Involvement of NF-κB and NFATc1 in benzo[a]pyrene-mediated effects on osteoclastogenesis.

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Introduction

Cigarette smoking is a risk factor for periodontal disease, dental implant failure, and impaired bone healing [1-3]. However, the mechanisms underlying the effect of cigarette smoke on bone remodeling are not known. Benzo[*a*] pyrene (BaP) is a typical polycyclic aryl hydrocarbon (PAH) present in cigarette smoke at high concentrations [4]. PAHs induce their toxic effects via the aryl hydrocarbon receptor (AhR), a cytosolic transcription factor that plays critical roles in the maintenance of homeostasis and embryonic development [5,6], resulting in the enhanced expression of numerous genes, such as phase I and phase II detoxification enzymes. Cytochrome P450 1A1 (CYP1A1) is the most studied enzyme in relation to PAH-induced gene regulation, however, other enzymes, such as CYP1B1, have been implicated as well. It had been demonstrated that inflammatory mediators inhibit the expression of these enzymes possibly as part of the host-defense response mechanism [7]. *In vivo* and *in vitro* studies have shown that cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon γ (IFN-γ), and lipopolysaccharides (LPS) decrease cytochrome P450 enzyme and activity levels [8-13]. Based on these results, it has been proposed that AhR and NF-κB physically interact and functionally modulate one another's activities [14]. Bone is a dynamic tissue that is constantly being remodeled. Two major cell types are responsible for this remodeling: osteoclasts, which resorb bone and osteoblasts, which form bone. Osteoblasts are derived from the cells of mesenchymal lineage, while osteoclasts are derived from hematopoietic precursors of the monocyte/macrophage lineage, which fuse to form multinucleated cells. Characteristics of osteoclasts include actin ring formation, expression of the calcitonin receptor (CR) and integrin αvβ3, and production of tartrate-resistant acid phosphatase (TRAP) [15]. Factors that promote bone resorption, such as inflammatory cytokines TNF-α, IL-1, IL-6; parathyroid hormone (PTH), and 1,25$(OH)_2$vitamin D3, stimulate osteoblasts and stromal cells to secrete receptor activator of nuclear factor κB ligand (RANKL), a cytokine necessary for osteoclast differentiation. RANKL binds to its cognate receptor RANK on the surface of the mononuclear osteoclast progenitors and activates a number of signal transduction cascades resulting in the formation of active multinucleated osteoclasts. RANKL is a membrane bound factor essential for osteoclast differentiation, activation, and survival [15]. It is a member of TNF superfamily and is produced by osteoblasts, stromal cells, activated synovial fibroblasts, chondrocytes, and activated T-cells. The binding of RANKL to the RANK receptor on the surface of osteoclast progenitors [16] is followed by a signal transduction cascade that involves the TNF receptor associated factor (TRAFs 1,2,3,5,6) adaptor proteins and downstream targets, such as nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), microphthalmia transcription factor (MITF), and NF-κB [15,17]. Several groups have investigated the effect of PAHs on bone remodeling. *In vitro* studies demonstrated that PAHs inhibits bone nodule formation, alkaline phosphatase activity, and osteocalcin expression probably through inhibition of osteodifferentiation [18,19]. *In vivo* studies demonstrated that PAHs dose-dependently inhibit bone growth, modeling and mechanical strength in rats [20], cause loss of bone mass and bone strength in ovariectomized rats [21], decrease osteoblast proliferation and differentiation, and delay ossification in mouse fetuses [22]. Most of the studies have focused on the effects of PAHs on bone formation, while the effects of PAHs on osteoclasts have been limited to one study. It was shown that 3-methylcholanthrene (3-MC), another aryl hydrocarbon, inhibited osteoclast formation by suppressing RANKL mRNA production by stromal cells [23]. Here we demonstrate that BaP directly inhibits osteoclastogenesis and investigate the involvement of NF-κB and NFATc1 in the mechanism of BaP-mediated effects.

Materials and Methods

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC). These cells, when cultured in the presence of RANKL, differentiate into osteoclasts [16]. The cells were maintained in DMEM containing 10% FBS, 100 µg/ml penicillin and streptomycin. The medium also contained 0-200 ng/ml recombinant glutathione S-transferase-soluble RANKL (GST-sRANKL), 0.1% DMSO (vehicle) and 10^{-5}-10^{-6} M BaP, depending on the experimental group. All cultures were incubated at 37°C in humidified air containing 5% CO_2. The cells were incubated for up to 5 days. To measure NF-κB p65 activation, the cells were plated in 100 mm Petri dishes, grown to confluence, and incubated in the presence of DMSO, 10^{-5}-10^{-6} M BaP, and either 25 ng/ml RANKL or 200 ng/ml RANKL for 30-60 minutes. The nuclear extracts were prepared and analyzed for p65 activation using Active Motif TransAM p65 ELISA kit. To measure NFATc1 activation, the cells were plated in 100 mm Petri dishes, allowed to attach overnight, incubated in the presence of DMSO, 10^{-5}-10^{-6} M BaP, and either 25 ng/ml RANKL or 200 ng/ml RANKL for 24 or 48 hours. The nuclear extracts were analyzed for NFATc1 activation using Active Motif TransAM ELISA NFATc1 kit. To observe the effect of BaP on NF-κB p65 nuclear translocation by immunofluorescence, the cells were plated on 4-well chamber slides, allowed to attach overnight, and then exposed to DMSO, 10^{-5}-10^{-6} M BaP, and 0 - 200 ng/ml RANKL for 30 minutes. The cells were fixed with 100% methanol at –20°C for 10 minutes, blocked in 1% normal goat serum in PBS for 1 hour, incubated with mouse monoclonal anti-p65 (Santa Cruz Biotechnology) overnight at 4°C, biotinylated secondary antibody for 2 hours, and fluorescein-conjugated streptavidin for 1 hour at room temperature (Vector Laboratories). The nuclei were counterstained with DAPI. To investigate the involvement of NF-κB on BaP-mediated gene expression, the experiments were performed in the presence of gliotoxin (Calbiochem), a NF-κB inhibitor. The cells were plated in 100 mm Petri dishes, allowed to attach overnight, pre-treated with gliotoxin for 30 minutes, and then exposed to DMSO, 10^{-5} M BaP and 7.5, 15, and 30 ng/ml gliotoxin for 20 hours. These concentrations of gliotoxin were selected after performing a dose response assay and measuring cell viability after 5 day incubation period. Total RNA was extracted using TRIzol reagent (Invitrogen) by following the manufacturer's protocol. The samples were analyzed for the CYP1B1, NFATc1, and GAPDH expression using HotStarTaq polymerase (Qiagen).

Results and discussion

We have previously demonstrated that treatment with 10^{-5} M BaP decreased osteoclast differentiation, TRAP activity levels, and resorption of bone-like substrata at 25 ng/mL RANKL, but not at 100 ng/mL and the inhibition could be reversed by either receptor antagonist (resveratrol) or high (100-200 ng/ml) concentrations of RANKL, suggesting interacting signaling pathways between RANKL and BaP. Moreover, RT-PCR indicated the presence of CYP1B1 mRNA in the group exposed to BaP only and that the levels decreased in the presence of increasing concentrations of RANKL. To test the hypothesis that BaP and RANKL signaling pathways interact, we examined the activation of NF-κB in our model system in the presence of low (25 ng/ml) and high (200 ng/ml) concentrations of RANKL. In the presence of 25 ng/ml RANKL and at 30 minutes of incubation, 10^{-5} M BaP suppresses the RANKL-mediated activation of NF-κB, while 10^{-6} M BaP has little or no effect compared to vehicle control. While RANKL-mediated NF-κB activation subsides by 45-60 minutes, the levels in 10^{-5} M BaP group remain above the levels of NF-κB in unstimulated cells and RANKL-stimulated cells. At 200 ng/ml RANKL, both 10^{-5} and 10^{-6} M BaP suppress RANKL-mediated NF-κB activation at 30 minutes of incubation, and, as in 25 ng/ml RANKL groups, the levels of RANKL-mediated NF-κB activation decrease by 60 minutes, while the levels of NF-κB in BaP groups remain high. To investigate whether BaP treatment affected NF-κB translocation, the cells were examined by immunohistochemistry. After 30 minutes of incubation in the presence of 200 ng/ml RANKL NF-κB translocated into the nucleus. 10^{-5} M BaP but not 10^{-6} M BaP blocked RANKL-mediated NF-κB translocation. No NF-κB translocation was observed in the absence of RANKL. BaP interfered with RANKL-mediated NF-κB activation by suppressing the activation at the peak (around 30 minutes) while sustaining the level of activation above the control levels beyond 60 minutes. To investigate the involvement of NF-κB in BaP-mediated gene expression, the cells were incubated in the presence of gliotoxin, an inhibitor of NF-κB, and the levels of CYP1B1 mRNA were determined by RT-PCR. CYP1B1 mRNA levels had decreased with increasing concentrations of gliotoxin, suggesting that NF-κB is involved in BaP-mediated CYP1B1 expression. To evaluate whether NFATc1 is involved in BaP-mediated inhibition of osteoclastogenesis, NFATc1 activation was measured by ELISA. 10^{-5} M BaP increased activation of NFATc1 in all groups compared to vehicle control in 0, 25, and 200 ng/ml RANKL, both after 24 hours and 48 hours of incubation. However, when analyzed by RT-PCR, there were no apparent differences in NFATc1 expression between the groups exposed to BaP and vehicle control, even though the levels of NFATc1 were increased in RANKL-containing groups, consistent with the literature [24]. The increase in NFATc1 activation in response to BaP is not surprising. NFATc1

belongs to the NFAT family of transcription factors under control of the Ca^{2+}-regulated phosphatase, calcineurin. RANKL induces Ca^{2+} oscillations and contributes to the sustained activation of NFATc1 via calcineurin-dependent mechanism [25]. At the same time, BaP and its metabolites cause a dose dependent increase in intracellular Ca^{2+} in several cell types [26,27]. It is possible that a BaP-mediated increase in cytosolic Ca^{2+} increases NFATc1 activation. In this paper, we present the preliminary results illustrating the involvement of two transcription factors NF-κB and NFATc1 in BaP-mediated inhibition of osteoclastogenesis. Our results suggest that while BaP inhibits NF-κB nuclear translocation, it increases NFATc1 activation and confirm our hypothesis that the RANKL and PAH signaling pathways interact.

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