BIOMEDICAL AND ENVIRONMENTAL SCIENCES **19**, 375-379 (2006)

Combined Effect of Fluoride and Arsenate on Gene Expression of Osteoclast Differentiation Factor and Osteoprotegerin

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Objective To study the combined effect of fluoride and arsenate on the expression of SD rat osteoblastic osteoclast differentiation factor (ODF) mRNA and osteoprotegerin (OPG) mRNA. **Methods** Osteoblasts were obtained by enzymatic isolation from newborn SD rats. A factorial experiment was performed. Osteoblasts were exposed to NaF (0.5 mmolF/L, 4 molF/L) and Na_3AsH_2 (12.5 μmolAs/L and 200 μmolAs/L) separately or F plus As and cultured for 48 h. The gene expression of osteoblastic ODF and OPG was observed by RT-PCR. **Results** The expression of ODF mRNA increased in $F_{0.5}$, F_4 groups compared with control group and two groups of $F_{0.5}As_{200}$, F_4As_{200} compared with As_{200} group, and decreased significantly in groups of $F_4As_{12.5}$, $F_{0.5}As_{200}$, and F_4As_{200}. The expression of OPG mRNA decreased in groups of F_4, As_{200}, $F_4As_{12.5}$, $F_{0.5}As_{200}$, and F_4As_{200}. **Conclusion** The joint effect of fluoride and arsenate on the gene expression of ODF is antagonistic, while the combined effect on the gene expression of OPG is synergistic. F_4, $F_4As_{12.5}$, and $F_{0.5}As_{200}$ promote bone resorption of rat osteoclasts, whereas $F_{0.5}As_{12.5}$ inhibits osteolytic effect of rat osteoclasts.

Key words: Fluoride; Arsenate; ODF; OPG; Combined effect

INTRODUCTION

Fluoride is an essential trace element in human bodies and is highly correlated with the metabolism of bone and tooth. But excessive exposure to fluoride for a long term leads to bone damage with complicated pathological changes such as osteoporosis and osteopetrosis. Arsenide is an environmental toxicant and a known carcinogen[1]. Inorganic arsenide is highly accumulated *in vivo*. Arsenate deposits in skeleton because it takes the place of phosphate in the apatite crystal of bone. Fluoride and arsenide in groundwater, air, and food in some areas of China as a result of specific geographical and geological environment and living habits of local people have tremendous impact on health of local residents. Great attention has been paid to the possible combined effect of fluoride and arsenide[3]. There are different reports about the role of arsenide in the bone damage caused by fluoride[4].

Both osteoblasts and osteoclasts are involved in bone damage. Studies indicate that the proliferation, differentiation, and maturity of osteoclasts are dependent on the existence of osteoblasts[5]. Osteoprotegerin (OPG) and osteoclast differentiation factor (ODF) are secreted by osteoblasts. The former is responsible for inhibiting osteoclastogenesis, while the latter plays an important role in stimulating the differentiation and maturity of osteoclasts, activating mature osteoclasts and inhibiting their apoptosis. So OPG and ODF may combine the functions of osteoblasts and osteoclasts, which is of great help to the study of the influence of toxicants on bone remodeling.

The aim of the present study was to investigate the joint effect of fluoride and arsenide on the process of bone remodeling at cellular level by observing the changes in gene expressions of OPG and ODF.

MATERIALS AND METHODS

Cell Isolation and Culture Procedure

Parietal bones were removed aseptically from newborn Sprague-Dawley rats and put into PBS after the rats were disinfected with 70% ethanol for 2-3 min. The periosteum and blood vessel were removed and the bones were washed twice with PBS. Bones

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0895-3988/2006
CN 11-2816/Q

were then cut into scraps of 1 mm^3 and incubated in 0.25% trypsin for 15-20 min at 37℃ to remove the fibroblasts. The cells were digested twice in the solution of 0.1% type Ⅱ collagenase (Sigma, USA), each for one hour, and centrifuged at 1500 g for 15 min. Then the cell conglomerations were mixed up and seeded into culture flasks in DMEM (Gibco, USA) containing 10% heat-inactivated newborn calf serum and 100 U/mL sodium penicillin and 100 U/mL streptomycin in a controlled atmosphere (5% CO_2/95% air, 37℃). When the cells reached confluence after 7-10 days, they were released with 0.25% trypsin solution and implanted in culture plates according to required density.

The fluoride and arsenate were divided into 9 groups according to the factorial design (Table 1).

TABLE 1

Experimental Groups

F (mmol/L)	As (μmol/L)		
	0	12.5	200
0	Control	$As_{12.5}$	As_{200}
0.5	$F_{0.5}$	$F_{0.5}As_{12.5}$	$F_{0.5}As_{200}$
4	F_4	$F_4As_{12.5}$	F_4As_{200}

MTT Colorimetric Assay

Released osteoblasts were implanted in 96-well plates (Falcon, Germany) at a density of 5000-6000/well. Four hours before measurement, 20 μL MTT was added to each well and incubated for additional 4 h at 37℃. DMSO (150 μL) was then added and the plates were shaken for 10 min. Finally the optical density (OD) of each well was measured using an ELISA plate reader, and the cell relative viability (= OD of experimental group/OD of control group) was assessed.

RNA Extraction

The procedure of RNA extraction was carefully carried out according to the manual (Molecular Research Center, Inc.). In brief, media were poured off and wells were washed with PBS after cells were cultured in 6-well plates with fluoride or arsenate and in combination for 48 h. Then 1 mL TRI reagent was added to every well and the cell lysate was passed several times through a pipette. The homogenates were stored for 5 min at room temperature, to which 0.2 mL chloroform was added, and were vigorously shaken for 15 s. The mixture was stored at room temperature for 10 min and centrifuged at 12 000 g for 15 min at 4℃. Then the aqueous phase was carefully transferred to a fresh tube and the RNA was precipitated by mixing with 0.5 mL isopropanol. After storage at room temperature for 10 min, the samples were centrifuged at 12 000 g (4℃) for 10 min. RNA precipitates formed a white pellet on the side or the bottom of the tube. The supernatant was removed carefully and RNA pellet was washed with 75% ethanol by vortexing, and then centrifuged at 7500 g for 5 min at 4℃. At the end of the procedure the ethanol was removed and the RNA pellet was air-dried for 20 min. The RNA pellet was dissolved with 20 μL DEPC-treated water for 20 min at 55℃ and stored at -80℃.

Semi-quantitative RT-PCR

The preparation of full-length first strand cDNA from RNA templates and the PCR amplification carefully followed the protocol described in the RevertAid™ first strand cDNA synthesis kit (Fermentas) and 2×PCR Master Mix (Fermentas), respectively. The PCR primers (Table 2) of β-actin and ODF were designed with the Primer 5 software according to the cDNA sequences from Http://www.ncbi.nlm.nih.gov/. Using the standard protocol of the manufacturer, after denaturation at 94℃ for 5 min, 35 cycles of amplification were performed, each consisting of denaturation at 94℃ for 30 s, annealing at 56℃ for 1 min for β-actin while at 54℃ for 1 min for ODF, and extension at 72℃ for 50 s, then a final extension at 72℃ for 10 min. OPG[6] (Table 2) underwent 5 min of denaturation at 94℃ with 30 cycles of denaturation for 1 min at 94℃, annealing elongation at 55℃ for 2 min and extension at 70℃ for 1 min, then a final extension at 72℃ for 7 min. After PCR, 5 μL products and 1 μL 6×loading dye were subjected to electrophoresis on 1.7% agarose gel with 0.01% ethidium bromide. The image was visualized and photographed under UV transillumination.

TABLE 2

Primers Used for PCR

Transcripts	Forward Primer	Reverse Primer	Amplification Length
β-actin	CCTCTATG CAACACAGTGC	GTACTCCTCTTGCTGATCC	210bp
ODF	TCGGGTTCCCA AAAGTC	GAAGCAAATGTTGGCGTA	138bp
OPG	TCCTGGCACCTACCTAATACAGCA	CTACACTCTCGGCATTCACTTTG	578bp

The intensity of each band was measured and analysed using the software of Quantity One and Bandscan. The relative abundance of each target band was normalized according to the housekeeping gene β-actin, calculated as the ratio of each target product to β-actin.

Statistical Analysis

The analysis of interaction was carried out with the software SPSS11.0. If there existed interaction, the one-way ANOVA was performed to test for the statistical significance of the means of all groups. $P<0.05$ was considered statistically significant.

RESULTS

Morphology

Osteoblasts first released from bones were round, suspending in the medium. After 24 h, most of them adhered tightly to the surface of the culture dishes and exhibited a variety of forms such as triangle and polygon. When the cells reached confluence, they connected with each other in the form of slabstones and even grew overlapped (Figs. 1 and 2).

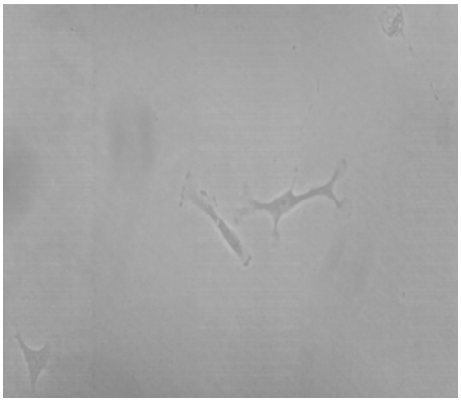

FIG. 1. Implanted cells after 24 h (×200).

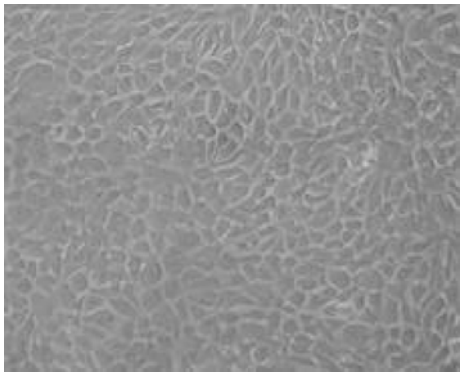

FIG. 2. Implanted cells reaching confluence (×200).

Cell Relative Viability

After exposure to fluoride or arsenate alone or their combination for 72 h and 120 h (Table 3), $F_{0.5}$and $As_{12.5}$ enhanced the proliferation of osteoblasts, while the group of F_4 and As_{200} inhibited osteoblast proliferation ($P<0.05$). Compared with $F_{0.5}$, $F_{0.5}As_{12.5}$ and $F_{0.5}As_{200}$ decreased cell viability ($P<0.05$). In addition, after treatment for 72 h, the group of F_4As_{200} compared with As_{200} group and the groups of $F_{0.5}As_{12.5}$ and $F_4As_{12.5}$ compared with $As_{12.5}$, inhibited osteoblast proliferation ($P<0.05$). Table 3 also demonstrates that after osteoblasts were exposed to $F_4As_{12.5}$ for 120 h, compared with the group of $As_{12.5}$, cell viability decreased significantly ($P<0.05$).

Combined Effects of Fluoride and Arsenate on Expressions of Osteoblastic ODF mRNA and OPG mRNA

The expression of ODF mRNA was induced when osteoblasts were exposed to fluoride or arsenate alone or their combination (Table 4, Fig. 3). When treated with fluoride alone, both $F_{0.5}$ and F_4 increased the expression of ODF mRNA ($P<0.05$). In addition, $F_{0.5}As_{200}$ and F_4As_{200} compared with As_{200} also enhanced the gene expression of ODF ($P<0.05$). However the

TABLE 3

Effects of Different Groups on the Proliferation of Osteoblasts ($\bar{x} \pm s$)

	Hours	F_0	$F_{0.5}$	F_4
As_0	72	1.00±0.14	1.34±0.07[c]	0.19±0.06[c]
	120	1.00±0.18	2.11±0.06[c]	0.18±0.09[c]
$As_{12.5}$	72	1.27±0.06[c]	0.91±0.04[a,b]	0.19±0.05[b]
	120	1.55±0.09[c]	1.14±0.11[a]	0.20±0.24[b]
As_{200}	72	0.4±0.06[c]	0.39±0.08[a]	0.18±0.07[d]
	120	0.18±0.11[c]	0.20±0.09[a]	0.20±0.17

Note. [a]$P<0.05$ vs $F_{0.5}$, [b]$P<0.05$ vs $As_{12.5}$, [c]$P<0.05$ vs control group, [d]$P<0.05$ vs As_{200}.

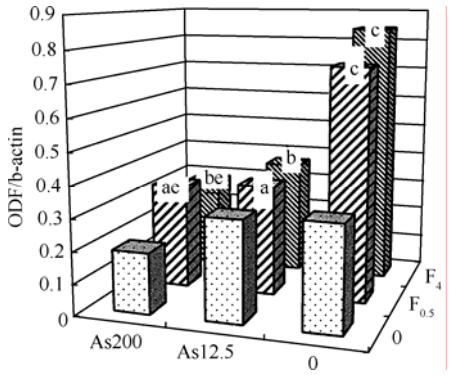

FIG. 3. Comparison of mRNA expression of ODF in osteoblasts among different groups. [a]$P<0.05$ vs $F_{0.5}$, [b]$P<0.05$ vs F_4, [c]$P<0.05$ vs control group, [d]$P<0.05$ vs $As_{12.5}$, [e]$P<0.05$ vs As_{200}.

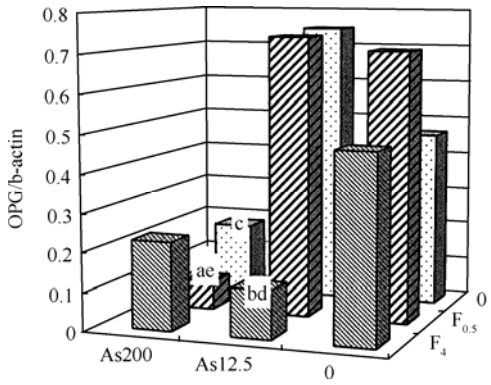

FIG. 4. Comparison of mRNA expression of OPG [a]$P<0.05$ vs $F_{0.5}$, [b]$P<0.05$ vs F_4, [c]$P<0.05$ vs control group, [d]$P<0.05$ vs $As_{12.5}$, [e]$P<0.05$ vs As_{200}. The values are geometric means.

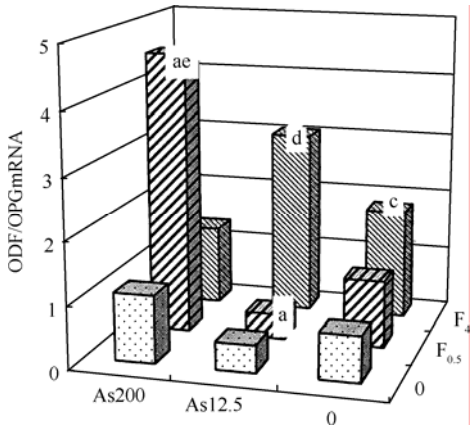

FIG. 5. Comparison of mRNA ratio of ODF and OPG among different groups. [a]$P<0.05$ vs $F_{0.5}$, [b]$P<0.05$ vs F_4, [c]$P<0.05$ vs control group, [d]$P<0.05$ vs $As_{12.5}$, [e]$P<0.05$ vs As_{200}. The values are geometric means.

decreased gene expressions of ODF were found in the groups of $F_{0.5}As_{12.5}$ and $F_{0.5}As_{200}$ (compared with $F_{0.5}$, $P<0.05$) and in the groups of $F_4As_{12.5}$ and F_4As_{200} (compared with F_4, $P<0.05$).

TABLE 4

Effects of Different Groups on the Gene Expression of Osteoblastic ODF and OPG ($\bar{x} \pm s$)

Groups	n	ODF / β-actin	OPG /β-actin	ODFmRNA/ OPGmRNA
Control	3	0.33±0.17	0.45±0.18	0.74±0.24
$As_{12.5}$	3	0.32±0.05	0.72±0.14	0.45±0.18
As_{200}	3	0.19±0.12	0.18±0.12[c]	1.09±0.22
$F_{0.5}$	3	0.74±0.05[c]	0.68±0.08	1.09±0.08
$F_{0.5}As_{12.5}$	3	0.35±0.17[a]	0.70±0.09	0.40±0.01[a]
$F_{0.5}As_{200}$	3	0.33±0.01[a,e]	0.07±0.05[a,e]	4.59±0.04[a,e]
F_4	3	0.84±0.06[c]	0.46±0.08	1.80±0.12[c]
$F_4As_{12.5}$	3	0.37±0.07[b]	0.12±0.36[b,d]	3.04±0.39[d]
F_4As_{200}	3	0.29±0.03[b,e]	0.22±0.12	1.30±0.14

Note. [a]$P<0.05$ vs $F_{0.5}$, [b]$P<0.05$ vs F_4, [c]$P<0.05$ vs control group, [d]$P<0.05$ vs $As_{12.5}$, [e]$P<0.05$ vs As_{200}.

The expression of OPG mRNA was observed when osteoblasts were treated with fluoride or arsenate alone or their combination (Table 4, Fig. 4). When treated with arsenate alone, osteoblasts exposed to As_{200} had low gene expression of OPG ($P<0.05$). In the combined groups, $F_{0.5}As_{200}$ could inhibit the gene expression in osteoblasts when compared with $F_{0.5}$ and As_{200}, respectively ($P<0.05$). The same situation could be found in the group of $F_4As_{12.5}$ compared with the groups of F_4 and $As_{12.5}$ treated with fluoride or arsenate alone ($P<0.05$).

We also calculated the ratio of ODF mRNA to OPG mRNA (Table 4, Fig. 5). In the groups treated with fluoride and arsenate, the ratio declined only in the group of $F_{0.5}As_{12.5}$. The increased ratio could be seen in the group of $F_{0.5}As_{200}$ compared with $F_{0.5}$ and As_{200}. In addition, osteoblasts exposed to $F_4As_{12.5}$ had a significantly higher ratio than those exposed to $As_{12.5}$ ($P<0.05$).

DISCUSSION

Lindgren *et al.*[2] found that the concentration of arsenic in organs tends to be higher after administration of arsenite than that of arsenate at all time points, and there is a significant accumulation of arsenate in bone with prolonging time. This is ascribed to arsenate having a structure analogous to phosphate which is substituted in the apatite crystal of bone. Therefore we used arsenate to study the effect of arsenide on bone in our experiments.

ODF is also called OPGL, which is an important osteoclast differentiation/activation factor responsible for bone modeling. Lacey *et al.*[7] found that ODF is

expressed in various cells such as osteoblasts and lymphocytes. Further experiments found that the mutant mice with OPGL exhibit severe osteopetrosis, delay of growth, and defect in tooth eruption[8]. However OPGL in these mice with haematopoietic precursors differentiate into phenotypically and functionally mature osteoclasts *in vitro* in the presence of recombinant OPGL and CSF-1, suggesting that ODF plays an important role in promoting the differentiation and activation of osteoclasts. In 1997, Simonet *et al*.[9] first identified a novel member of the tumor necrosis factor receptor (TNFR) superfamily during a cDNA-sequencing project of a fetal rat, and named this factor osteoprotegerin (OPG) because they found that it decreases osteoclastogenesis *in vitro* and blocks the loss of bone induced by ovariectomy. The OPG transgenic mice suffered severe yet nonlethal osteopetrosis with a defect of osteoclast differentiation in later stages. *In vitro*, recombinant OPG protein inhibits differentiation of osteoclasts in a dose-dependent manner. In the same year, Eisuke Tsuda *et al.*[10] isolated a factor from the conditioned medium of human embryonic fibroblasts (IMR-90), which could inhibit osteoclast development and was named osteoclastogenesis inhibitory factor (OCIF). All these findings show that OCIF is a new factor that specifically inhibits osteoclasogenesis. Sequence analysis of OCIF cDNA revealed that OCIF is identical with OPG [9].

In our experiment, osteoblasts exposed to fluoride or arsenide alone or their combination induced the gene expression of ODF and OPG. But fluoride stimulated the expression of ODF mRNA and promoted the function of osteoclasts, whereas arsenate played an important role in decreasing the expression of ODF mRNA and inhibiting the function of osteoclasts. On the whole, the inhibitory role of arsenate exceeded the promoting role of fluoride, so in the combined groups, the gene expression of ODF decreased with declining bone resorption of osteoclasts. The combined effect of fluoride and arsenate was antagonistic. The gene expression of OPG decreased significantly in the groups treated with higher doses of fluoride and arsenate. The expression of OPG mRNA was markedly decreased when osteoblasts were treated with F_4 or As_{200} alone and $F_4As_{12.5}$, $F_{0.5}As_{200}$, F_4As_{200} in combination, indicating that the combined effect of fluoride and arsenate is mainly synergistic.

Some scholars believe that the ratio of ODF mRNA to OPG mRNA directly influences bone remodeling. The increased ratio shows that the role of osteoclasts enhanced by ODF predominates in bone resorption[11]. We found that in the groups of F_4, $F_4As_{12.5}$, and $F_{0.5}As_{200}$, the ratios increased significantly, suggesting that the resorption function of osteoclasts is promoted. In contract, osteoblasts exposed to the group of $F_{0.5}As_{12.5}$ had a amazingly decreased ratio value, which could protect osteoblasts against bone injury. All these show that $F_{0.5}As_{12.5}$ increases the gene expression of OPG while $F_{0.5}As_{12.5}$ decreases the expression of ODF mRNA, thus reducing the resorption of osteoclasts.

In summary, fluoride and arsenate may influence the process of bone remodeling by changing the gene expressions of OPG and ODF of osteoblasts, which cause bone damages. However, *in vivo*, a number of factors are involved in bone metabolism including transforming growth factor (TGF)-β, colony-stimulating factor-1 (CSF-1 or MCSF), interleukin (IL)-1, tumor-necrosis factor (TNF)-α besides ODF and OPG, all of which may influence the dynamic balance between formation and resorption of bone. The roles of fluoride and arsenide in the osteoporosis and fluorosis need to be further studied.

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(*Received January* 20, 2005 *Accepted December* 10, 2005)