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Doxycycline affects gene expression profiles in aortic tissues in a rat model of vascular calcification

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ABSTRACT

Vitamin D_3-induced vascular calcification (VC) in rats shares many phenotypical similarities with calcification occurring in human atherosclerosis, diabetes mellitus and chronic kidney disease, thereby it is a reliable model for identifying chemopreventive agents. Doxycycline has been shown to effectively attenuated VC. This study aimed to explore the effects of doxycycline on gene expression profiles in VC rats. The model of VC in rats was established by subcutaneous injection of vitamin D3 for 3 days. Doxycycline at 120 mg kg^{-1} day^{-1} was given via subcutaneous injection for 14 days. Rat pathological changes, calcium deposition and calcium content in aortic tissues were measured by Hematoxylin–eosin, von Kossa staining and colorimetry, respectively. The gene change profile of aortic tissues after doxycycline treatment was assessed by Gene Microarray analysis using the Agilent Whole Rat Genome Oligo Microarray. The results showed that doxycycline significantly decreased the deposition of calcium, reduced the relative calcification area and alleviated pathological injury in aortic tissues. In addition, doxycycline treatment altered 88 gene expressions compared with untreated VD group. Of these, 61 genes were down-regulated and 27 genes were up-regulated. The functions of differentially expressed (DE) genes were involved in neutrophil chemotaxis, chronic inflammatory response, negative regulation of apoptotic process, cellular response to mechanical stimulus and immune response, etc. In conclusions, this study might provide the potential novel insights into the molecular mechanisms of doxycycline on VC.

1. Introduction

Vascular calcification (VC) is often associated with the common pathological phenomenon in patients with atherosclerosis, diabetes mellitus and chronic kidney disease, especially those with end-stage renal disease on hemodialysis (Chistiakov et al., 2014; Kraus et al., 2015; Tolle et al., 2015). VC is also thought to be a key contributor to cardiovascular morbidity and mortality, and calcium deposits are highly common in advanced atherosclerotic plaque lesions (Rennenberg et al., 2009). VC means the deposition of calcium phosphate in the form of apatite in the media of arteries and typically associated with elastin. It leads to increase in arterial stiffness, which in turn increases systolic blood pressure and decreases diastolic blood pressure, thereby increase afterload favoring left ventricular hypertrophy and compromise coronary perfusion, ultimately resulting in hypertension, aortic stenosis, cardiac hypertrophy and congestive heart failure (Demer and Tintut, 2008). Therefore, a better understanding of the mechanisms controlling VC is required to address the burgeoning unmet clinical needs of these diseases.

Previously considered the passive deposition of calcium (Ca)-phosphate (Pi) crystals, VC is now regarded as an active cell-mediated and highly regulated process of complex pathology, involving the reprogramming and transdifferentiation of vascular smooth muscle cells (VSMCs) to osteoblast-like cells, apoptosis of VSMCs, loss of osteogenic inhibition, matrix vesicle release, and extracellular matrix degradation (Chistiakov et al., 2013; Dai et al., 2013; Johnson et al., 2006; Leopold, 2015; Shroff et al., 2008). However, the precise mechanism of vascular calcification has not yet been well elucidated.

Evidence is mounting that matrix metalloproteinases (MMPs) and elastin degradation are implicated in the VC process. MMPs are a large group of zinc-dependent endopeptidases. It has been thought that MMPs function chiefly in the degradation of extracellular matrix molecules (Galis and Khatri, 2002). However, it is now clear that MMPs may also liberate bioactive fragments from extracellular matrix molecules and, through these effects, influence cellular behavior. One such molecule is elastin–a key constituent of the extracellular matrix in elastic arteries (Mott and Werb, 2004). Elastin degradation is observed in almost all types of VC (Proudfoot and Shanahan, 2001), and is commonly associated with MMPs activities. The contribution of MMPs to disease conditions justified the search for MMP inhibitors. Doxycycline is a well-known tetracycline class antibiotic and apart from its antibiotic properties, it also has been found to have inhibitory effects on the

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MMPs (Cena et al., 2010; Fiotti et al., 2009). Our previous study has shown that doxycycline can significantly attenuated VC both in vivo and in vitro (Qin et al., 2006). Bouvet et al. demonstrated concordant findings that blocking MMP synthesis and activity with doxycycline prevents calcification in a rat model of medial elastocalcinosis caused by warfarin treatment (Bouvet et al., 2008). However, the mechanism that underlies the beneficial effects of doxycycline treatment in VC is still obscure. Therefore, to gain additional insight into the functional role of doxycycline treatment in VC, in the present study, we explored global gene expression changes in the aorta tissues of VC rats undergoing 2 weeks of doxycycline treatment by using microarray methodologies. Our aim was to perform a comprehensive analysis of changes induced by doxycycline treatment, which may reveal new targets for the molecular mechanisms underlying doxycycline action.

2. Materials and methods

2.1. Animals

The Animal Care and Use Committee of Guangxi Medical University approved all protocols in this experiment. Seven-week-old, male Sprague–Dawley (SD) rats were purchased from the Experiment Animal Center of Guangxi Medical University and housed under the following conditions: 12 h/12 h light/dark photocycle and 50% to 70% humidity, Food and water were supplied ad libitum. For animal adaptability, all experimental animals had been fed for one week before the experiment.

2.2. Main reagents

Vitamin D_3 (cholecalciferol), doxycycline and Olive oil were purchased from Sigma Aldrich (St. Louis, MO, USA). Trizol reagent, the first-strand cDNA synthesis kit and SYBR Green Master Mix were obtained from Invitrogen, Co. Takara and Roche Ltd., respectively. Low Input Quick Amp Labeling Kit, Agilent Whole Rat Genome Oligo Microarray (4 × 44 K), Gene Expression Hybridization Kit and Gene Expression Wash Buffer Kit came from Agilent technologies Inc. (Santa Clara, US).

2.3. Induction of vascular calcification in rats and doxycycline treatment

The rats were divided randomly into 3 groups ($n = 10$ for each group): Control group (Con group), Vitamin D3-induced model group (VD group) and doxycycline treatment group (VD + Dox group). In order to induce rat model of VC, the VD and VD + Dox groups were given subcutaneous injection of Vitamin D_3 (600,000 IU/kg) once daily from day 1 to day 3. The Con group underwent the same procedure, except that animals received subcutaneous injection of D5W. Vitamin D_3 solution for injection was prepared as described by Price (Price et al., 2000). A stock solution was made by dissolving 100 mg vitamin D_3 in 600 μL ethanol and suspended in 5.2 mL of Olive oil before diluting it to 1.65 mg/mL (66,000 IU/mL) in 60 mL D5W. Meanwhile, VD + Dox group were additionally received doxycycline (120 mg/kg, dissolved in 0.9% physiological saline) subcutaneous injection once daily for 2 weeks. The dosage and duration of doxycycline administration were calculated considering the maximum therapeutic dose in humans and based on our previous study (Qin et al., 2006). The Con and the VD group were injected in the same way with saline as a vehicle control. All rats were killed by exsanguination while under general anesthesia (intraperitoneal sodium thiopental) on day 15 and the aorta from the arch to the iliac bifurcation was harvested for further evaluation. Some fresh aortic tissues were maintained immediately in liquid nitrogen, and others were fixed in formalin solution for pathological analysis.

2.4. Histopathological examination

In order to insure consistency, the infrarenal aorta was used routinely for histologic evaluation. The aorta was fixed with 10% neutral buffered formalin for 24 h, then embedded in paraffin, cut into serial sections of 3-μm, deparaffinized and stained by hematoxylin-eosin (HE) and von Kossa method. Von Kossa staining was used to observe calcium deposits in aorta. Briefly, sections were immersed in 1% silver nitrate for 1 h under an intense sunbeam, and were then washed 3 times with deionized water. Subsequently, 5% sodium thiosulfate was added for 5 min to remove un-reacted silver, finally sections were counterstained with hematoxylin. Slides were observed under a microscope (Olympus BX53F, JPN) by 2 investigators blinded to treatment conditions.

2.5. Calcium content measurements

The thoracic aortic segments were first dried at 55 °C and weighed, then dissolved in HNO_3, dried in an oven and re-dissolved with a blank solution (27 nmol/L KCl, 27 μmol/L $LaCl_3$, in deionized water). Calcium levels were determined by colorimetry through a reaction with o-cresolphthalein complexon. The data were normalized to aortic tissue dry weight.

2.6. Microarray experiment

RNA extraction and gene array preparation were commercially outsourced to Shanghai Southgene Technology Co., Ltd. (Shanghai, China). Abdominal aortic tissues samples of VD group and VD + Dox group ($n = 20$) were shipped on dry ice. Total RNA was extracted from aortic tissues samples using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was quantified by measuring absorbance ratio (A260/A280) using a Spectrophotometer (NanoDrop-2000, Technologies, USA). Its quality and integrity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) and 1% agarose gel electrophoresis.

The RNA samples were amplified and transcribed into fluorescent cRNA using the Low Input Quick Amp Labeling Kit (Agilent, p/n 5190-2305) following the manufacturer's protocol. Then the labeled cRNA from each sample was hybridized to Agilent Whole Rat Genome Oligo Microarray (Agilent, 4 × 44 K) using the Gene Expression Hybridization Kit (Agilent, p/n 5188-5242). Briefly, 1.65 μg Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight (17 h, 65 °C) to Agilent Whole Rat Genome Oligo Microarray using Agilent recommended hybridization chamber (Agilent, p/n G2534A) and oven (Agilent, p/n G2545A). After hybridization and washing, fluorescence signals of the hybridized Agilent microarrays were detected using an Agilent microarray scanner (Agilent, p/n G2565BA), the scanned images were analyzed by Agilent Feature Extraction software (version 10.7).

2.7. Data analysis

Quantile controlization and subsequent data processing used GeneSpring Software 12.6.1 package (Agilent Technologies). Gene expression profiles of VD group were compared to those from VD + Dox group. Differentially expressed genes were identified by fold-change, *t*-test and *P*-value screening between the two groups. The changing was selected by the criteria: $P < 0.05$ and fold changes ≥ 2 or ≤0.5. The roles of differentially regulated genes were identified by KEGG Pathway and GO Analysis.

2.8. qRT-PCR validation

To validate microarray data, the expression patterns of several genes of interest (e.g. S100a8 and Hspa1a) were determined by qRT-PCR analysis. The first strand cDNA was synthesized using Prime Script RT