Bone 81 (2015) 683–690

Contents lists available at ScienceDirect

Bone

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

Original Full Length Article

Androgen receptors and experimental bone loss — an *in vivo* and *in vitro* study

Joao Paulo Steffens [a,b,c,*], Leila Santana Coimbra [a], Carlos Rossa Jr [d], Alpdogan Kantarci [b], Thomas E. Van Dyke [b], Luis Carlos Spolidorio [a]

[a] Department of Physiology and Pathology, Univ Estad Paulista — UNESP, School of Dentistry at Araraquara, 1680 Humaitá Street, 14801-903 Araraquara, SP, Brazil
[b] Department of Applied Oral Sciences, The Forsyth Institute, 245 First Street, 02142 Cambridge, MA, USA
[c] Department of Specific Formation, Universidade Federal Fluminse — UFF, School of Dentistry at Nova Friburgo, 22 Doutor Sílvio Henrique Braune Street, 28625-650 Nova Friburgo, RJ, Brazil
[d] Department of Diagnosis and Surgery, Univ Estad Paulista — UNESP, School of Dentistry at Araraquara, 1680 Humaitá Street, 14801-903 Araraquara, SP, Brazil

ARTICLE INFO

Article history:
Received 16 May 2015
Revised 18 September 2015
Accepted 2 October 2015
Available online 9 October 2015

Keywords:
Testosterone
Androgens
Receptors, androgen
Androgen receptor antagonists
Periodontitis
Bone and bones

ABSTRACT

Testosterone is a sex hormone that exhibits many functions beyond reproduction; one such function is the regulation of bone metabolism. The role played by androgen receptors during testosterone-mediated biological processes associated with bone metabolism is largely unknown. This study aims to use a periodontal disease model *in vivo* in order to assess the involvement of androgen receptors on microbial-induced inflammation and alveolar bone resorption in experimental bone loss. The impact of hormone deprivation was tested through both orchiectomy and chemical blockage of androgen receptor using flutamide (FLU). Additionally, the direct effect of exogenous testosterone, and the role of the androgen receptor, on osteoclastogenesis were investigated. Thirty male adult rats (n = 10/group) were subjected to: 1-orchiectomy (OCX); 2-OCX sham surgery; or 3-OCX sham surgery plus FLU, four weeks before the induction of experimental bone loss. Ten OCX sham-operated rats were not subjected to experimental bone loss and served as healthy controls. The rats were euthanized two weeks later, so as to assess bone resorption and the production of inflammatory cytokines in the gingival tissue and serum. In order to study the *in vitro* impact of testosterone, osteoclasts were differentiated from RAW264.7 cells and testosterone was added at increasing concentrations. Both OCX and FLU increased bone resorption, but OCX alone was observed to increase osteoclast count. IL-1β production was increased only in the gingival tissue of OCX animals, whereas FLU-treated animals presented a decreased expression of IL-6. Testosterone reduced the osteoclast formation in a dose-dependent manner, and significantly impacted the production of TNF-α; FLU partially reversed these actions. When taken together, our results indicate that testosterone modulates experimental bone loss, and that this action is mediated, at least in part, *via* the androgen receptor.

1. Introduction

Hypothalamic GnRH is responsible for regulating the release of interstitial cell-stimulating hormone in men, which in turn stimulates the production of testosterone [1]. Production of GnRH declines with age, lowering testosterone levels and indicating the closure of an individual's reproductive period [2]. Male hypogonadism, a condition associated with a reduced libido, loss of body hair, small or shrinking testes, low bone mineral density and other ill effects, is more likely to happen secondary to low serum total testosterone levels (generally less than 300 ng/dL) [3]. The prevalence of hypogonadism is variable; prevalence varies between 2.1–18.4%, with higher values for older individuals [4,5]. This suggests that aging has an impact on testosterone levels.

Declines in testosterone have consequences that extend beyond reproduction. Cross-sectional and prospective studies suggest that low testosterone levels are related to increased serum sIL-6r in older men; however, there remains some controversy about the effect of IL-6 on testosterone [6,7]. Testosterone replacement therapy in hypogonadal men has been seen to significantly decrease serum pro-inflammatory cytokines of IL-1β and TNF [8]. A cross-sectional study demonstrated that MIP-1α, MIP-1β and TNF are negatively associated with total testosterone in young men, suggesting that low testosterone levels

Abbreviations: GnRH, Gonadotropin-releasing hormone; sIL6r, Soluble interleukin-6 receptor; IL-6, Interleukin-6; IL-1β, Interleukin-1β; TNF, Tumor necrosis factor; MIP, Macrophage inflammatory protein; DHT, Dihydrotestosterone; GPR30, G-protein coupled receptor-30; Body weight, b.w.; ELISA, Enzyme-linked immunosorbent assays; RANKL, Receptor Activator of nuclear factor kappa-B ligand; microCT, Micro-computed tomography; ROI, region of interest; BV/TV, Bone volume fraction; EDTA, Ethylenediamine tetraacetic acid; FBS, Fetal bovine serum; RANTES, Regulated on activation normal T cells expressed and secreted; CEJ, Cement–enamel junction; TRAP, Tartrate-resistant acid phosphatase; TIMPs, Tissue inhibitor of metalloproteinases; LPS, Lipopolysaccharyde; IL-1ra, Interleukin-1 receptor antagonist.

* Corresponding Author at: Rua Humaita, 1680, Araraquara, SP 14801-903, Brazil.
E-mail address: joaopaulosteffens@hotmail.com (J.P. Steffens).

http://dx.doi.org/10.1016/j.bone.2015.10.001
8756-3282/

may also promote a pro-inflammatory status independent of aging [9]. These findings highlight the role male hormones play in inflammatory responses.

Research suggests that testosterone may impact bone tissues and cells [10–14]. Testosterone can act directly, through binding to the androgen receptor, or indirectly, by its conversion to a more potent hormone, DHT. Testosterone can also be converted to estradiol by the aromatase enzyme. Many of the effects testosterone has on bone tissues have been attributed to its conversion to estradiol, which acts through estrogen receptors α and β, and possibly GPR30 [15–17]. Other studies, however, support the direct role of the androgen receptor, or an estrogen-independent participation of testosterone on bone turnover and regulation [10,18,19].

Periodontitis is a microbial-mediated chronic inflammatory disease affecting the supporting structures of teeth. Periodontitis is characterized by an overproduction of innate immune cytokines, such as IL-1β, IL-6, and TNF [20]. This overproduction leads to tissue breakdown and the consequent resorption of the alveolar process, a bone structure consisting of outer cortical plates of compact bone, a central spongiosa (trabecular bone), and bone lining the alveolus (alveolar bone) [21]. Our previous studies suggest that both low and high testosterone levels regulate experimental bone loss in rats [13,14]. This study tests the hypothesis that testosterone suppression increases inflammation-induced bone loss through the reduction of androgen receptor activation. This hypothesis is tested through the use of a model of experimental periodontitis in rats. The objectives of this study were as follows: to compare the impact of orchiectomy with that of chemical inhibition of androgen receptor *in vivo* through the assessment of associated bone markers and cytokine expression, and to investigate the impact of testosterone and its associated androgen receptor activation on osteoclastogenesis *in vitro*.

2. Materials and methods

2.1. Animals

Forty male adult Holtzman rats weighing 300–400 g (approximately 3 months old) were kept in cages under similar conditions (controlled temperature 23 ± 2°°C, humidity 65–75% and 12-h light–dark cycles). Food and water were provided *ad libitum*. Randomization of animals was performed using a raffle method. All experimental protocols were approved by the Institutional Ethics Committee for Animal Experimentation (protocol #25/2010) and performed in accordance with the guidelines of the Brazilian Society of Science on Laboratory Animals (SBCAL). This study conforms to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

2.2. Androgen deprivation model

After 1 week of acclimatization, ten rats received orchiectomy to suppress testosterone production. Briefly, a scrotal incision was performed for bilateral testicular removal and the incision was sutured under anesthesia using ketamine (1 mL/kg b.w.) and xylazine (0.4 mL/kg) under sterile conditions. The rats were given acetaminophen (300 mg/kg; orally) for postoperative pain relief and an intramuscular dose of penicillin and streptomycin (1 mL/kg). After the procedure, the animals were kept in separate cages for recovery for 7 days. Thirty other rats received the same surgical procedure, except for the testicular removal (sham operation).

2.3. Chemical inhibition of androgen receptors

Ten sham-operated rats received flutamide (Sigma-Aldrich, Saint Louis, MO, USA; 50 mg/kg), an androgen receptor antagonist, which was administered intragastrically every other day, until sacrifice. Flutamide was diluted to 25 mg/mL in distilled water and Tween-20. The treatment was started three days following sham surgery [22].

2.4. Induction of experimental periodontal bone loss

Four weeks after orchiectomy (or sham surgery), 30 rats (n = 10/group) were anesthetized as described above. A 3.0 cotton ligature was placed in a subgingival position around the lower first molar tooth to enable bacterial accumulation, leading to inflammation and bone loss. The other 10 sham-operated animals did not receive ligatures and served as healthy controls. The ligatures were maintained for 2 weeks at which time all rats were euthanized.

2.5. Assessment of testosterone and local/systemic markers

A blood sample was collected from every animal at the end of the experiment. After clotting for 45 min at room temperature, the sample was centrifuged for 20 min at 20,000 × *g* to obtain serum. Each serum sample was analyzed for total testosterone levels using a chemiluminescence-based immunoassay (Immulite 2000, Diagnostic Products Corporation, Gwynedd, UK); and for the detection of estradiol, IL-1β and IL-6 using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

The mucogingival tissues around the first molars of 5 animals per group were removed and processed for concentrations of IL-1β and IL-6 using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions. Total protein was extracted in T-Per lysis buffer (Pierce, Thermo Scientific, Rockford, IL, USA) supplemented with proteinase inhibitors (cOmplete, Roche Diagnostics, Mannheim, Germany) and determined using the Bradford method. The results were used for data normalization.

2.6. microCT analyses

For quantitative three-dimensional analysis of the alveolar bone, mandibles of 5 animals per group were scanned using a microCT system (Skyscan, Aartselaar, Belgium). The specimens were scanned at a resolution of 18 μm in all three spatial dimensions. CTan/CTvol software (Skyscan) was used for imaging and analysis. A standardized rectangular ROI measuring 1.13 × 0.97 mm was positioned at the furcation area (region among the roots of the teeth), in a slice-based method. The histogram settings were standardized at 90–130 and 101 serial slices were selected in each sample. Bone volume fraction was analyzed by the CT-scan software [14].

The linear distance between CEJ, an anatomical reference on the tooth, and alveolar bone crest was measured in the mesial surface of the first molars using software (Dataviewer 1.4.3, Skyscan). The measurement was performed three times by a calibrated individual (JPS) who was blinded to the treatment groups under the same background conditions. The mean of all three measurements was considered one sample and used for statistical analysis [16].

2.7. Histologic analysis

After the rats were euthanized, 5 hemi-mandibles per group were used for histologic analysis. The samples were fixed in 10% formalin for 48 h and demineralized for 10 h using a rapid demineralization chemical agent containing EDTA, sodium and potassium tartrate, chloric acid and deionized water (Alkimia, Campinas, SP, Brazil). Serial sections (5 μm) were obtained in a buccal-lingual orientation and the slides were stained using hematoxylin and eosin or TRAP-staining kit (Sigma-Aldrich), according to the manufacturer's instructions.

In order to perform stereometric analysis, a 1000 × 500 μm grid was positioned vertically upon the connective tissue in histologic images (200× magnification), with its lower border in contact with the alveolar bone crest and the vertical border in contact with the root cementum.