EFFECT OF FLUORIDE ON EXPRESSION OF PURA GENE AND CaM GENE IN NEWBORN RAT OSTEOBLASTS

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SUMMARY: To explore the effect of fluoride (F) on the expression of purine-rich element-binding protein (PURA) gene and calmodulin (CaM) gene in osteoblasts of newborn rats, parietal calvaria bone osteoblast cultures of 48-hr-old rats were treated for 48 hr with sodium fluoride (NaF) at concentrations of 0 (control), 0.5, 2, and 8 mg/L. The expression of PURA gene and CaM gene was determined by reverse transcription polymerase chain reaction (RT-PCR). The results indicated that F significantly enhanced ($p<0.05$ to $p<0.01$) the expression levels of the two genes in the osteoblast cultures with increasing F concentrations compared with the control group.

Keywords: Calmodulin (CaM) gene; COL2A1 gene; Fluoride and gene expression; Newborn rat osteoblasts; Purine-rich binding protein (PURA) gene; Reverse transcription polymerase chain reaction (RT-PCR).

INTRODUCTION

Besides causing skeletal damage,[1-3] fluoride (F) is well-known for its ability to induce dental, brain, heart, liver, renal, and thyroid damage.[4-5] F is also known to affect cell cycle distribution[6] and to increase levels of undesirable free radicals.[7] At high concentrations, F adversely affects the health of osteoblasts,[8-9] and at certain concentrations induces apoptosis and causes G_0/G_1 arrest.[8] However, the molecular mechanisms of these effects are not clear.

Recently, DNA damage by F in sufficient concentrations has been detected in osteoblasts of newborn rats by single-cell gel electrophoresis assay (SCGE).[10] Industrial F pollution has also been found to induce elevated expression of the type II collagen (COL2A1) gene in rib cartilage of Inner Mongolia cashmere goats.[11] In view of these new findings, we have undertaken further research on DNA gene damage by F to help elucidate the complex molecular mechanisms involved.

In the present study, reverse transcription polymerase chain reaction (RT-PCR) was used to quantify effects of F on mRNA in the expression of purine-rich element-binding (PURA) and calmodulin (CaM) genes in newborn rat osteoblasts. PURA gene has been implicated in diverse cellular functions, including transcriptional activation and repression, translation, and cell growth.[12] CaM is a very important thing in cell pathway signaling involving calcium transport. Earlier studies have shown that PURA gene exerts its function through CaM.[13] Our new findings presented here indicate that F effects on PURA and CaM genes may play a role in skeletal fluorosis.

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MATERIALS AND METHODS

Materials: Three male and two female newborn Wistar rats, 48-hr old, were provided by Laboratory Animal Center of Guiyang Medical College of China. Dulbecco's Modified Eagle Medium (DMEM), trypsin, and fetal bovine serum (FBS) were supplied by Gibco Company of USA. HEPES buffer was obtained from Hyclone Company of America. NaF was supplied by Shanghai Biochemistry Pharmacy of China. Trizol Reagent came from the Invitrogen Company of USA, and RT-PCR Kit from the Fermentas International Company of Lithuania. Primers of PURA and CaM were supplied by Takara of China. Marker, which included the following six bands of base pairs: 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, and 600 bp, obtained from Tian Wei Shi Dai Biocompany of China. The Syngene Analysis System was provided by Gene Company of China.

Osteoblasts separation and culture: Primary osteoblasts cultures were obtained from the parietal bones of calvaria of five 48-hr-old newborn Wistar rats. The cells were adjusted to 10^6/mL and cultured in 25-mL tissue culture flasks plated with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. When the cells had been grown to confluence, 0.25% trypsin was used for the secondary culture. The cell number was adjusted to a density of 1°¡10^6 cells/mL (Figure 1).

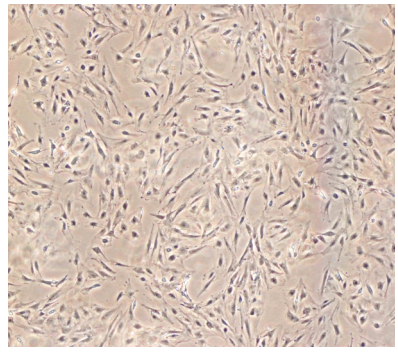

Figure 1. Secondary cultured osteoblasts grown to 70% confluence.

Fluoride treatment: When the cells had again grown to confluence, they were exposed to 0, 0.5, 2, and 8 mg NaF/L for 48 hr. The cells were then washed twice in HEPES buffer.

RNA Isolation: Total cellular RNA was extracted using Trizol reagent (1 mL Trizol per (5–10)×10^6 osteoblasts). The RNA pellet that remained at the end was air-dried and dissolved in RNAse-free water. Using a spectrophotometer (Shimadzu, Japan), the RNA samples had $A_{260/280}$ ratios between 1.8 and 2.0. Finally, gel electrophoresis with 1.5% agarose was used to detect the RNA (Figure 2).

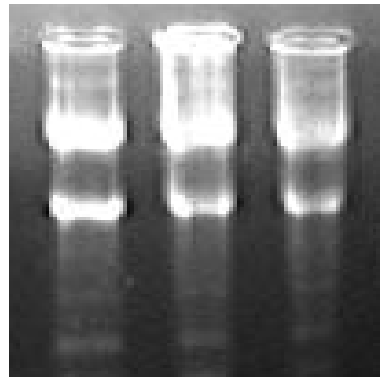

Figure 2. PCR electrophoresis imaging of total RNA. The upper bands were 28S rRNA, while the bands below were 18S rRNA.

Reverse transcription and synthesis of first strand cDNA: 0.1 μg – 5 μg template RNA was used to reverse transcription and synthesize the first strand cDNA. When the reaction was complete, the mixture was chilled on ice.

Polymerase chain reaction (PCR) analysis: The primers used to amplify PURA gene were: 5'-GAT GTG GGC TCC AAC AAG TAC GGC-3' and 5'-GTG AAG

CTG CTC ACA GGC AGC CCG-3'.[14] Primers sets for CaM gene were obtained from Takara Company of China. The sequences were: 5'-GGC ATC CTG CTT TAG CCT GAG-3' and 5'-ACA TGC TAT CCC TCT CGT GTG AC-3'. In addition, primers used to amplify β-actin gene were 5'-Tgg AgA AAA TCT ggC ACC AC-3' and 5'-gAg gCg TAC Agg gAT AgC AC-3'. Each PCR was performed in a total volume of 50 μL with 10 ng of cDNA template.

The three thermocycling parameters of PURA, CaM, and β-actin gene were identical to each other. After an initial denaturing at 95°C for 6 min, further steps were as follows: 30 cycles of denaturing for 30 s at 95°C, annealing for 40 s for 60°C, and elongation for 40 s at 72°C, and finally, an extension of 7 min at 72°C. At the end, 5 μL of each PCR reaction was separated on a 1.5% agarose gel and stained with ethidium bromide.

Quantity of PURA mRNA and CaM mRNA expression: The quantities of PURA mRNA and CaM mRNA were divided by the quantity of β-actin mRNA to obtain a normalized value expression. Each sample analysis was repeated three times.

Statistical analysis: An independent sample T-test was performed to analyze differences in PURA and CaM gene expression levels between the fluorosed group and the control group. Results are expressed as Mean±SEM. Differences with $p<0.05$ were considered statistically significant.

RESULTS

Secondary Cultured Osteoblasts: After culturing for 48 hr, the active osteoblasts adhered to the walls of the flasks, and showed a spindly appearance (Figure 1).

RNA extracted from Osteoblasts: The abundances of 28SrRNA/18SrRNA were all about 2:1, which indicate the integrity of the total RNA. However, the presence of white bands around the three gel holes indicates that the RNA that was extracted still contains DNA. We acknowledge that this indicates there were shortcomings in the separation of RNA in our procedure (Figure 2).

Expression of PURA gene: The expression levels of the 0.5, 2, and 8 mg NaF/L groups of PURA mRNA were higher than the level of the control group using our Syngene Analysis System with rat β-actin gene as a control (Figures 3 and 4). In Figure 3, other bands can be seen at the top of lanes 2–4, which also indicates a shortcoming of our procedure.

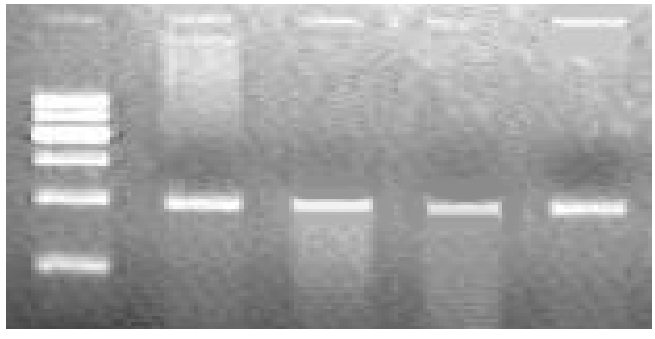

Figure 3. PCR electrophoresis imaging of β-actin gene expression. Lane 1 is the Marker. Lanes 2–5 are the control, 0.5, 2, and 8 mg NaF/L group, the bands of which indicate the expression levels of β-actin gene in the respective groups. The band sequences were 190 bp. The abundances of fluorosed groups were not distinctly different from that of the control group.

With higher concentrations of F, the percentages of PURA mRNA calculated in comparison with β-actin mRNA also increased. The expression levels of the 0.5, 2, and 8 mg NaF/L groups divided by the control group were 105% ($p<0.05$), 114% ($p< 0.05$), and 126% ($p< 0.01$).

Expression of CaM gene: The expression levels of the 0.5, 2, and 8 mg NaF/L groups of CaM mRNA were also higher than the level of the control group using our Syngene Analysis System with rat β-actin gene as a control. Again, we acknowledge that there are shortcomings in our procedure because other bands can be seen at the top of lanes 2 and 3 (Figure 5).

With increasing concentration of F, the ratio of CaM mRNA to β-actin mRNA also increased. The percentage of expression levels of 0.5, 2, and 8 mg NaF/L groups divided by the control group were 110% ($p<0.05$), 119% ($p< 0.01$), and 128% ($p< 0.01$).

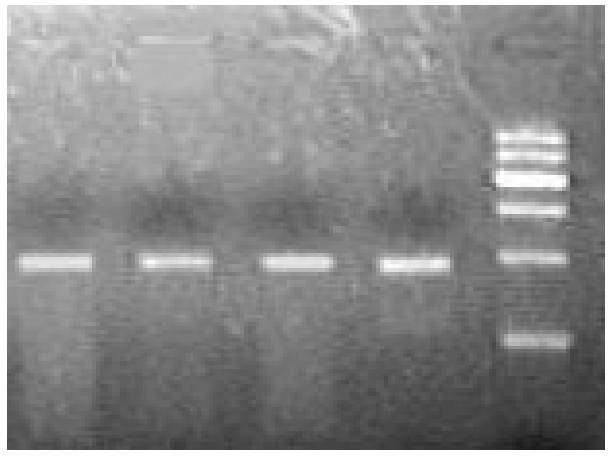

Figure 4. PCR electrophoresis imaging of PURA gene expression. Lanes 1–4 are the control, 0.5, 2, and 8 mg NaF/L group, the bands of which indicate the expression levels of PURA gene in the respective groups. The sequences were 188 bp. Lane 5 indicates the Marker. The band abundances of fluorosed groups were greater than the control group.

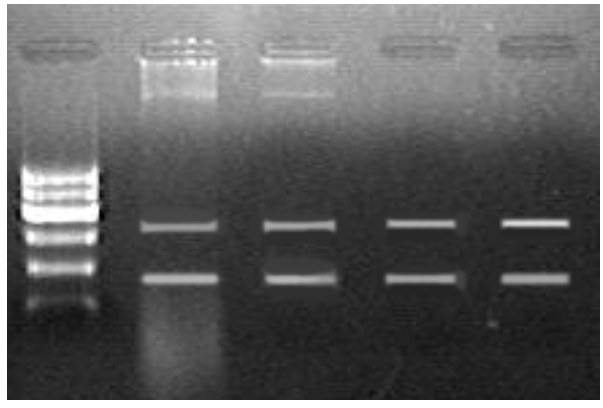

Figure 5. PCR electrophoresis imaging of CaM gene expression. Lane 1 is the marker. Lanes 2–5 are the control, 0.5, 2, and 8 mg NaF/L group. The upper band sequences that indicate the expression levels of CaM were 328 bp and the band abundances of fluorosed groups were greater than the control group. The lower bands indicate the expression levels of β-actin gene as control, the sequences of which were 190 bp.

DISCUSSION

PURA protein encoded by PURA gene is a sequence-specific, single-stranded nucleic acid-binding protein, and is a member of the highly conserved PUR family. PURA is both a DNA- and RNA-binding protein, and it has been implicated in processes as diverse as DNA replication, gene transcription, RNA transport, and mRNA translation.[12,15] PURA is involved in cell growth and mediates the progress of cell cycle.[12] Levels of PURA decline precipitously in the G_1 phase of the cell cycle, and remain low in the early S phase.[16] Levels

subsequently recover throughout the late S phase and G_2 phases of the cell cycle to peak at mitosis and remain maximal through cytokinesis and re-entry into early G_1 phase.[16]

PURA functions through its binding to PUR elements of CaM.[13] CaM also enhances the expression level of COL2A1 gene[17] and inhibition of CaM in chondrogenic cells to reduce the expression of the major cartilage matrix gene COL2A1.[18] Thus we suggest that the COL2A1 gene also exerts its function through CaM.

CaM levels are regulated as cells progress through the cell cycle and decrease in the first few hours after mitogenic stimulation. They then increase 2- to 4-fold at the G1/S boundary.[19] In mammalian cells, overexpression of CaM causes an acceleration of cell proliferation.[20] In contrast, reduction of CaM levels using anti-CaM drugs results in a transient inhibition of cell proliferation.[21] Thus CaM plays a key role in cell cycle and cell growth.

Earlier research by others has shown that F at certain concentrations affects the proliferation of osteoblasts and induces apoptosis with DNA fragmentation.[8,9] Recently, F has been found to induce DNA damage in rat osteoblasts.[10] Changes in cell cycle have also been reported in osteoblasts.[8] F caused G_0/G_1 arrest and this led to fewer cells in the S phase and the G_2/M phase.[8] However, the molecular mechanisms of these changes are not yet clear. Accordingly, we designed our study to investigate the two genes that regulate cell cycle to explore how they are associated with fluorosis in osteoblasts.

The results of our study show that the expression levels of PURA gene and CaM gene were significantly different between the control and fluorosed group. According to our findings and the known character of PURA and CaM, we conclude that F first affects CaM gene, which then affects the expression of PURA gene. Hence, the two genes may play important roles in skeletal fluorosis. The results of the work reported here may therefore help elucidate the complex mechanism of skeletal fluorosis.

ACKNOWLEDGEMENT

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