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Low testosterone elevates interleukin family cytokines in a rodent model: a possible mechanism for the potentiation of vascular disease in androgen-deficient males

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ABSTRACT

Background: Androgen deficiency (AD) is associated with increased risk of atherosclerosis, cardiovascular, and peripheral arterial disease. Although the biochemical and molecular mechanisms underlying this risk remain unclear, higher testosterone (TST) levels correlate to significant immunoprotective molecular and cellular responses. Our group has previously demonstrated that female sex hormones influence vascular pathogenesis *via* inflammatory-modulated matrix metalloproteinase (MMP) regulation. Here we investigated the role of AD and androgen replacement therapy in the modulation of these hormonally responsive pathways that could be playing a role in the development of vascular pathogenesis.

Methods: Aged orchiectomized male rats underwent TST supplementation per controlled release pellet implantation (0–150 mg). Young and aged intact groups served as controls. Serum was collected at 0–4 wk and analyzed by enzyme-linked immunosorbent assays, qualitative cytokine screening, and quantitative multiplex analyses. Human aortic smooth muscle cells were treated with 4,5α-dihydrotestosterone (DHT; 0–3000 nM) before or after interleukin 1β (IL-1β; 5 ng/mL) stimulation. Quantitative polymerase chain reaction and in-gel zymography was used to assay the effect on MMP expression and activity.

Results: Subphysiological, physiological, and supraphysiological levels of TST were achieved with 0.5, 2.5, and 35 mg TST pellet implants *in vivo*, respectively. Inflammatory arrays indicated that interleukin cytokines, specifically IL-2, IL-6, IL-10, IL-12, and IL-13, were elevated at subphysiological level of TST, whereas TST supplementation decreased interleukins. Supraphysiological TST resulted in a significant increase in MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) *in vivo*. Pretreatment with IL-1β slightly increased membrane type 1-MMP (MT1-MMP) and MMP-2 expression at low to mid-level DHT exposure *in vitro*, although these trends were not statistically significant.

Conclusions: Here we demonstrate AD is a proinflammatory modulator and indicate that MMP-independent mechanisms may play a role downstream of AD-induced inflammatory

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signaling in dysfunctional vascular remodeling. Future *in vivo* studies will examine AD and TST supplementation in acute inflammatory response to vascular injury and in MMP-modulated vascular disease.

1. Introduction

Androgen deficiency (AD) is an increasingly prevalent diagnosis among today's aging male population. Androgen replacement therapy (ART) in patients with idiopathic AD has seen growing popularity with the advent of multiple exogenous replacement forms and is largely aimed at health maintenance and improving quality of life. However, we may underappreciate the benefits of ART in AD subjects regarding cardiovascular and vascular disease [1–5]. Likewise, we may not fully appreciate the consequences of androgen deprivation therapy routinely used in the treatment of patients with high-risk prostate cancer [6].

Regardless of whether a physiological consequence of primary testicular failure or a result of a medical or surgical intervention in the management of prostate cancer, AD has been associated with an increased risk of atherosclerosis, cardiovascular, and peripheral arterial disease [7–11]. Peripheral arterial disease is a major risk factor for cardiovascular and cerebral ischemic events, is often debilitating with claudication and loss of mobility, and can ultimately lead to limb loss. In spite of the vast magnitude of clinical and epidemiologic research investigating their correlation, the molecular and biochemical mechanisms underlying the role of AD in vascular disease remain unclear.

Both clinical and experimental studies have shown AD is associated with various autoimmune diseases [12–14] and that higher testosterone (TST) levels correlate to significant immunoprotective molecular and cellular responses [15]. Additionally, low TST levels have been linked to increased inflammatory markers in hypogonadal men [16]. It is well established that cytokines, growth factors, stress, and inflammation affect the regulation of matrix metalloproteinases (MMPs), enzymes largely responsible for vascular remodeling [17–20]. Others and we have shown that female sex hormones influence vascular pathogenesis *via* inflammatory-modulated signaling and that there is a positive correlation with dysfunctional MMP regulation [21–26]. Additionally, we have demonstrated a role for TST in the modulation of these MMP regulatory mechanisms and in the cellular processes of hyperplasia development *in vitro* [27]. Here we aim to identify other critical mechanisms modulated by AD that could be playing a role in the development of vascular pathogenesis. Our working hypothesis is that AD is acting as a proinflammatory modulator contributing to dysfunctional vascular remodeling.

2. Methods

2.1. Animal groups, induction of AD, and ART experimental design

Male Sprague–Dawley rats, beginning the study at the age of 8–10 mo (weighing 425–525 g) to best represent the older male population affected by the pathology of this study, were orchiectomized when obtained from Charles River Laboratories, Inc (Wilmington, MA). After 4 wk of hormone deficiency, each aged orchiectomized rat was randomly assigned to one of seven treatment groups with $n = 6$. Subcutaneous insertion of a 90-d slow-release water-soluble placebo pellet (Plac; 10 mg) or TST pellet (0.5, 2.5, 10, 35, 75, or 150 mg) was performed. ART by the sustained release pellets continued for 4 wk. An aged group with their testicles intact served as the orchiectomized negative control (aged intact [AI]) with no pellet placement. A group of young rats at the age of 1–3 mo (weighing 400–425 g) with their testicles intact, served as the young negative control (young intact [YI]) with no pellet placement. Pellets were obtained from Innovative Research of America, Inc (Sarasota, FL). All animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee, certified by the American Association of Accreditation of Laboratory Animal Care.

2.2. Blood and tissue collection

Five hundred microliters of whole blood was collected at the time of pellet implantation (baseline) and at 2 d, 1 wk, 2 wk, and 4 wk of therapy. The blood was centrifuged at 1500*g* for 5 min, serum aliquoted, and frozen at −80°C until analysis. After 4 wk of ART, animals were euthanized by CO_2 overdose. Prostate and seminal vesicles were collected at the time of euthanization and their weights were recorded as an indicator of systemic hormone exposure.

2.3. Androgen and inflammatory cytokine immunoassays

Serum TST levels were measured in duplicate by a rat-specific sandwich enzyme-linked immunosorbent assay (ELISA; DRG Diagnostics, Germany) to assay baseline and circulating TST in time interval dose response to ART conditions. Serum levels of inflammatory cytokines were measured by qualitative cytokine screening arrays and quantitative multiplex analyses after 2 wk of ART. Cytokine Rat Membrane Antibody Arrays (Abcam, Cambridge, UK) were used to qualitatively screen 34 cytokine serum antigens. Serum aliquots (25 μL) from each experimental sample were pooled together by treatment group and ran in batch on independent membranes according to the manufacturer's instructions. Milliplex Rat Cytokine and Chemokine Magnetic Bead Panel (EMD Millipore Corp, Billerica, MA) was used to quantitatively measure 27 cytokine serum antigens using the Luminex 200 multiplexing immunoassay system (Life Technologies Corp, Carlsbad, CA). Serum was diluted 1:2 and ran independently in duplicate according to the manufacturer's instructions. Rat-specific Quantikine ELISA kits were purchased commercially for MMP-2, MMP-9, and tissue inhibitor of