Life Sciences 93 (2013) 423–428

Contents lists available at ScienceDirect

Life Sciences

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

Preventive and therapeutic anti-TNF-α therapy with pentoxifylline decreases arthritis and the associated periodontal co-morbidity in mice

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ARTICLE INFO

Article history:
Received 25 May 2013
Accepted 19 July 2013

Keywords:
Pentoxifylline
TNF-α
Arthritis
Periodontal disease
Periodontitis
Alveolar bone loss

ABSTRACT

Aims: The association between rheumatoid arthritis (RA) and periodontal disease (PD) has long been studied and some reports suggest that treating RA may improve the associated PD, and vice versa. This study aimed to evaluate the effects of an anti-tumor necrosis factor (TNF)-α therapy with pentoxifylline (PTX) in an experimental model of RA-associated PD.
Main methods: Male C57BL/6 mice were subjected to chronic antigen-induced arthritis (AIA) and daily treated with PTX (50 mg/kg, i.p.) using preventive (Pre-PTX) or therapeutic (The-PTX) strategies. Fourteen days after the antigen challenge, mice were euthanized and knee joints, maxillae and serum were collected for microscopic and/or immunoenzymatic analysis.
Key findings: AIA triggered significant leukocyte recruitment to the synovial cavity, tissue damage and proteoglycan loss in the knee joint. Pre-PTX and The-PTX regimens decreased these signs of joint inflammation. The increased levels of TNF-α and IL-17 in periarticular tissues of AIA mice were also reduced by both PTX treatments. Serum levels of C-reactive protein, which were augmented after AIA, were reduced by the PTX regimens. Concomitantly to AIA, mice presented alveolar bone loss, and recruitment of osteoclasts and neutrophils to periodontal tissues. Pre-PTX and The-PTX prevented and treated these signs of PD. PTX treatment also decreased TNF-α and increased IL-10 expression in the maxillae of AIA mice, although it did not affect the expression of IFN-γ and IL-17.
Significance: The current study shows the anti-inflammatory and bone protective effects of preventive and therapeutic PTX treatments, which decreased the joint damage triggered by AIA and the associated periodontal co-morbidity.

Introduction

The medical history suggests an association between rheumatoid arthritis (RA) and periodontal disease (PD) since the 19th century (reviewed in Persson, 2012). Thereafter, several clinical and experimental reports have shown a relationship for these two bone-resorbing, chronic inflammatory conditions. It has been shown that the likelihood of a RA patient to develop PD, and vice versa, is increased (Farquharson et al., 2012; Pablo et al., 2009; Park et al., 2011; Pischon et al. 2008; Trombone et al., 2010). Although there is no definite evidence for a causal theory to explain such an association, infectious-induced PD and auto-immune-mediated RA share a number of pathogenic aspects, as the cascade of inflammatory events that characterize the bursts of activity of both disorders. In this regard, the expression of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) is widely related to the pathogenesis of RA (McInnes and Schett, 2007) and PD (Garlet, 2010).

TNF-α amplifies the expression of other cytokines during inflammatory conditions (Cunha et al., 2005), activates osteoclasts, and promotes bone resorption, the hallmark of RA and PD (Garlet et al., 2006; Redlich et al., 2002). Indeed, the use of anti-TNF-α "biological agents" is among the gold therapies in the treatment of RA and anti-TNF-α antibodies favorably result in the relief of RA-associated PD (Mayer et al., 2009; Miranda et al., 2007; Pers et al., 2008; Queiroz-Junior et al., 2011, 2012). Despite such evidence, these drugs own important adverse effects related to secondary infections, potential risk for neoplasias, besides their high cost, which may limit their use in long-term therapies (Furst et al., 2003).

In this regard, pentoxifylline (PTX) is a well known TNF-α inhibitor. Originally used as a haemorheological drug to treat intermittent

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0024-3205/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.lfs.2013.07.022

claudication, with minor adverse effects (Ward and Clissold, 1987), PTX significantly reduces TNF-α and potentially increases IL-10 expression (Fernandes et al., 2008; Schmidt-Choudhrury et al., 1996; Van Furth et al., 1997; Zabel et al., 1993). These anti-inflammatory effects account for the benefits of PTX in the relief of experimental inflammatory pain (Vale et al., 2004), prevention of neutrophil migration (Bombini et al., 2004) and decrease in oxidative stress (Maiti et al., 2007). PTX also reduces the inflammatory joint damage triggered by adjuvant arthritis (Silva et al., 2000), the experimental signs of oral mucositis (Lima et al., 2005) and the alveolar bone loss induced by ligature in rats (Lima et al., 2004). Therefore, PTX may also be efficient in decreasing arthritis-associated co-morbidities, such as PD.

The aim of the current study was to evaluate the effects of preventive and therapeutic treatment strategies with PTX in an experimental model of arthritis-associated periodontal disease in mice.

Materials and methods

Mice

Forty 6-week-old male C57BL/6 mice, obtained from the Instituto de Ciências Biológicas at Universidade Federal de Minas Gerais, Brazil, were used in the current study. Mice were housed under standard laboratory conditions and had free access to commercial chow and water. All experiments were approved by and conducted according the guidelines of the local Institutional Committee for Animal Care and Use.

Chronic antigen-induced arthritis

In the current study, an experimental model of chronic antigen-induced arthritis (AIA) in mice was used. This model is known to be associated with clinical signs of periodontal disease without any manipulation of the oral microbiota (Queiroz-Junior et al., 2011, 2012). Briefly, mice were immunized twice with 100 μg mBSA (s.c.; Methylated Bovine Serum Albumin, Sigma-Aldrich, Saint Louis, MO, USA) in Freund's complete and incomplete adjuvant (Sigma–Aldrich), and 200 ng *Bordetella pertussis* toxin (i.p.; Calbiochem, La Jolla, CA, USA). Fourteen days after immunization, mice were challenged by intra-articular knee joint injection with mBSA (100 μg mBSA in 20 μL phosphate buffered saline, PBS) and, 30 days later, re-challenged with mBSA in the same knee joint to induce chronic AIA. The euthanasia was conducted 14 days later. Non-immunized and immunized mice joint-challenged with PBS did not present any differences in arthritis indices and periodontal parameters and, thus, constituted the Control group.

Pentoxifylline

The effects of pentoxyfylline (PTX; Sigma-Aldrich) in AIA and AIA-associated PD were evaluated using two treatment strategies: 1. Preventive (Pre-PTX, $n = 10$ mice), in which daily i.p. injections of 50 mg/kg PTX were performed from the fifth day before AIA joint re-challenge until the end of the experiment (14 days after re-challenge); 2. Therapeutic (The-PTX, $n = 10$ mice), in which daily i.p. injections of 50 mg/kg PTX were performed from the fifth day after AIA joint re-challenge until the end of the experiment. The dose of PTX used here was based on previous studies (Bombini et al., 2004; Lima et al., 2004, 2005). The vehicle group ($n = 10$ mice) comprised mice with chronic AIA injected daily with PBS (i.p.).

Evaluation of knee joints

Fourteen days after knee joint re-challenge with mBSA, five mice/group were euthanized and their knee cavities were washed with PBS (2 × 5 μL). The total number of leukocytes was counted in a Neubauer chamber. Differential counting was obtained from cytospin preparations (Shandon III, ThermoShandon, Frankfurt, Germany) stained with May–Grunwald–Giemsa. After PBS wash, the periarticular tissue was removed from the joint and used for immunoenzymatic assays (described below).

Knee joints of other 5 mice/group were also collected and histologically processed for routine H&E or toluidine blue (TB) staining. Two sections per knee joint were microscopically examined by a single examiner (CM Queiroz-Junior) and scored in a blind manner for different parameters: intensity and extension of inflammatory infiltrate, severity of synovial hyperplasia and bone erosion (arthritis index; range: 0–8). TB stained slides were used to estimate joint proteoglycan content using the Image J software (National Institute of Health, Bethesda, MD, USA) (Queiroz-Junior et al., 2011). Cartilage proteoglycan content is reported as the percentage of the TB-stained area *versus* the total evaluated cartilage.

Evaluation of maxillae

The maxillae of mice euthanized for knee joint evaluation were also collected, mechanically dissected and stained with 0.3% methylene blue for blinded quantification of alveolar bone loss (ABL). ABL comprised the area between the cement–enamel junction (CEJ) and the alveolar bone crest (ABC) in the 1st molar, measured using the Image J software (NIH). The maxillae were also collected and used for immunoenzymatic assays (described below) and for quantification of osteoclasts. To that end, the hemi-maxillae were processed for routine histological techniques and stained for tartarate resistant acid phosphatase (TRAP, Sigma–Aldrich). Three distinct sections were used for counting osteoclasts in the coronal region of the alveolar bone adjacent to the 1st molar in five consecutive microscopic fields (400×) per section. Samples were analyzed under a microscope Axioskop 40 (CarlZeiss, Göttingen, Germany) adapted to a digital camera (PowerShot A620, Canon, Tokyo, Japan).

Myeloperoxidase and N-acetylglucosaminidase

The neutrophil enzyme marker myeloperoxidase (MPO) and the macrophage enzyme marker N-acetylglucosaminidase (NAG) were quantified colorimetrically in whole maxillary specimens, which included upper molars, alveolar bone, buccal and palatal gingival tissues, and knee joint tissues, respectively, by enzymatic reactions as described earlier (Barcelos et al., 2005). The MPO activity was assessed in whole tissue maxillary samples, which were weighed, homogenized in phosphate buffer (0.1 M NaCl, 0.02 M Na_3PO_4, 0.015 M NaEDTA, pH4.7), and centrifuged (8946 g) at 4 °C for 10 min. The pellet was subjected to hypotonic lysis (0.2% NaCl solution for 30 s followed by addition of an equal volume of 1.6% NaCl and 5% glucose) and, after further centrifugation, it was resuspended in 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% hexa-1,6-bisdecyltrimethylammonium bromide (HTAB, Sigma Chemical Co., USA). The suspensions were freeze thawed thrice and centrifuged (8946 g) for 10 min at 4 °C. MPO activity was assayed in 25 μL of the resulting supernatant by incubating it (at 37 °C for 5 min) to 25 μL of 3,3-5,5-tetramethylbenzidine (TMB, Sigma Chemical Co., USA, 1.6 mM) in dimethylsulfoxide (DMSO, Merck, USA), and 100 μL H_2O_2 (0.003% (v/v)) in 0.5% HTAB. The reaction was terminated by adding 100 μL 1 M H_2SO_4 and was quantified colorimetrically at 450 nm in a spectrophotometer (SPECTRAmax PLUS-Molecular Devices). NAG activity was assessed in the periarticular joint tissues of mice, which were weighed, homogenized in phosphate buffer, centrifuged, subjected to hypotonic lysis and further centrifuged, similarly to the above described MPO protocol. Thereafter the pellet was resuspended in 0.9% saline containing 0.1% (v/v) Triton X-100 (Sigma–Aldrich) and further centrifuged. NAG activity was assayed in 100 μL of the resulting supernatant by incubating it (37 °C for 10 min) with 100 μL *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma Chemical Co.) in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4, pH 4.5). The reaction was terminated by adding 100 μL 0.2 M glycine