Effects of the Toll-like Receptor 4 Antagonist Eritoran on Necrotic Bone-Induced Inflammation and Osteoblast Differentiation

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INTRODUCTION:
Osteonecrosis of the femoral head (ONFH) is a progressive disease often affecting young adults, leading to femoral head collapse and severe hip pain, for which no effective non-surgical treatment exists. The pathogenesis involves an inflammatory response to necrotic bones, where damage-associated molecular patterns (DAMPs) released from necrotic cells activate innate immune response, inducing inflammation and perturbed bone regeneration.Toll-like receptor 4 (TLR4), a key innate immune receptor, is implicated in this process by recognizing DAMPs, such as High Mobility Group Box 1 (HMGB1), and promoting inflammation and subsequent bone resorption._Accordingly, the TLR4 antagonist Eritoran developed for the treatment of severe sepsis, can be a potential therapeutic agent for ONFH. This study aimed to examine the hypothesis that TLR4 inhibition with the specific antagonist Eritoran could prevent bone fragility in ONFH by suppressing inflammation and promoting osteogenesis.

METHODS:
All animal experiments were approved by the Nagoya University Institutional Animal Care and Use Committee. All surgical procedures were conducted under usual sterile conditions.
Effects on inflammation and bone metabolism in a simulated osteonecrotic environment: Necrotic bone fluid (NBF) containing DAMPs such as HMGB1 was produced from repeated freeze-thawed bones of C57BL/6J mice according to the method by Deng et al. Mouse bone marrow-derived mesenchymal stromal stem cells (MSCs) were cultured with NBF in the presence or absence of Eritoran (20 ng/mL). The effects on inflammation and osteogenesis were assessed by qPCR (IL-6, Runx2). Mineralization was evaluated using osteoblast-like MC3T3-E1 cells with Alizarin Red S staining. Osteoclastogenesis was assessed using RANKL-stimulated RAW264.7 cells.
Therapeutic effects in a murine model of osteonecrosis: Osteonecrosis was induced in 6-week-old male C57BL/6J mice (n = 12) by coagulating four nutrient vessels of the femoral condyle as reported by Kamiya et al. An initial study evaluated Eritoran (20 mg/kg, i.p.) administered for 1-week post-surgery, with evaluation at 3 weeks. Mice were given intraperitoneal injection of Eritoran (20 mg/kg) or vehicle three times during the first week postoperatively (on days 1, 4, and 7). All mice were sacrificed at 3 weeks, and the knees were extracted.To investigate the role of sustained TLR4 signaling, TLR4 KO mice (n = 4) and wild-type (WT) C57BL/6J mice (n = 6) underwent the same surgical procedure without drug treatment. Male mice were used in this proof-of-concept study to reduce hormonal variability. Bone structural changes were evaluated using micro-computed tomography (μCT) and histology (H&E, TRAP, IL-6).
Statistical Analysis: Data were analyzed using ANOVA followed by Tukey's post-hoc test or Student's t-test, with $p < 0.05$ considered significant.

RESULTS:
Effects on inflammation and bone metabolism in a simulated osteonecrotic environment: NBF stimulation significantly increased IL-6 expression in MSCs, and this increase was significantly suppressed by co-treatment with Eritoran ($p < 0.01$) (Fig. 1). NBF inhibited osteogenic differentiation and mineralization of MC3T3-E1 cells, but co-treatment with Eritoran significantly rescued and promoted mineralization (Fig. 2). Eritoran had no significant effect on RANKL-induced osteoclastogenesis in RAW264.7 cells.
Therapeutic effects in a murine model of osteonecrosis: Short-term Eritoran administration for 1 week did not result in significant differences in μCT parameters or histology at 3 weeks post-surgery compared to the vehicle group. In the TLR4 KO model, there were no significant differences in μCT parameters between KO and WT mice at 3 weeks. However, at 6 weeks post-surgery, all TLR4 KO mice exhibited severe articular collapse and bone destruction, precluding quantitative μCT analysis, whereas WT mice maintained their joint structure (Fig. 3).

DISCUSSION: Our in vitro results indicate that Eritoran effectively suppresses inflammation and promotes osteoblastic differentiation in a simulated osteonecrotic environment. The lack of effect in the in vivo study at 3 weeks may be because the observation period was insufficient to detect structural changes. The catastrophic joint collapse observed in TLR4 KO mice at a later stage suggests that sustained TLR4 inhibition may interfere with bone repair and remodeling. A limitation of this study is the use of a condyle model, which is subjected to different mechanical loading compared to the femoral head.

SIGNIFICANCE/CLINICAL RELEVANCE: This study challenges the simplistic paradigm of continuous TLR4 inhibition for ONFH treatment. It proposes a novel and clinically applicable strategy of acute-phase immunomodulation, which may suppress initial damage without compromising long-term healing.

REFERENCES:
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・Kamiya N, Yamaguchi R, Aruwajoye O, Adapala NS, Kim HK. Development of a mouse model of ischemic osteonecrosis. Clin Orthop Relat Res. 2015;473(4):1486-1498.

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IMAGES AND TABLES:
Figure. 1

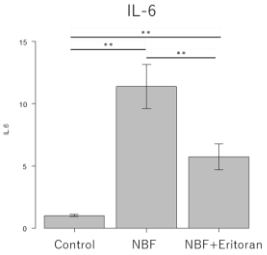

Figure. 2

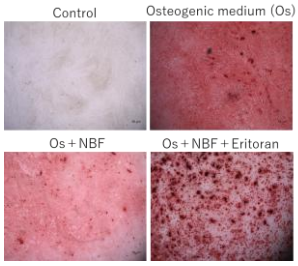

Figure. 3