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Effect of lead on IL-8 production and cell proliferation in human oral keratinocytes

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ABSTRACT

Objective: To investigate the effect of lead on the production of IL-8 and cell proliferation in normal human oral keratinocytes (NHKs). **Methods:** NHKs were prepared as outgrowths from normal human buccal mucosa. The cells were treated with three concentrations of lead glutamate (4.5×10^{-5}M, 4.5×10^{-6}M and 4.5×10^{-7}M). NHKs grown in glutamic acid were used as control. The amounts of IL-8 secreted in the culture supernatants were evaluated at 12 and 24 h using enzyme-linked immunospecific assay (ELISA). Cell proliferation was determined by the MTT colorimetric assay. Three cultures were used for each experiment, and three independent experiments were performed. Analysis of variance and Duncan's multiple range tests were used for statistical analysis. **Results:** An elevation of IL-8 in culture supernatants of NHKs treated with lead at all concentrations at 12 and 24 h after exposure in a dose-dependent manner was revealed. A significant increase in cell numbers was observed only at 24 h exposed to 4.5×10^{-5}M lead glutamate. **Conclusions:** The capacity of NHKs, to secrete IL-8, enhanced by lead glutamate, is demonstrated here. Induction of cell proliferation is revealed only after exposure to high lead concentration. The elevation of secreted IL-8 is a probable initial sign for the acute inflammatory response and may be involved in the pathogenesis of lead stomatitis.

1. Introduction

Lead is a heavy metal occurring naturally as a sulfide in galena. It is a soft, bluish-white, silvery-gray metal prevalent in water and soil. Generally, lead compounds are emitted into the atmosphere from three sources; gasoline-powered vehicles, industrial processes, and incineration[1, 2]. Human exposure occurs primarily through air, drinking water, diet, and dust from industrial and motor vehicle sources[3]. More than 95% of lead is inorganic and occurs as a lead salt[4]. Food can be contaminated by lead from lead solder in cans or other food containers. It is also found in paints and glazes used in making pottery and ceramic ware.

Lead poisoning can be classified into acute and chronic types. Most cases of acute poisoning occur among specific groups such as workers renovating old buildings[5]. The classical clinical features of acute poisoning are abdominal colic and constipation. These symptoms are largely due to irritation of the alimentary tract. If sufficient lead is retained after a single exposure, a syndrome identical to chronic intoxication may develop with symptoms including leg cramps, muscle weakness, paresthesia, CNS depression, coma, and death within one to two days. Chronic lead poisoning has an impact on more populations than acute type as a result of environmental pollution. Besides the non-specific symptoms described above, chronic lead poisoning has been shown to affect many organ systems, for example, the hematopoietic, reproductive, central and peripheral nervous systems and renal tissue[1]. In the oral cavity, a blue gingival lead line (Burton's line) has been shown to be a valuable clinical sign of moderate chronic lead poisoning[6]. Other oral manifestations of lead poisoning include a heavy coating on the tongue and tremor of the tongue on thrusting, advanced periodontal disease, excessive salivation, metallic taste and ulcerative stomatitis [7].

Ulcerative stomatitis may develop when the oral mucosa has a long term direct contact to lead. It is an inflammation condition of the oral mucosa characterized by ulcers on the cheek, tongue and lips. During inflammation, several cytokines and chemokines produced by a variety of cell

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types such as macrophages, fibroblasts and keratinocytes are involved in this process. Among these mediators, interleukin-8 (IL-8) is one of the best studied chemokines involved in ulcerative stomatitis[8,9]. One study has demonstrated the elevation of serum IL-8 in patients with recurrent aphthous ulcer (RAU), a common type of painful ulcerative stomatitis affecting the oral mucosa[8]. In addition, IL-8 is considered to be a marker in monitoring the disease's activity[10,11].

Since the epithelium is the first barrier of oral mucosa exposed to lead, it is important to study how keratinocytes, the main cell type of the epithelium, respond to this toxic metal, particularly their ability to produce IL-8. To the best of our knowledge, the effect of lead on normal human oral keratinocytes (NHKs) has not been studied. Therefore, the purpose of the present study was to investigate the effect of lead on the production of IL-8 in NHKs. Moreover, the effect on cell proliferation was also examined since the level of IL-8 may be related to the number of cells.

2. Materials and methods

2.1. Lead preparation

Lead glutamate was used in this study. A stock solution, containing 4.5×10^{-2} M lead nitrate (Sigma Chemical Company, St. Louis, USA) and 5×10^{-2} M glutamic acid in deionized water, was prepared.

2.2. Cell culture

NHKs were prepared as outgrowths from normal human buccal mucosa collected from the Department of Oral Surgery, Faculty of Dentistry, Mahidol University. The preparation of the cells from explant cultures was modified from that described by Freshney[11]. Briefly, the tissue was finely chopped into small pieces and placed in 25 cm^2 culture flasks. Then keratinocyte growth medium (KGM) was gently added. KGM consisted of 1 g/mL minimum essential medium (Gibco BRL, NY, USA), 2.2 mg/mL $NaHCO_3$ (Merck KgaA, Darmstadt, Germany) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone (Gibco BRL), 1.8×10^{-4} M adenine, 0.5 μg/mL hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin and 10 ng/mL epidermal growth factor (all obtained from Sigma). This medium was further supplemented with 10% fetal calf serum (Gibco BRL).

After one week, the outgrowth of cells was observed and the medium was changed. Cultures were fed every 2 to 3 days for 18 to 21 days until subconfluent cell monolayers were formed. The cells were used within two passages.

2.3. Treatment

The cultured NHKs were seeded in 96-well culture plates (8×10^3 cells/mL) with serum-free medium supplemented with antibiotic and fungizone; the cells were incubated overnight. On the following day, the medium was changed and supplemented with three concentrations of lead glutamate: 4.5×10^{-5} M, 4.5×10^{-6} M, and 4.5×10^{-7} M. NHKs grown in 5×10^{-5} M glutamic acid were used as control.

2.4. IL-8 determination

The level of IL-8 secreted in the culture supernatants was evaluated at 12 and 24 hours. The amount of IL-8 was assessed by quantitative sandwich enzyme immunoassay technique (IL-8 ELISA, Biosource International, Belgium) according to the manufacturer's protocol.

2.5. Cell proliferation study

Cell proliferation was evaluated by the MTT colorimetric assay. The culture media was removed at 12 and 24 hours after inoculation with lead glutamate. A 50 μL of MTT solution (3-(4, 5- Dimethylthiazole-2-yl) - 2, 5-diaphenyltetrazolium bromide) (Sigma) was added to the culture wells. The amount of formazan produced was assayed by measuring the density at 570 nm using a μ-Quant microplate reader (Biotek Instruments, Inc., USA).

2.6. Statistical analysis

Three cultures were used for each experiment, and three independent experiments were performed. Statistical analysis was carried out by one-way ANOVA. Tests of differences of the treatments were analyzed by Duncan's test and a value of $P<0.05$ was considered as statistically significance.

3. Results

NHKs were exposed to three concentrations of lead glutamate (4.5×10^{-5} M, 4.5×10^{-6} M, and 4.5×10^{-7} M). Rapid accumulation of IL-8 was detected in culture supernatants of NHKs treated with lead at all concentrations after 12 hour of exposure, and this elevation was also observed at 24 hour incubation period in a dose-dependent manner (Table 1). The optimal concentration for IL-8 stimulation in this study was 4.5×10^{-5} M lead glutamate. Compared to the control, a significant increase in cell numbers was observed only after 24 hour exposure to 4.5×10^{-5} M lead glutamate ($P<0.05$) (Table 2). No significant differences were found for the 4.5×10^{-6} M and 4.5×10^{-7} M lead glutamate groups.

4. Discussion

The results presented here demonstrates that in a primary cell culture system NHKs have the capacity to secrete IL-8. This capacity can be enhanced by lead glutamate at concentrations between 10^{-5} M to10^{-7} M corresponding to those of unsafe levels ($\geqslant$ 0.5 μM)[6] and the levels (0.5-3 μM) in human blood with varied effects on brain function[12]. Induction of cell proliferation was observed only after 24 hour exposure to high lead concentration (10^{-5} M). Thus, we propose that the lead induced elevation of IL-8 observed in

Table 1

IL-8 production in culture supernatants of NHKs after treatment with various concentrations of lead glutamate.

Lead exposure time (h)	IL-8 (pg/mL)			
	control	Lead (M)		
		4.5×10^{-5}	4.5×10^{-6}	4.5×10^{-7}
12	652.30±35.29	917.70±50.35*	886.84±88.15*	885.11±73.16*
24	1099.90±92.81	1334.17±25.42*	1235.12±33.17*	1190.05±42.56*

Data are expressed as mean + SD of triplicate wells. *statistically significant difference from control (P<0.05).

Table 2

The number of NHKs after treatment with various concentrations of lead glutamate.

Lead exposure time (h)	Cell number ($\times10^3$ cell)			
	control	Lead (M)		
		4.5×10^{-5}	4.5×10^{-6}	4.5×10^{-7}
12	7.21±1.39	7.35±0.76	7.51±1.25	7.13±1.42
24	7.78±0.23	8.89±0.51*	7.97±0.76	8.10±1.57

Data are expressed as mean + SD of triplicate wells, * statistically significant difference from control (P<0.05).

this study is not related to an increase of cell proliferation.

IL-8 is an important mediator of host response to injury and inflammation[13]. It possesses diverse functions as a neutrophil activator and a chemo-attractant for neutrophils, T cells, and basophils[14]. IL-8 is produced by a variety of cell types including monocytes/macrophages, T cells, neutrophils, endothelial cells, fibroblasts, and keratinocytes [9,13,15]. In healthy tissues, IL-8 is barely detectable, but is rapidly induced by 10- to 100-fold in response to pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or IL-1β, bacterial or viral products, and cellular stress[16]. A high level of IL-8 in serum has been found to be significantly associated with the presence of oral ulcer[8,9] and IL-8 activated neutrophils is believed to be a major source of enzymes involved in tissue destruction[17]. Therefore, the elevation of IL-8 secreted by oral keratinocytes may be the initial sign of the acute inflammatory response following lead exposure to oral mucosal surfaces and may reflect the pathogenesis of lead stomatitis in the oral cavity. It appears that IL-8 plays an important role both in lead-induced stomatitis and RAU, thus the treatment of RAU may be applied to lead-induced stomatitis.

In the present study, the ability of lead at high concentration to increase oral keratinocyte proliferation is similar to our previous study in dental pulp cells[18]. However, the mechanism underlying this effect remains unknown. Based on our result, the induction of keratinocyte proliferation observed at 10^{-5} M lead glutamate is not likely to be relevant to the elevation of IL-8 since cell numbers did not increase in many conditions that IL-8 production was up-regulated. It should be noted that several growth factors and cytokines such as epidermal growth factor, fibroblast growth factor-2, transforming growth factor-beta, IL-1, IL-6 or TNF-α can promote keratinocyte proliferation[19]. Additionally, cell proliferation can be influenced by other mechanisms such as up-regulation of growth factor receptors or cytokine receptors and inhibition of the apoptosis pathway[20]. Interestingly, the lead-induced cell proliferation may be associated with the carcinogenic mechanisms of lead. The issue of potential carcinogenic effects of lead has received renewed focus, leading to the classification Group 2A (probably carcinogenic to humans) by IARC and Group 2 (considered to be carcinogenic to humans) by the German MAK Commission[21, 22]. These classifications were mainly based on animal experiments, where increased tumor incidences were observed in multiple organs, including the brain and kidney. In mammalian AS52 cells, lead chloride induced mutations in a dose-dependent manner, starting at the non-cytotoxic concentration of 10^{-7} M[23]. Furthermore, it has been demonstrated that lead at high concentrations has the ability to bind to DNA, change DNA conformation[24] and break nucleic acids[25]. Recent research suggested that lead may act as a human carcinogen by permitting or enhancing carcinogenic events at cellular or molecular levels[26]. Taken these evidences with our results showing that lead at high level could induce cell proliferation, it is possible that lead may be related to the pathogenesis of oral squamous cell carcinoma particularly in people who has been exposed to lead. However, further studies are needed to determine the exact role and mechanisms of this lead-induced hyperproliferation in NHK.

In summary, the elevation of IL-8 production by oral keratinocytes was observed at various lead concentrations. However, induction of cell proliferation was demonstrated only at a high lead concentration. These results indicate that the increased production of IL-8 might be involved in the initiation or pathogenesis of lead stomatitis found in the oral cavity while at high concentration, induction of cell proliferation may be an evidence of lead carcinogenicity.

Conflict of interest statement

We declare that we have no conflict of interest.

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