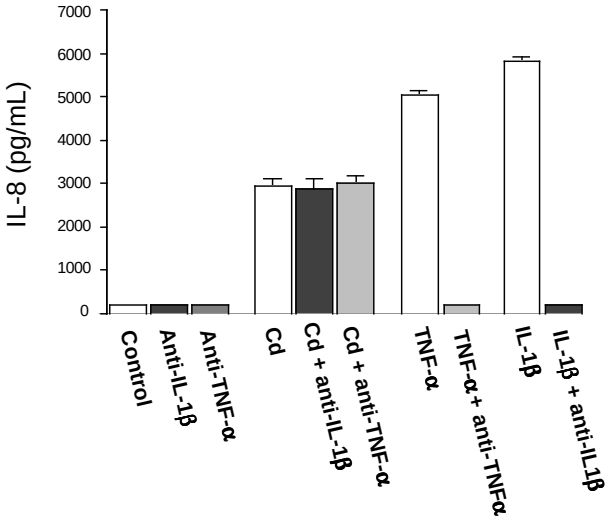


Fig. 7. The effect of anti-TNF-α and anti-IL-1β antibodies. Cd (50 μM), TNF-α (10 U/mL) and IL-1β (10 U/mL) were added 30 min before the antibodies (100 U/mL), and IL-8 in the cell cultures was measured after 24 h incubation. Values represent mean ± SD of 6 cultures obtained from representative experiments.

genesis. Centrilobular emphysema associated with cigarette smoking is the most common form and represents air space enlargement of respiratory bronchioles. Cd is a contaminant of cigarette smoke and it has been suggested that it plays an important role in cigarette smoking emphysema, because repeated exposure to subtoxic concentrations of Cd can induce destructive peribronchial lesions in rat lungs resembling human centrilobular emphysema (Snider et al., 1973, 1988). However, the mechanism of this has not been elucidated even until the present time.

Neutrophils are accepted now as the predominant effector cells for emphysema in cigarette smokers. Neutrophils increase in number in bronchoalveolar lavage fluids (BALF) and lung tissues in smokers (Hunninghake et al., 1979; Hunninghake and Crystal, 1983) and smoking increases the activity of neutrophil elastase in BALF (Fera et al., 1986). Snider and colleagues (1973, 1988) also observed that pulmonary neutrophilia occurs immediately after Cd exposure in rats.

IL-8, a potent chemotactic cytokine, was recently reported to increase in the airway secretion of patients with emphysema (Yamamoto et al., 1996), and it has been suggested to enhance the recruitment of neutrophils in the lung (Baggliony et al., 1989). Although the alveolar macrophages are major producers of IL-8 (Cooper et al., 1973), alveolar type II cells also produce IL-8 within the alveoli (Standiford et al., 1991). In emphysema, type II cells are important as a source of IL-8, because hyperplasia of these cells has been observed in the alveolar walls of human emphysematous lesions (Nagai and Konno, 1993) and proliferation of type II cells also occurred after repeated Cd inhalation in the rat lung (Hayes et al., 1976).

Since type II cells were difficult to obtain in pure culture, we investigated the effect of Cd on the IL-8 production using A549, type II cell line. Cells of the A549 cell line, at low and high passage levels, have properties of typical alveolar epithelial type II cells (Leiber et al., 1976). However, between the 20th and 27th sublines of A549, a different Cd-cytotoxic response was observed (Kang et al., 1990). In our preliminary studies, there was no difference in Cd cytotoxicity among the 82nd to 86th passages of the A549 sublines which we used, and cytotoxic threshold dose of these sublines were higher than those of younger sublines.

In the present study, dose- and time-dependent IL-8 production was found in 1, 5 and 10 μM of Cd until 48 h postexposure, and in 50 μM Cd up to 24 h without any significant decrease in cell viability (Figs. 2 and 3A). There was no difference in the level of IL-8 induced by 50 μM Cd at 24 and 48 h. This may be due to presecreted IL-8 remaining in the supernatant without decay. The marked reduction of the IL-8 level at 100 μM Cd was probably due to the severe

cytotoxic effect of Cd, because MTS activity was reduced after 16 h postexposure. These results indicate that 50 μM Cd is optimal for inducing IL-8 secretion without cytotoxic effect.

Up to 24 h, 50 μM Cd-treated cells showed significantly high MTS activity and marked IL-8 production (Figs. 2A and 3A), although Cd-exposed viable cells did not increase in number, determined by trypan blue exclusion (Fig. 2B). MTS activity represents mitochondrial metabolization of living cells. In alveolar macrophages, Cd was reported to be bound strongly to mitochondrial dehydrogenases and behave as an inhibitor of electron transport (Mustafa and Cross, 1971). On the other hand, numerous mitochondria and their hypertrophy have been observed in type II cells from Cd-treated rats (Hart et al., 1990; Murthy and Holovack, 1991). The high MTS activity of cells exposed to 50 μM Cd in the present study may be explained by the phenomenon of hormesis, the elevation of cellular metabolic responses at subtoxic concentrations of cell toxicants (Swisher, 1989). Accelerated mitochondrial functions may contribute to high MTS activity in our study.

To evaluate the total production of IL-8, intracellular IL-8 contents were also examined by triton X and freeze-thaw treatments (Fig. 3B). The levels of IL-8 obtained with these treatments was negligible compared with those of supernatants of Cd-exposed cells. Accordingly, the amount of extracellular IL-8 secretion was thought to be nearly equal to total IL-8 production.

Chemotactic activity for human neutrophils was examined to determine whether IL-8 induced by Cd in the present study was biologically active. It is reported that Cd itself inhibits chemotactic activity (Ward et al., 1986). Since, the 50 μM Cd-containing medium also allowed few neutrophil to migrate in our preliminary studies, ultrafiltration was performed to remove Cd before the assay. These supernatants induced potent neutrophil chemotactic activity, and the addition of anti-IL-8 inhibited this activity significantly (Fig. 5). These results indicate that Cd-induced IL-8 is a major neutrophil chemotactic factor in the present study. Therefore, neutrophil infiltration in the lung following Cd exposure may be mainly due to the action of IL-8.

For a further demonstration that endotoxin contaminants are not responsible, but Cd itself induced IL-8 production, we examined the effects of a chelating agent for divalent cations, EDTA. Cd was a stronger stimulant for IL-8 production than LPS in A549 cells. Cd-induced IL-8 production was inhibited by EDTA, and this was blocked by the addition of $CuCl_2$. Cu^{2+} binding affinity for EDTA is stronger than that of Cd^{2+}. Uncoupling Cu^{2+} may displace Cd^{2+} and the effect of Cd^{2+} liberated from EDTA during IL-8 production was revived when the EDTA binding capacity was saturated by Cu^{2+}. These results suggest that the induction of IL-8 could be ascribed mainly to this uncoupling cation, Cd^{2+}.

TNF-α and IL-1β were reported to be mainly generated by alveolar macrophages, and were potent stimuli for the induction of IL-8 by type II cells (Kunkel et al., 1991). IL-8 production by A549 cells and bronchial epithelial cells has been shown to be induced by TNF-α and IL-1β (Standiford et al., 1991; Cromwell et al., 1992). Moreover, pulmonary tumors produce a variety of growth factors and cytokines that may act in both autocrine and paracrine fashion (Wang et al., 1996). To determine whether A549, a characterized human lung adenocarcinoma-cell line, produced TNF-α or IL-1β in response to Cd, neutralizing experiments of TNF-α and IL-1β were conducted using the monoclonal antibodies. As shown in Fig. 7, co-incubation of Cd with these monoclonal antibodies did not diminish the level of IL-8 concentration, indicating that A549 produced little TNF-α or IL-1β in response to Cd.

In summary, the present study demonstrates that Cd increased the production of biologically active IL-8 by A549, alveolar epithelial type II cells, suggesting that Cd plays an important role in the development of cigarette smoking emphysema.

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