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Natural uranium impairs the differentiation and the resorbing function of osteoclasts

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ABSTRACT

Background: Uranium is a naturally occurring radionuclide ubiquitously present in the environment. The skeleton is the main site of uranium long-term accumulation. While it has been shown that natural uranium is able to perturb bone metabolism through its chemical toxicity, its impact on bone resorption by osteoclasts has been poorly explored. Here, we examined for the first time in vitro effects of natural uranium on osteoclasts.
Methods: The effects of uranium on the RAW 264.7 monocyte/macrophage mouse cell line and primary murine osteoclastic cells were characterized by biochemical, molecular and functional analyses.
Results: We observed a cytotoxicity effect of uranium on osteoclast precursors. Uranium concentrations in the μM range are able to inhibit osteoclast formation, mature osteoclast survival and mineral resorption but don't affect the expression of the osteoclast gene markers *Nfatc1*, *Dc-stamp*, *Ctsk*, *Acp5*, *Atp6v0a3* or *Atp6v0d2* in RAW 274.7 cells. Instead, we observed that uranium induces a dose-dependent accumulation of SQSTM1/p62 during osteoclastogenesis.
Conclusions: We show here that uranium impairs osteoclast formation and function in vitro. The decrease in available precursor cells, as well as the reduced viability of mature osteoclasts appears to account for these effects of uranium. The SQSTM1/p62 level increase observed in response to uranium exposure is of particular interest since this protein is a known regulator of osteoclast formation. A tempting hypothesis discussed herein is that SQSTM1/p62 dysregulation contributes to uranium effects on osteoclastogenesis.
General significance: We describe cellular and molecular effects of uranium that potentially affect bone homeostasis.

1. Introduction

Uranium (U) is a radioactive heavy metal naturally present in soils, air, water and therefore in animal and human diet. Its extensive use in civil and military activities has led to an increased risk of exposure to U, not only for workers engaged in milling and mining, but also for populations. Naturally occurring U is composed of 99.27% ^{238}U, 0.72% ^{235}U and 0.006% ^{234}U and is found, in most environmental systems, in its oxocationic form $\{U(VI)O_2^{2+}\}$ (called uranyl form and referred to as U(VI) below). Due to the very low specific activity of its main component ^{238}U, radiological toxicity of natural U(VI) is minimal while its chemical toxicity is well established. Individuals can be exposed to U(VI) by ingestion, inhalation and dermal contact. In all cases, most of U(VI) entering the body is eliminated in feces and only a small part reaches the bloodstream. 80 to 90% of the uranium present in the blood is filtered through the kidneys and cleared within a few days in urine [58]. The remainder is mostly deposited in bones and kidneys, as assessed by animal studies [4,43] and follow-up of human exposure [23,24,29,53].

In bones, U(VI) accumulates mainly near vascularized areas and sites of active calcification [3,6,49]. With a half-life retention of 70 to 200 days the removal of uranium stored in the bone is slow [3]. Thus a fraction of the absorbed uranium remains trapped in bones for several decades and the skeleton is considered as the major site of long-term storage of U(VI) [4,33,56]. Therefore, several animal and human studies have explored the effect of U(VI) on bone metabolism. Acute as well as chronic exposure to U(VI) have been shown to inhibit bone formation in rats [7,10,16,17,55,57]. Consistently, *in vivo* and *in vitro* studies have demonstrated that both the number and the activity of osteoblasts, the cells responsible for bone formation and mineralization, were altered by U(VI) [7,10,38,54]. More recently, our team has shown that U(VI) perturbs osteoblastic functions by reducing mineralization capacity. In addition, our results suggest that U(VI) exerts its toxicity in osteoblasts in part through the inhibition of autophagy, a major cellular catabolic process [46].

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While a number of studies have focused on the effect of U(VI) on bone formation and osteoblasts [2], the impact of U(VI) on bone resorption has been poorly explored. Ubios et al. have observed an increase in bone resorption of the alveolar bone after intraperitoneal injection of uranyl nitrate in Wistar rats [55]. Analysis of histomorphometric parameters of mouse metaphyseal bone, after oral administration of a lethal dose of uranyl nitrate, has also revealed an extension of resorption surfaces compared to untreated animals [7]. These results are not confirmed by those of Fukuda et al. [15]. Indeed, after an intramuscular injection of depleted uranium to rats, the authors have failed to detect any significant modification in bone resorption of proximal tibial metaphyses [15]. Besides animal studies, several epidemiological investigations have addressed the question of the health effects of naturally occurring uranium in drinking water [59]. Among them, the study from Kurttio et al. [30] provided some evidence of a positive association (only in males) between uranium exposure and serum levels of carboxy-terminal telopeptide, an indicator of bone resorption. The aforementioned *in vivo* studies have led to the proposal that U(VI) could promote bone resorption. Nonetheless, as far as we know, the cellular and molecular mechanisms underlying this possible effect of uranium have never been explored. Bone resorption is performed by large multinuclear cells, called osteoclasts. These cells result from the fusion of precursor cells of hematopoietic origin and are unique in their ability to solubilize both the mineral and organic components of the bone matrix [20].

In the present study, we examined for the first time the effect of natural uranium on osteoclast differentiation and function. Moreover, we sought to identify molecular signaling pathways disrupted by exposure of osteoclastic cells to uranium.

2. Materials and methods

2.1. Uranium exposure

Uranium solutions were prepared extemporaneously in conditions allowing the control of uranium speciation in exposure cell culture media [39]. First, an uranyl acetate stock solution ($[UO^{2+}] = 100$ mM, pH 4) was diluted to 10 mM in a cold sodium bicarbonate aqueous solution ($[HCO_3^-] = 100$ mM, Sigma-Aldrich, #S8761), thus bringing the pH to 7.0. This intermediate solution was equilibrated for 3 h at room temperature. Next, appropriate amounts of the 10 mM uranyl solution were diluted drop by drop to desired working concentration in the following basic culture medium: alpha modified Minimum Essential Medium (αMEM, Lonza, #BE12-169F) with 2 mM L-Glutamine (Sigma-Aldrich, #G7513) and supplemented or not with 5% HyClone fetal bovine serum (Thermo Scientific, #SH30071.03). A control medium was prepared simultaneously by adding the amount of bicarbonate used in the most concentrated U(VI)-treated condition, to the basic medium. Resulting control and uranium-containing media were then incubated for 3 h at room temperature before being added to cells.

2.2. Care of animals

The mice were housed and bred in the Faculty of Medicine animal facility, University of Nice, France. The procedures for the care and sacrifice of the animals were in accordance with the EU Directive 2010/63/EU for animal experiments and approved by the local experimentation committee. For bone marrow culture, 3 month-old C57BL/6 mice were killed by cervical dislocation. Femur and tibia were then dissected out and processed in sterile conditions as described below.

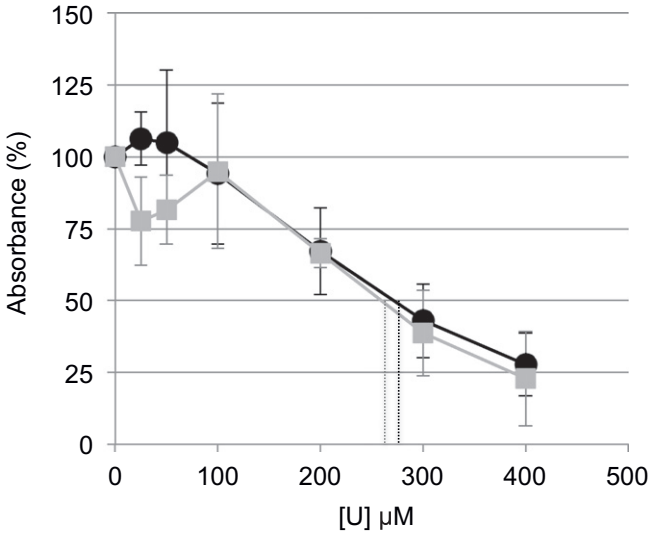

Fig. 1. Uranium (VI) cytotoxicity in the RAW 264.7 cell model. RAW 264.7 cells were exposed for 24 h to the indicated concentrations of U(VI) in culture medium containing (black circle) or not (grey square) 5% of fetal bovine serum. The colorimetric MTT assay was used to evaluate cytotoxicity. Relative absorbance values presented are the means ± standard deviation of 5 independent experiments each performed in triplicate. Mean absorbance in the control condition (without U) is set as 100%.

2.3. Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (#TIB-71) and cultured in Dulbecco's modified Eagle medium (DMEM, Lonza, #BE12-604F/12) supplemented with 5% HyClone serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, Sigma-Aldrich, #P4333). Cells were grown in 75 cm^2 flasks and passed by mechanical scrapping. Only cells from passages 5 to 9 were used in our experiments. Where indicated, cells were incubated for 2 h with 100 nM of Bafilomycin-A1 (Sigma-Aldrich, #B1793).

Bone marrow cells were flushed-out from cleaned long bones with Dulbecco's phosphate buffered saline modified (Sigma-Aldrich, #D8537) dispensed by a 2.5 ml syringe with a 26-gauge needle. After the red blood cells were removed with ACK buffer (0.01 mM EDTA, 0.011 M KHCO3, and 0.155 M NH4Cl, pH 7.3), the bone marrow cells were suspended in alpha-MEM containing 10% HyClone fetal bovine serum complemented with 2 mM L-Glutamine and cultured for 24 h in the presence of 10 ng/ml M-CSF (PeproTech, #315–02). The nonadherent cells were collected and used as bone marrow-derived macrophages (BMM) for osteoclast differentiation experiments.

Table 1
Primer sequences.

Gene	Accession number	Forward primer	Reverse primer
Rplp0	NM_007475.5	5′-TCCAGGCTTTGGGCATCA-3′	5′-CTTTATCAGCTGCACATCACTCAGA-3′
Nfatc1	NM_016791.4	5′-TGAGGCTGGTCTTCCGAGTT-3′′	5′-CGCTGGGAACACTCGATAGG-3′′
Acp5	NM_007388.3	5′-TGCCTACCTGTGTGGACATGA-3′′	5′-CACATAGCCCACACCGTTCTC-3′′
Dc-stamp	NM_029422.4	5′-AAGCGGAACTTAGACACAGGG-3′	5′-AAGCGGAACTTAGACACAGGG-3′
Ctsk	NM_007802.4	5′-CAGCAGAGGTGTGTACTATG-3′′	5′-GCGTTGTTCTTATTCCGAGC-3′′
ATP6v0d2	NM_175406.3	5′-CCTTTGTTTGACGCTGTCGG-3′	5′-ATTGCCTGTTGAATGCCAGC-3′
ATP6v0a3	NM_001167784.1	5′-GGACCATATCCCTTT GGCATT-3′	5′-AAAGCTCAGGTGGTTCGTGG-3′