European Journal of Pharmacology 679 (2012) 132–138

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

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

Immunopharmacology and Inflammation

A structural modulator of tumor necrosis factor type 1 receptor promotes bone formation under lipopolysaccharide-induced inflammation in a murine tooth extraction model

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ARTICLE INFO

Article history:
Received 17 June 2011
Received in revised form 15 December 2011
Accepted 28 December 2011
Available online 20 January 2012

Keywords:
TNF-α antagonist
Tooth extraction model
Bone formation
Inflammation
Bone destruction

ABSTRACT

Recently an increase in the serum levels of a bone formation marker after anti-tumor necrosis factor (TNF)-α therapy in rheumatoid arthritis patients has been reported. However, there remains no direct evidence that TNF-α antagonist could accelerate bone formation under inflammatory condition. Cavity-induced allosteric modification (CIAM) compound, F002, is a newly developed-TNF-α antagonist, which was designed by using the structure of TNF type 1 receptor, thus preventing TNF-α-induced signaling. In this study, we examined whether the CIAM compound can promote bone formation under inflammatory condition induced by lipopolysaccharide (LPS). Thirty-six 10-week-old male mice (C57BL/6J) were used. Half of the mice received 10 mg/kg LPS, while the other half received PBS. Thereafter, incisor extraction was performed at 4 days after either LPS or PBS injection. Intraperitoneal injections of 2 mg/kg/day, 4 mg/kg/day CIAM, or vehicle (10% DMSO) were performed once a day from day 0 to day 7 after incisor tooth extraction. The mice were sacrificed at 21 days after the extraction. The regenerated bone mineral density (BMD) in the tooth socket, and the mineral apposition rate and the bone formation rate, direct evidences of bone formation, were significantly decreased in the LPS-injected group compared to the PBS-injected group. CIAM compound dose-dependently prevented the decrease of BMD under the LPS-injected condition, and promoted both the mineral apposition rate and the bone formation rate significantly compared to the LPS-injected group. We did not observe any significant differences among the PBS-injected groups. Taken together, TNF-α antagonist CIAM compound, was found to accelerate bone formation under LPS-induced inflammatory condition.

1. Introduction

Anti-tumor necrosis factor (TNF)-α monoclonal antibodies and a soluble receptor for TNF-α are major therapeutic agents used for the treatment of rheumatoid arthritis (Landewe et al., 2006; Lipsky et al., 2000; Tanaka et al., 2008; Weinblatt et al., 1999), and the inhibitory effects of these agents on inflammatory bone destruction are so prominent that the responders to these agents can show a decrease in the bone erosion score of peripheral bones in rheumatoid arthritis patients determined by radiography (Sharp score), while the traditional disease-modifying antirheumatic drugs like methotrexate cannot (Isaacs, 2010; van der Heijde et al., 2006; Weinblatt et al., 1999). The effects of anti-TNF-α treatment on and the mechanism for inhibition of bone destruction, however, have not been clarified well.

The prevention of bone resorption by blocking the actions of TNF-α may underlie the preservation of the peripheral bone around the inflammatory sites, including joints and alveolar bone, by the TNF-α antagonists (Assuma et al., 1998; Delima et al., 2001; Di Paola et al., 2007; Saito et al., 2007). On the other hand, several lines of evidence show that the treatment with an anti-TNF-α antibody increases the osteocalcin level in serum, which is a marker of bone formation (Seriolo et al., 2006). However, there remains no direct evidence that TNF-α antagonist could accelerate bone formation under inflammatory condition.

The cavity induced allosteric modification (CIAM) compound, F002, is a newly developed TNF-α antagonist (Murali et al., 2005) that was designed to fit the cavity of the critical loop on the TNF type 1 receptor.

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doi:10.1016/j.ejphar.2011.12.046

It has already been demonstrated that the CIAM compound blocks TNF-α activity by modifying the conformation of the ligand recognizing loop on the TNF type 1 receptor (Murali et al., 2005). In addition, the CIAM compound inhibited osteoclast differentiation and blocked inflammatory bone resorption in a murine rheumatoid arthritis model (Murali et al., 2005), however, the effects of the CIAM compound on bone formation have not been reported yet.

In this study, we used a murine tooth extraction model, which was designed to evaluate the bone forming ability of reagents. In this model, the serum level of TNF-α is elevated after the subcutaneous injection of lipopolysaccharide (LPS), which promotes the production of other pro-inflammatory cytokine IL-6 (Tomomatsu et al., 2009). Osteoclasts and the eroded bone surface due to bone resorption have not yet appeared in the bone regeneration area of the tooth socket during the first 21 days after tooth extraction, thereby allowing the effects of compounds on bone formation to be more easily observed. In the present study, we evaluated the effects of the CIAM compound, a structural modulator of the TNF receptor, on bone formation, and examined whether the compound could accelerate bone formation in this tooth extraction model. The evidence to show the increase of bone formation by a TNF-α antagonist would clarify the mechanism of bone destruction in rheumatoid arthritis and the other bone diseases accompanied by inflammatory bone destruction.

2. Materials and methods

2.1. Reagents

LPS (Salmonella; Re595), calcein, and demeclocycline hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Villanueva's bone stain solution was obtained from Maruto Instrument (Tokyo, Japan). The CIAM compound, F002, was kindly provided by Dr. Mark I. Greene (University of Pennsylvania, PA, USA).

2.2. Animals

The 36 male C57BL/6J mice (10 weeks old) were obtained from CLEA Japan (Tokyo, Japan), and divided into 6 groups (6 mice per group) taking care that the average of the body weight was similar among the groups. The mice were maintained under control conditions as described elsewhere (Alles et al., 2009; Soysa et al., 2010; Tomomatsu et al., 2009). The experimental procedures were reviewed and approved by the Animal Care and Use Committee of Tokyo Medical and Dental University (Tokyo, Japan).

2.3. Murine tooth extraction model

The murine tooth extraction model was prepared as described elsewhere (Tomomatsu et al., 2009). Briefly, the left mandibular incisor was cut at the gingival margin to make the tooth extraction smooth under anesthesia. At the same time, half of the mice received subcutaneous injections of 10 mg/kg LPS onto the calvariae, and the other half received PBS as a vehicle control for LPS. The left incisor was extracted 4 days after the tooth cutting (on the day of the PBS or the LPS injections).

2.4. CIAM compound treatment

The LPS-treated and PBS-treated mice were divided into 3 groups each, depending on the dose of CIAM compound which was injected soon after tooth extraction. Intraperitoneal injections of 2 mg/kg/day or 4 mg/kg/day of the CIAM compound, or vehicle (10% DMSO, Vh) were carried out from the day of tooth extraction. The injections were performed once a day around 4 PM for 8 days. The six experimental groups were abbreviated as follows. 1) PBS and 10% DMSO-injected group [PBS/Vh], 2) PBS and 2 mg/kg/day CIAM-injected group [PBS/LD-CIAM], 3) PBS and 4 mg/kg/day CIAM-injected group [PBS/HD-CIAM], 4) LPS and 10% DMSO-injected group [LPS/Vh], 5) LPS and 2 mg/kg/day CIAM-injected group [LPS/LD-CIAM], 6) LPS and 4 mg/kg/day CIAM-injected group [LPS/HD-CIAM]. The mice were sacrificed on day 21 after tooth extraction.

To investigate the bone formation marker in serum, one more set of experiment using the same protocol shown above was performed, and the 54 mice (n = 9 per one group) were sacrificed on day 7 after tooth extraction. The blood serum was collected from the orbital vein under the anesthesia on the day of sacrifice.

2.5. Radiological assessment

After sacrificing the mice, the mandibles were dissected, and the soft tissue was roughly removed. The bone was fixed in a PBS-buffered glutaraldehyde (0.5%)-formalin (3.7%) solution (pH 7.4) for 2 days at 4 °C and washed with PBS for further study.

The mandibular bone was scanned by μCT (ScanXmate-E090; Comscan, Yokohama, Japan) and three-dimensional reconstruction images of the tooth socket in the mandible were obtained at the site of the mesial root of the first molar in a 300 μm thickness by using the image analyzing software program (TRI/3D-VIEW; Ratoc System Engineering, Tokyo, Japan). The region of interest (ROI) for analyzing the newly formed bone area to the total tissue area (total area in the tooth socket) ratio (NfB. Ar/Tt. Ar), the newly formed bone number (NfB. N), the newly formed bone thickness (NfB. Th), and the newly formed bone separation (NfB. Sp) was set inside the tooth socket of the reconstruction images. The equations used to calculate these structural parameters are shown in Table 1. All of the structural parameters used in this study were defined based on the definitions of trabecular bone (BV/TV), trabecular bone thickness (Tb. Th), trabecular bone number (Tb. N), and trabecular bone separation (Tb. Sp) (Parfitt et al., 1987).

The bone mineral density (BMD) was measured by peripheral quantitative computed tomography (pQCT, XCT Research SA+; Stratec Medizintechnik GmbH, Pforzheim, Germany) as described elsewhere (Nonaka et al., 2006; Tomomatsu et al., 2009). Briefly, 5 slices of the pQCT scanning were performed from the first slice, which was set at the mesial root of the first molar of the mandible (Fig. 3A), and another 4 slices of the scanning were performed every 0.25 mm toward the posterior region from the first slice. The ROI for measuring BMD were set as shown in Fig. 3B. The BMD of a sample was shown as the average of the 5 slices.

2.6. Histological preparation and bone histomorphometry

For *in vivo* fluorescent labeling, subcutaneous injections of calcein (20 mg/kg) were performed on days 3 and 13 after tooth extraction. Demeclocycline hydrochloride (20 mg/kg) was administered on days 8 and 18 after tooth extraction. For histological preparation, left mandibles were immersed in Villanueva's bone stain solution for 7 days after radiological assessments, and embedded in methyl methacrylate (MMA) as described elsewhere (Baron et al., 1983; Tomomatsu et al., 2009). Undecalcified grinding sections (10 μm) were prepared from the embedded bone blocks using an automicrotome (SP1600; Leica,

Table 1
Structural parameters in the region of the regenerated bone of the tooth socket.

Parameters	Abbreviations	Formula	Unit
Newly formed bone area/total tissue area	NfB. Ar/Tt. Ar	100× NfB. Ar/Tt. Ar	%
Newly formed bone thickness	NfB. Th	NfB. Ar/(NfB. Pm\/2)	μm
Newly formed bone number	NfB. N	(NfB. Ar/Tt. Ar)/NfB. Th	/mm
Newly formed bone separation	NfB. Sp	2× (Tt. Ar–NfB. Ar)/NfB. Pm\	μm

\ NfB. Pm: Newly formed bone perimeter.