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Influence of testosterone and a novel SARM on gene expression in whole blood of *Macaca fascicularis*☆

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ABSTRACT

Anabolic hormones, including testosterone, have been suggested as a therapy for aging-related conditions, such as osteoporosis and sarcopenia. These therapies are sometimes associated with severe androgenic side effects. A promising alternative to testosterone replacement therapy are selective androgen receptor modulators (SARMs). SARMs have the potential to mimic the desirable central and peripheral androgenic anabolic effects of testosterone without having its side effects.

In this study we evaluated the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood in an non-human model. The regulated genes can act as potential blood biomarker candidates in future studies with AR ligands.

Cynomolgus monkeys (*Macaca fascicularis*) were treated either with testosterone or LGD2941 for 90 days in order to compare their effects on mRNA expression in blood. Blood samples were taken before SARM application, on day 16 and on day 90 of treatment.

Gene expression of 37 candidate genes was measured using quantitative real-time RT-PCR (qRT-PCR) technology.

Our study shows that both testosterone and LGD2941 influence mRNA expression of 6 selected genes out of 37 in whole blood. The apoptosis regulators CD30L, Fas, TNFR1 and TNFR2 and the interleukins IL-12B and IL-15 showed significant changes in gene expression between control and the treatment groups and represent potential biomarkers for androgen receptor ligands in whole blood.

1. Introduction

Over the last decades the proportion of elderly people in the population has increased [1]. This is the reason why the incidence of age-related conditions like sarcopenia and osteoporosis is rising and becoming one of the major topics in health care. Sarcopenia is the loss of muscle mass during the aging process that may lead to frailty [2–5]. Sarcopenia is commonly associated with osteoporosis, which is the age-related loss of bone mineral density. The combination of sarcopenia and osteoporosis results in a high incidence of bone fractures relating to accidental falls, which is a significant cause of morbidity and mortality in the elderly population.

Both conditions are associated with a decrease in the endogen production of anabolic hormones, including testosterone [4]. Testosterone treatment has been proposed as a therapy for osteoporosis and frailty in both men and women [6,7]. However, the androgen therapies available today are associated with androgenic side effects, such as skin virilization in women and prostate hypertrophy in men [8–10].

A promising alternative for testosterone replacement therapy is the development of selective androgen receptor modulators (SARMs) [6]. SARMs are synthetic molecules that bind to the androgen receptor exhibiting tissue-selective effects. An “ideal” SARM is an orally active compound that provides an increase in muscle mass and strength and has an anabolic effect on bone density without inducing undesirable androgenic side effects [6]. LGD2941 is a novel non-steroidal, orally active SARM, which has shown potent anabolic activity on bone and muscle in rats and monkeys, but reduced effects on the prostate [7].

It is already known that androgens cause changes in the biochemical pathways of different organs and tissues. Specific enzymes, receptors and cytokines can be activated or suppressed on the cellular mRNA expression level. Using appropriate specific and sensitive quantification methods, like quantitative real-time RT-PCR, such mRNA expression changes are measurable.

The aim of this study was to evaluate the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood samples. Whole blood is chosen because samples can easily been taken from the living organism. Furthermore there is evidence in the literature that androgens affect gene

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expression of the different blood cells. The regulated genes have the potential to act as blood biomarkers in future studies with AR ligands.

2. Materials and methods

2.1. Animal experiment

24 male cynomolgus monkeys (*Macaca fascicularis*) were separated to four groups of six animals each. All animals were 5–6 years old, skeletally mature and had an average body weight of 6± kg. The treatments were group 1 (control or oral vehicle group), group 2 (reference group, testosterone group) 3.0 mg/kg Testosteronenanthate as Testoviron®-depot-250 (Schering, Berlin, Germany), dosed biweekly by intramuscular injection, group 3 (intermediate concentration group, SARM1) 1 mg/kg SARM LGD2941 daily and group 4 (high concentration group, SARM10) 10 mg/kg SARM LGD2941 daily. The oral vehicle control and the SARM were dosed once daily for 90 days.

Whole blood samples were taken at three time points. Predose samples were taken after study start without prior treatment. Further samples were taken at day 16 and day 90 of treatment. Duplicate blood samples (2.5 mL each) were transferred into PAXgene blood RNA tubes (BD, Heidelberg, Germany) gently shaken, incubated at room temperature for two hours and stored at −20 °C.

The animal attendance and blood sampling were done by Covance Laboratories GmbH (Münster, Germany) and was conducted with permission from the local veterinary authorities and in accordance with accepted standards of Humane Animal Care.

2.2. RNA preparation and qRT-PCR

RNA from blood samples was extracted using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To quantify the amount of total RNA extracted, optical density (OD) was measured with the Biophotometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. RNA purity was calculated with the $OD_{260/280}$ ratio.

RNA integrity and quality control was performed via capillary electrophoresis in the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Eukaryotic total RNA Nano Assay (Agilent Technology) was taken for sample analysis and the RNA Integrity Number (RIN) served as RNA quality parameter. Agilent Bioanalyzer 2100 calculated the RIN value based on a numbering system from 1 to 10 (1 being the most degraded profile, 10 being the most intact) for all samples. A RIN ≥ 6 should be achieved to assure good results in qRT-PCR [11,12].

Candidate genes were chosen by screening the respective literature for androgen and inflammation-related effects on blood cells. Their expression was investigated using listed primers (Table 1). All primers were designed using published human nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer design and optimization was done with primer design program of MWG Biotech (MWG, Ebersberg, Germany) and primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with regard to primer dimer formation, self-priming formation and a constant primer annealing temperature of 60 °C. Newly designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany) or Invitrogen (Karlsruhe, Germany). Primer performance testing was done with six optional untreated samples and a no template control (NTC contains only RNAse free water) for each primer set.

Quantitative real-time RT-PCR was performed using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA) by a standard protocol, recommended by the manufacturer. With the kit the master mix was prepared as follows: for one sample it is 5 μL 2× SYBR Green Reaction Mix, 0.5 μL forward primer (10 pmol/μL), 0.5 μL reverse primer (10 pmol/μL) and 0.2 μL SYBR Green One-Step Enzyme Mix (Invitrogen, Carlsbad, USA). 6.2 μL of the master mix was filled in the special 100 μL tubes and 3.8 μL RNA (concentration 1 ng/μL respectively 10 ng/μL) was added. Tubes were closed, placed into the Rotor-Gene 3000 and Analysis Software v6.0 was started (Corbett Life Science, Sydney, Australia). The following one-step qRT-PCR temperature cycling program was used for all genes: Reverse transcription took place at 55 °C for 10 min. After 5 min of denaturation at 95 °C, 40 cycles of real-time PCR with 3-segment amplification were performed consisting of 15 s at 95 °C for denaturation, 30 s at primer dependent temperature for annealing and 20 s at 68 °C for polymerase elongation. The melting step was then performed with slow heating starting at 60 °C with a rate of 0.5 °C per second up to 95 °C with continuous measurement of fluorescence.

Take off points (Ct) and melting curves were acquired by using the "*Comparative quantitation*" and "*Melting curve*" program of the Rotor-Gene 3000 Analysis software v6.0. Only genes with melting curves showing a single peak and no primer dimers were taken for further data analysis. Samples that showed irregular melting peaks were excluded from the quantification procedure.

2.3. Selection of target genes

Candidate genes that might be biomarkers in blood were chosen by screening the respective literature for androgen and inflammation-related effects on blood cells. Androgens are known to down-regulate proliferation of lymphocytes [13,14]. Therefore the different pro- and anti-inflammatory interleukins (IL) IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12B, IL-13 and IL-15 and the growth factors tumor growth factor β (TGF-β), insulin growth factor 1 receptor (IGF-1R) were selected for analysis. It was already shown that testosterone influences the rate of apoptotic blood cells [15–17]. Therefore different apoptosis regulators were chosen for analysis: the TNF receptor superfamily member 6 (Fas), its ligand FasL, tumor necrosis factor receptor (TNFR) 1 and 2, their ligand tumor necrosis factor α (TNF-α), B-cell CLL/lymphoma 2 (BCL-2), BCL2-like 1 (BCL-XL), Caspase 3 (Casp 3), Caspase 8 (Casp 8), CD30 Ligand (CD30L), the inflammatory factor nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFκB) and its inhibitor IκB. To determine if the treatment also has an influence on the amount of the different white blood cells, the expression of the cell specific CD Antigens CD4 (T helper cells), CD8 (cytotoxic T cells), CD11b (granulocytes), CD14 (monocytes), CD20 (B-cells), CD25 (activated T cells) and CD69 were measured. Further leukocyte genes that were measured are androgen receptor (AR), tumor necrosis factor β (TNF-β) and CD27 Ligand (CD27L). As genes expressed in reticulocytes, haemoglobin alpha (α-globin), haemoglobin beta (β-globin) and their transcription factors and stabilization factors transcription factor CP2 (CP2), acid phosphatase 1 (αCP1) and upstream transcription factor 1 (USF-1) were chosen. As reference gene candidates β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured, whereas β-Actin and GAPDH were chosen as best reference genes by using GenEx Ver 4.3.3 Software (multiD Analyses AB, Gothenburg, Sweden).

2.4. Data analysis and statistics

Statistical description of the expression data as well as statistical tests were produced with SAS v. 9.1.3 for Windows. The raw data were the Ct values obtained from each qPCR sample. Each qRT-PCR sample was associated with a blood sample whereas for each experimental animal two blood samples were analysed. Since the