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Role of direct estrogen receptor signaling in wear particle-induced osteolysis

Christophe Nich [a,b,c,*], Allison J. Rao [a], Roberto D. Valladares [a], Chenguang Li [a], Jane E. Christman [a], Joseph K. Antonios [a], Zhenyu Yao [a], Stefan Zwingenberger [a,d], Hervé Petite [b], Moussa Hamadouche [b], Stuart B. Goodman [a]

[a] Department of Orthopaedic Surgery, Stanford University School of Medicine, 300 Pasteur Drive, R105, Stanford, CA 94305, USA
[b] Laboratoire de Biomécanique et Biomatériaux Ostéo-Articulaires – UMR CNRS 7052, Faculté de Médecine, Université Paris 7, Paris, France
[c] Department of Orthopaedic Surgery, European Teaching Hospital, Assistance Publique – Hôpitaux de Paris, Université Paris 5, Paris, France
[d] Department of Orthopaedics, University Hospital Carl Gustav Carus at Technical University Dresden, Fetscherstraße 74, 01307 Dresden, Germany

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ABSTRACT

Estrogen withdrawal following surgical ovariectomy was recently shown to mitigate particle-induced osteolysis in the murine calvarial model. Currently, we hypothesize that estrogen receptors (ERs) were involved in this paradoxical phenomenon. To test this hypothesis, we first evaluated polyethylene (PE) particle-induced osteolysis in the murine calvarial model, using wild type (WT) C57BL6J female mice, ERα deficient (ERαKO) mice, and WT mice either treated with 17β-estradiol (E2) or with the ER pan-antagonist ICI 182,780. According to micro-CT and histomorphometry, we showed that bone resorption was consistently altered in both ERαKO and ICI 182,780 treated mice as compared to WT and E2 groups. Then, we demonstrated that ER disruption consistently decreased both PE and polymethylmethacrylate (PMMA) particle-induced production of TNF-α by murine macrophages *in vitro*. Similar results were obtained following ER blockade using ICI 182,780 in RAW 264.7 and WT macrophages. ER disruption and pre treatment with ICI 182,780 resulted in a consistent down-regulation of particle-induced TNF-α mRNA expression relative to WT macrophages or untreated RAW cells. These results indicate that the response to wear particles involves estrogen receptors in female mice, as part of macrophage activation. Estrogen receptors may be considered as a future therapeutic target for particle-induced osteolysis.

1. Introduction

Aseptic loosening secondary to periprosthetic osteolysis represents the leading cause of failure of total joint replacements in the long-term [1]. The development of periprosthetic osteolysis is driven by adverse reactions from the host toward particulate wear debris delivered from an articulating surface of a total joint replacement. Upon activation by wear particulate debris, macrophages release an array of cytokines and pro-inflammatory mediators that result in the recruitment, multiplication, differentiation, and maturation of osteoclast precursors, leading to bone resorption and eventual implant failure [2,3].

Although it is widely accepted that generation of polyethylene particles by the bearing couple correlates with the risk for revision due to aseptic loosening [4,5], a high variability in the incidence of aseptic loosening and size of the periprosthetic osteolytic lesions has been observed between individuals with similar PE wear rates [6]. Hence, the potential role for individual factors such as genetic background [7,8] and genetically determined obesity [9] to influence the development of particle-induced osteolysis has been advocated.

Recently, we evaluated the bone response to particulate wear debris in an ovariectomy (OVX) mouse model [10]. We found a significant decrease in osteolysis in this model, indicating that OVX, although known as an experimental model of menopause, could be protective against wear debris-induced osteolysis. Furthermore, we showed the biologic pathway leading to particle induced bone resorption, driven by cytokines such as IL-1β, IL-6, and TNF-α, was consistently altered in estrogen deficient mice [11], suggesting a regulation at the cellular level.

Most biological effects of estrogens are mediated by two subtypes of nuclear receptors, described as estrogen receptor (ER) α and ERβ, both being members of the nuclear steroid hormone receptor gene superfamily [12–14]. Nuclear estrogen-bound ERs

* Corresponding author. Department of Orthopaedic Surgery, Stanford University School of Medicine, 300 Pasteur Drive, R105, Stanford, CA 94305, USA. Tel.: +1 650 814 3822; fax: +1 650 725 6631.
E-mail address: chrnich@gmail.com (C. Nich).

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act as transcription factors regulating expression of specific target genes [15–18]. Although the molecular role of bone ERs in the osteoprotection induced by estrogen remains unclear, it has been suggested that the inhibitory action of estrogens on bone resorption is mediated by the osteoclastic ERα, through the shortened lifespan of osteoclasts in female mice. In male bones, beneficial effects of estrogens could be predominantly mediated by the osteoblastic ERα [19]. Interestingly, macrophages, among other cells of the immune system, also express estrogen receptors. *In vivo*, their activation enhances their ability to produce inflammatory mediators and cytokines in conjunction with the activation of the Toll-Like Receptor (TLR) pathway [20], which has been recently reported to play a role in the recognition of wear particles [21].

Considering these observations, we hypothesized that ERs could be involved in the biologic response to wear debris in female mice. To assess the role of ERs in particle-induced osteolysis, we employed the murine calvarial model in wild-type mice treated with supra-physiological doses of estrogen or ER antagonist, and ERα knock out mice. In an attempt to shed light on the importance of macrophages, we secondly evaluated the *in vitro* inflammatory response to wear debris by macrophages subjected to either ER disruption, stimulation or blockade.

2. Materials and methods

2.1. Preparation of wear debris particles

For the *in vivo* study, we used conventional non-highly cross-linked UHMWPE (PE) particles obtained from joint simulator tests and isolated according to an established protocol [22]. Briefly, the particles were isolated by density gradient centrifugation and incubated with 95% ethanol overnight for sterilization. Then, frozen aliquots of the particles containing solution were lyophilized for 4–7 days. The dried material was digested in 5 M sodium hydroxide at 70 °C for 2 h. The digested particle suspension was centrifuged through a 5% sucrose gradient at 40 K rpm at 10 °C for 3 h. The collected particles were ultrasonicated and centrifuged again. Particles were then re-suspended in 95% ethanol, which was evaporated completely, and ultimately washed in 70% ethanol. This treatment resulted in negative testing for endotoxins using a quantitative Limulus Amebocyte Lysate (LAL) Assay (BioWhittaker, Walkersville, MD), at a detection level of <0.05 EU/ml. The mean diameter of the particles was 1.0 ± 0.1 μm (mean ± SEM) measured by electron microscopy. For the purpose of the *in vivo* study, PE particles were re-suspended in phosphate buffered saline prior to implantation. The concentration of PE particle stock solution was 30 mg/ml, and using the density value of 0.94 g/ml, we calculated the appropriate volume to administer 4×10^8 particles to the calvaria of each animal.

For the purpose of the *in vitro* study, we used both PE particles, prepared as described above, and PMMA particles, since these particles have been used extensively by our group and others, and have been well documented for their ability to activate macrophages to release pro-inflammatory cytokines *in vitro* [21]. Briefly, PMMA particles (Polysciences, Warrington, PA, USA) 1–10 μm in diameter, were washed with 70% ethanol and incubated overnight with shaking at 4 °C. The particles were then washed extensively with phosphate-buffered saline (PBS) and resuspended to obtain a concentrated 5% v/v stock solution. Also, the particles were free of endotoxin according to the LAL Assay (BioWhittaker).

2.2. Animal surgery and in vivo protocol

Forty wild-type (WT) C57BL/6J female mice, aged 9–10 weeks, were used. To evaluate the consequences of estrogen receptor alpha depletion on particle-induced osteolysis, we also obtained 10 estrogen receptor alpha knockout (ERαKO) age-matched female mice (strain B6.129P2-*Esr1*^{tm1Ksk}/J). The generation of ERαKO mice has been described previously [23]. All animals were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed in quarantine 48 h prior to experiment under local vivarium conditions (24 °C and 12 h/12 h light/dark cycle). All mice were handled according to international guidelines for care and use of laboratory animals. All experiments followed protocols approved by the Animal Facilities of Stanford University.

Surgical implantation of PE particles has been previously described in details [9]. Briefly, all mice were operated on general anesthesia via inhaled isoflurane 2–3% at 1 l/min. A 0.5×0.5 cm^2 area of periosteum was exposed using a direct approach over the shaved and cleaned calvaria. The periosteum was preserved in all cases. In each experimental group, 5 animals received 100 μl of the PE particle suspension and another 5 animals received saline alone (100 μl), and were defined as "internal controls". The incision was closed using 3–0 Ethylon sutures (Ethicon Inc., Somerville, NJ, USA).

At the time of surgery, 10 WT mice (5 saline-implanted, 5 PE-implanted) were implanted in the lateral side of the neck with subcutaneous hormone pellets (Innovative Research of America, Sarasota, FL, USA) designed for slow release of 8 μg/day of exogenous 17β-estradiol (E2) for a total of up to 15 days. The 8-μg/day E2 dose was chosen to assess the impact of supra-physiological levels of E2 on particle-induced osteolysis. Ten WT mice (5 saline-implanted, 5 PE-implanted) were implanted with placebo vehicle pellets.

To test further the implication of estrogen receptors signaling in particle-induced osteolysis, the synthetic ER pan-antagonist fulvestrant (Faslodex®) ICI 182,780 (Sigma–Aldrich, St. Louis, MO, USA) was applied in 10 WT mice (5 saline-implanted, 5 PE-implanted). Currently, we employed ICI 182,780, an ER antagonist that, unlike other anti-estrogens such as tamoxifen, has no known estrogen agonist effect. After binding, ICI 182,780 induces a conformational change of the ER thus reducing its ability to modulate gene transcription and increasing ER degradation [24]. The ER antagonist ICI 182,780 was applied according to a protocol previously used at our Institution [25]. Starting the day of particle implantation, 10 μl of a 100 μM solution ICI 182,780 (in a suspension containing 0.9% normal saline, 0.01% DMSO) was injected subcutaneously in the animal's back, 5 days a week. Vehicle only (0.9% normal saline, 0.01% DMSO) was injected in 10 littermates (5 saline-implanted, 5 PE-implanted), which were defined as "WT controls". Table 1 shows the treatment of the different experimental groups of mice. The results are reported in four mice groups, described as "WT control", "ERαKO", "E2" and "ICI 182,780" groups.

After the procedure, the animals were returned to their cages where water and food were given *ad libitum*. Sacrifice was done 2 weeks after the procedure by cervical dislocation following isoflurane anesthesia. The calvariae were dissected after sacrifice. The specimen was freed of all soft tissues, and fixed in 4% neutralized paraformaldehyde prior to micro-Computed Tomography (micro-CT) and histological analyses. Also, the entire uterus was collected to assess the consequences of *in vivo* exposure to E2 or ER depletion or blockade. After the serosal fat was removed, the uterus was transected at the junction of the uterine body and cervix, and the uterine weight was recorded.

2.3. Micro-computed tomography imaging and volumetric osteolysis analysis

Calvariae were scanned using a high-resolution micro-CT system (MicroCAT II, Siemens Preclinical Solutions, Knoxville, TN, USA). The scans were individually corrected to a calibration piece placed in each scan to control for any inter-scan variability. The radiographic projections were acquired at 80 kV and 500 μA with an exposure time of 200 ms. After scanning, 3D images were reconstructed with a voxel average size of 40 μm using COBRA reconstruction interface software (Exxim computing corporation, Pleasenton, CA). Qualitative and quantitative data were analyzed with a global fixed threshold [26]. A cylindrical volume of interest (VOI of $6 \times 6 \times 2$ mm) with the intersection of the sagittal suture and the coronal suture in its center was defined. For quantitative analysis of PE particle-induced osteolysis (bone volume fraction, BV/TV) within the VOI, we used eXplore MicroView V.2.0 imaging software (GE Medical, Raleigh, NC), an open source 3D volume viewer and analysis software package. To address bone tissue mineralization following estrogen receptor stimulation, depletion or blockade, we obtained the bone mineral density (BMD, mg/cc) in saline-implanted calvariae.

2.4. Histologic evaluation of osteolysis

After scanning, calvarial bones were decalcified in 2 successive ethylene diamine tetra acetic acid (EDTA) baths, for seven days each. Calvariae were then embedded in Optimal Cutting Temperature Compound (OCT, Tissue-Tek, Sakura Finetek USA, Torrance, CA) and flash frozen in liquid nitrogen and stored at −80 °C. Frozen sections (7 μm) in the coronal plane were obtained at the depth where the presence of particles was detected within the calvarial tissue using a cryostat (Cambridge Instruments, Buffalo, NY). These sections were stained with hematoxylin and eosin (H&E, Sigma–Aldrich) and a serial section stained for tartrate-specific acid phosphatase-positive (TRAP) osteoclasts (Acid Phosphatase kit, Sigma–Aldrich). Using a magnification of 20×, the histomorphometric analysis of each calvaria cap was performed on the most central section and on four adjacent sections. The region of interest was defined as previously recommended [9]. The sagittal suture area (SSA) was determined by tracing the area of soft tissue between the parietal bones. To determine bone thickness, sections were divided using a digital caliper in four 100 μm steps to the left and right sides of the midline suture respectively. The

Table 1
Experimental mouse groups.

	WT control	ERαKO	E2	ICI 182,780	Total (n)
PE (−)	5	5	5	5	20
PE (+)	5	5	5	5	20
Total (n)	10	10	10	10	40

WT, wild-type; ERαKO, estrogen receptor α knockout; E2, 17β-estradiol; ICI 182,780, estrogen receptor antagonist; PE (−), saline-implanted; PE (+), PE-implanted; * the WT Control group refers to the group of C57BL/6J mice that received vehicle only.