Molecular Immunology 85 (2017) 27–34

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Anthocyanin suppresses CoCrMo particle-induced osteolysis by inhibiting IKKα/β mediated NF-κB signaling in a mouse calvarial model☆

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ARTICLE INFO

Article history:
Received 22 November 2016
Received in revised form 20 January 2017
Accepted 6 February 2017

Keywords:
Osteolysis
Inflammation
CoCrMo particles
Anthocyanin
NF-κB pathway

ABSTRACT

Wear particle-induced osteolysis and bone resorption have been identified as critical factors of implant failure and total joint revision, in which nuclear factor kappa B (NF-κB) signaling and chronic inflammation have been shown to play key roles. Although anthocyanin is known to have anti-inflammatory function *via* blocking NF-κB pathway, it is still unclear whether anthocyanin has a protective effect on particle-induced osteolysis. In the present study, we aimed to investigate the detailed effects and the underlying mechanism of anthocyanin on CoCrMo particle-induced osteolysis in a mouse calvavial model. One hundred and twelve male BALB/c mice were divided randomly into four groups: sham group (sham operation and injection with PBS), vehicle group (CoCrMo particle treatment and injection with PBS), low-dose anthocyanin group (CoCrMo particle treatment and injecting anthocyanin with 0.1 mg/g/day), and high–dose anthocyanin group (CoCrMo particle treatment and injecting anthocyanin with 0.4 mg/g/day). Mice were sacrificed after two weeks, harvesting the calvariae tissue for in depth analysis by micro-CT, histomorphometry, immunohistochemical and molecular biology analysis. As expected, anthocyanin markedly inhibited CoCrMo particle–induced inflammatory infiltration and decreased bone loss *in vivo*. Anthocyanin also reversed the increase in the ratio of receptor activator of nuclear factor kappa B ligand (RANKL)/osteoproteger (OPG) and suppressed osteoclast formation in CoCrMo particle–stimulated calvaria. Additionally, anthocyanin significantly reduced the expression and secretion of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the calvaria of CoCrMo-stimulated mice. Furthermore, we confirmed that anthocyanin attenuated osteolysis by blocking NF-κB pathway *via* inhibiting inhibitor of nuclear factor kappa-B kinase α/β (IKKα/β) phosphorylation. In conclusion, our study demonstrated that anthocyanin can protect against CoCrMo particle–induced inflammatory osteolysis *via* inhibiting the IKKα/β-NF-κB pathway, and have a potential therapeutic effect on the treatment of wear particle–induced osteolysis.

1. Introduction

Total Joint Replacement (TJR) has been proved the most efficient and widely applicable option for the treatment of end–stage articular diseases, such as osteoarthritis, femoral head necrosis and rheumatoid arthritis. It can dramatically relieve patients' pain, recover joint function and improve their quality of life, resulting in an increased demand of these procedures (Della Valle et al., 2009; Teeny et al., 2003). However, a major concern is the *peri*-prosthetic osteolysis and secondary aseptic loosening, which can cause implant failure only with the solution of total joint revision, which remains a common procedure despite material development and technique improvement (St Pierre et al., 2010; Gallo et al., 2013). Peri-prosthetic osteolysis is induced by the organic response to particular wear particles generated at the bone–implant interfaces, such as ultrahigh molecular weight polyethylene particles, titanium particles and CoCrMo particles (Athanasou, 2016). There is a lack of efficient treatments, presenting only expensive and complicated revisions in order to protect patients from suffering particle-induced osteolysis with a failure of clinical application of bisphosphonates to aseptic loosening (Goodman et al., 2014).

☆ This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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http://dx.doi.org/10.1016/j.molimm.2017.02.003
0161-5890/

Therefore, there is an urgent need to develop novel pharmaceutical intervention in order to reduce osteolysis.

It is widely believed that wear particles lead to local bone loss and osteolysis by activating nuclear factor kappa B (NF-κB) signal channel, triggering the release of pro-imflammatory cytokines, and inducing osteoclast activation (Goodman et al., 2014). Many researchers have investigated targeting one particular pro-inflammatory cytokine like tumor necrosis factor-α (TNF-α) or induce osteoclast apoptosis for the treatment of particle-induced osteolysis, showing unsatisfactory results (Schwarz, 2008; Schwarz et al., 2000). NF-κB is a key regulator of many kinds of inflammation responses and bone remodeling (Lin et al., 2014). Goodman et al. suggested that the inhibition of NF–κB could be a viable strategy for mitigating the undesirable effects of wear particles (Goodman et al., 2014). Furthermore, inhibitor of nuclear factor kappa-B kinase α/β (IKKα/β) is an essential kinase for the activation of NF–κB combined with inhibition of κB (IκB) in the occurrence of induced activation signals. Therefore, it is a potential therapeutic target of IKKα/β to attenuate particle–induced osteolysis.

Anthocyanin, a class of flavonoids, has the advantages of anti-inflammatory properties and widely exists in many fruits and vegetables, including blueberries, radishes, currants or blackberries, among others (Veitch and Grayer, 2011). These have been consistently shown to attenuate the expression and secretion of multiple pro-inflammatory cytokines by inhibiting the activation of NF–κB and the phosphorylation of its upstream kinase (Kim et al., 2013; Tsoyi et al., 2008; Hecht et al., 2006). Furthermore, Afaq found that anthocyanin-rich pomegranate fruit extract blocked phosphorylation of IκB-α in a dose–dependently by inhibiting IKKα activation (Afaq et al., 2005). However, it is unclear whether anthocyanins can prevent particle–induced osteolysis by suppressing IKKα/β-NF–κB pathway *in vivo*.

We hypothesized that anthocyanin could protect against particle–induced osteolysis through NF–κB signaling blocking by inhibiting IKKα/β activation. In this study, we investigate the effect of anthocyanin on particle –induced osteolysis by administering low and high concentrations of anthocyanin to CoCrMo particle–stimulated mice for 2 weeks. Expression of osteoclast–specific genes and protein level of pro-inflammatory cytokines and RANKL/OPG ratio were detected by RT-PCR and ELISA. The degree of bone osteolysis was analyzed by micro-CT and immune–histochemical staining.

2. Materials and methods

2.1. CoCrMo particle preparation

CoCrMo particles, a kind gift from Sandvik (Stockholm, Sweden), had a mean diameter size 3.77 μm (range 0.02–11.57 μm). Samples were imaged with a scanning electron microscopy Vega3LMU (Tescan, Czech) at 1000× magnification to detect particle size characteristics and distribution. Before usage, CoCrMo particles were washed in 75% ethanol solution to remove endotoxin and were confirmed negative for endotoxin using a commercial limulus amebocyte lysate assay kit (Biowhittaker, Walkersville, MD, USA) as previously described (Geng et al., 2011; Wang et al., 2015). Sterile particles were suspended in phosphate buffered saline (PBS) and stored at 4 °C until usage.

2.2. Animals and grouping

All animal experiments were approved by the Ethics committee of Shanghai Jiaotong University Affiliated Sixth People's Hospital and the principles for care and use of the Laboratory Animals were carefully observed. Eight to ten week–old healthy male BALB/c mice (n = 112) were obtained from the Experimental Animal Center of Shanghai Sixth People's Hospital. Mice were randomly assigned into four groups (n = 28 in each group): sham group (sham operation and injection with PBS), vehicle group (CoCrMo particle treatment and injection with PBS), low-dose anthocyanin group (CoCrMo particle treatment and injecting anthocyanin with 0.1 mg/g/day), and high-dose anthocyanin group (CoCrMo particle treatment and injecting anthocyanin with 0.4 mg/g/day).

2.3. Mouse cavarial osteolysis model

As previously described (Zhai et al., 2014; Liu et al., 2014; Tian et al., 2014a), we prepared a mouse calvarial osteolysis model to investigate the inhibiting effects of anthocyanin on CoCrMo particle–induced osteolysis *in vivo*. Briefly, mice were anesthetized with 90 mg/kg chloral hydrate by intraperitoneal injection. An incision was made on the head of mouse from the midpoint of two ears to the eyes in the length of 1 cm and the periosteum was separated from the sagittal suture of calvarium to both sides. In the vehicle, low and high groups, 20 mg of CoCrMo particles were embedded on the surfaces of the calvarial bones, whereas the incision in the sham group was closed without intervention of CoCrMo particles. Anthocyanin was injected intraperitoneally with the doses of 0.1 mg/g/day in the low-dose group or 0.4 mg/g/day in the high-dose group for 2 weeks, respectively. Penicillin was intraperitoneally injected for 3 days post-operation. After 2 weeks, the mice were sacrificed with overdose anesthetics and the calvariae were harvested for further analysis.

2.4. Micro-CT scanning

Samples were harvested and fixed in formalin for 48 h, analyzing them (n = 7/group) using a high-resolution micro-CT scanner SkyScan1176 (software = Version 1.1 (build 6), Bruker, Kontich, Belgium). Wear particles were cleared before scanning to avert metal artifacts as Zhai described (Zhai et al., 2014). The scanning protocol was set at an isometric resolution at 9 mm and X-ray energy settings of 80 kV and 80 mA with an exposure time of 100 ms as previously described (Wedemeyer et al., 2007). After scanning, 3D images were reconstructed using NRecom software (Version:1.6.9.8). Bone mineral density (BMD), bone volume to tissue volume ratio (BV/TV), total porosity and the number of pores were measured and analyzed using CT Analyser Software (Version: 1.15.4.0+, Bruker) after selecting a cylindrical region of interest (ROI; 3 × 3 × 1 mm) centered around the intersection of the sagittal and coronal sutures as previously described (Kauther et al., 2013).

2.5. Histological and immunohistochemical analysis

After fixed in formalin for 48 h, specimens (n = 7/group) were decalcified in 10% ethylenediaminetraacetic (EDTA) for 14 days and then embedded in paraffin. The samples were sectioned in the coronal plane, mounted on glass slides and then deparaffinated in dimethylbenzene. Sections were firstly stained with H&E and then photographed under a high-quality light microscope centered on the middle suture at a magnification of 100×. Histomorphology was analyzed in the ROI of five consecutive sections from each calvaria using Image Pro Plus software 6.0 (Media Cybernetics, Bethesda, MD, USA). Eroded surface area was measured within the ROI of the middle suture encircled as described previously (Wedemeyer et al., 2007; Kauther et al., 2013).

For detection of osteoclasts, sections were stained with a commercial tartrate–specific acid phosphate (TRAP) kit. TRAP-positive cells presented claret–red stained granules within the edge of resorption lacuma. The number of TRAP-positive multinucleated osteoclasts was determined by the count method proposed by