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Research paper

Effect of glucocorticoids on miRNA expression spectrum of rat femoral head microcirculation endothelial cells

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ABSTRACT

The study profiled the differential miRNA expression from femoral head bone microvascular endothelial cells (BMECs) between model group and control group to explore the pathogenesis of steroid-induced osteonecrosis of femoral head (ONFH). Twenty 8-week-old Female Sprague-Dawley (SD) rats were randomly divided into control and model groups. Rats in model group received an intraperitoneal injection of 20-μg/kg lipopolysaccharide (LPS) at an interval of 24 h. Then, 24 h later, rats received three doses of 40-mg/kg methylprednisolone by intramuscular injection at intervals of 24 h. In control group, rats received the same volume of normal saline. After 4 weeks, the femoral heads were sectioned to confirm the establishment of the model. To replicate the animal model *ex vivo*, BMECs were isolated. Different miRNAs were screened using Agilent Gene Spring GX software, and real-time quantitative polymerase chain reaction (qPCR) was used to confirm the results of miRNA microarray analysis. The differentially expressed miRNA were assessed by bioinformatics analysis. Four differentially expressed miRNAs were identified (two upregulated: miR-132-3p, miR-335 and two down regulated: miR-466b-2-3p, let-7c-1-3p). qPCR results were consistent with the gene-chip results. Steroid-induced ONFH may cause miRNA changes in BMSCs. miR-132-3p and miR-335 may be important in steroid-induced ONFH.

1. Introduction

Osteonecrosis of the femoral head (ONFH) is a common disease in orthopedics, and is characterized by pathological processes including ischemic changes in cellular constituents such as endothelial, bone, hematopoietic and adipose cells (Yamasaki et al., 2012; Yuan et al., 2015). It is a devastating disease and if left untreated it can lead to the destruction and dysfunction of hip joints that require artificial joint replacement. In patients with total hip replacement, high levels of glucocorticoid-induced ONFH are observed (Weinstein, 2012; Moya-Angeler et al., 2015). The pathogenesis of non-traumatic ONFH is unclear. Some studies suggest that femoral head microvascular endothelial cell (MEC) injury and dysfunction may play an important role in the pathogenesis of femoral head necrosis (Kang et al., 2008). Li et al. (2004) also demonstrated that endothelial cell (EC) damage, coagulopathy and decreased fibrinolytic function were possible pathologic mechanisms of glucocorticoid-induced osteonecrosis. We speculated that femoral head bone microvascular endothelial cell (BMEC) damage might be an initiating and key factor that participates in ONFH.

MicroRNAs (miRNAs) are a group of small 18–25-nt-long single-stranded RNAs involved in many physiological and pathological processes, such as regulating the function of cells and tissues, and various orthopaedic diseases, such as bone tumors, osteoarthritis, and rheumatoid arthritis (Yuan et al., 2015; Wang et al., 2015). miRNAs have tissue and cell specificity, which are characterized by the expression of selection (Lagos-Quintana et al., 2002). It was reported that many miRNAs and key genes are highly expressed in endothelial cells (Harris et al., 2008). We investigated whether femoral head BMEC damage is

Abbreviations: BMECs, bone microvascular endothelial cells; ONFH, osteonecrosis of femoral head; SD, Sprague-Dawley; LPS, lipopolysaccharide; qPCR, quantitative polymerase chain reaction; MEC, microvascular endothelial cell; EC, endothelial cell; miRNAs, MicroRNAs; HE, hematoxylin and eosin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FC, fold change; qRT-PCR, quantitative real-time polymerase chain reaction; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; vWF, von Willebrand factor; eNOS, endothelial nitric oxide synthase; SOD2, superoxide dismutase 2; FoxO, Fork head box O

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an initiating and key factor in ONFH, and whether changes in BMEC miRNAs are induced by glucocorticoids, which might help explore the mechanism of ONFH further.

In this study, we established a rat model of ONFH by isolating and culturing BMECs, which were screened for differences in BMEC miRNA expressions, and predicted their gene targets using bioinformatics analysis. Additionally, the mechanisms of miRNA regulation in steroid-induced ONFH were also investigated to help understand the pathogenesis of ONFH.

2. Materials and methods

2.1. Ethical statement and animals

This study was approved by the Bioethics Committee of our Hospital, and all experimental procedures strictly adhered to the international rules and regulations provided by CPCSEA and World Medical Association Declaration of Helsinki on Ethical Principles. Overall, 26 8-week-old Female Sprague-Dawley (SD) rats (Beijing HFK Bioscience Co., LTD; Beijing, China) were used in the study. Rats were maintained under specific pathogen-free laboratory conditions for 1 week before experimentation (temperature 20 °C, 12 h light/dark cycle, 48% humidity). Standard rodent diet was fed *ad libitum*.

2.2. Animal model of ONFH

Each model and control group consisted of 10 rats. The model group of rats was induced by the intraperitoneal injection of 20-μg/kg lipopolysaccharide (LPS) (Sigma, San Francisco, CA, USA). Then, 24 h later, the rats received three doses of intramuscular injection of 40-mg/kg methylprednisolone (Pfizer Inc., Ascoli Piceno, Italy) at intervals of 24 h, as previously described (Dong et al., 2015). In the control group, the rats received the same volume of normal saline. After 4 weeks, the rats were sacrificed (*via* cervical dislocation) and the femurs were collected for analyses. The femoral specimens were fixed for 3 days with 10% neutral formalin (Guduo Biotechnology Corporation, Shanghai, China), and subsequently placed in 5% nitric acid solution (ShangHai Rongchuang Biological Technology Co. Ltd., Shanghai, China) at room temperature to decalcify for 7 days. The femoral heads were considered completely demineralized when the bone was easily pierced with a pin. The samples were dehydrated by a series of graded ethanol washes, placed in xylene (Rongbai Biotechnology Co. Ltd., Shanghai, China) for 2 h at room temperature, embedded in paraffin (ToYongBio, Shanghai, China) and sliced into 4-μm coronal tissue sections. The tissue sections were processed for hematoxylin and eosin (HE) staining for morphological evaluation.

2.3. Isolation, culture, and identification of BMECs

Rats were sacrificed by cervical dislocation. After a soak disinfection step in 75% ethanol for 10 min, the femoral head was dissected on a clean bench and stored in a 50-ml beaker. The femoral head was then cut into 1-mm^3 pieces and transferred to a 50-ml plastic centrifuge tube containing 10 ml of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) at 4 °C. The bone debris was washed twice with 4 °C DMEM for approximately 3 to 5 min to remove the fat tissue and blood cells. After removing the supernatant, the bone debris was incubated in 0.2% collagenase I (1-ml 1% collagenase I mixed with 4-ml DMEM) in a 37 °C thermostat water bath for 30 min, prior to the addition of 3-ml 0.25% trypsin to the centrifuge tube for continued digestion at 37 °C in the thermostat water bath for 5 min. The enzyme solution was then inactivated by adding DMEM containing 10% fetal bovine serum (FBS) (San Diego, CA, USA). After filtration through a 70-μM cell strainer, the solution was centrifuged at 430 ×*g* for 6 min and the supernatant removed. The harvested microvessels and cells were then suspended in a conical tube with ECM and seeded into a 35-mm tissue culture dish. Cells were cultured in an incubator at 37 °C, with 95% humidity, and 5% CO_2. After 48 h, the cells were washed twice with PBS to remove the nonadherent cells and impurities, and cultivated with fresh medium. Then medium was replaced every 3 days to ensure the continued provision of nutrients to cells. On reaching approximately 80% confluence, cells were detached by 0.25% trypsinization. The harvested cells were suspended in a conical tube, and were centrifuged again at 430 ×*g* for 6 min. Expression levels of the markers CD31, CD133 and vWF (Beijing Biosynthesis Biotechnology Co. Ltd., Beijing, China) were confirmed by immunofluorescence.

2.4. Microarray and data processing

Each experimental and control group consisted of three rats. The cells from every rat were pooled and cultured together. Total RNA was extracted from primary BMECs using TRIzol reagent (Thermo Fisher Scientific, MA, USA), according to the manufacturer's guidelines. A spectrophotometer (NanoDrop ND-1000) was used to quantify the samples. The quality of total RNA was tested by formaldehyde denaturing gel electrophoresis. A MirVana™ miRNA Isolation Kit (AM1561) was used to purify genes and then total RNA was quantified again. Subsequently, a total of 200-ng purified RNA from each sample was used to perform dephosphorylation and analyze genetic markers using an miRNA Complete Labeling and Hyb Kit (Agilent Technologies, CA, USA), according to the manufacturer's instruction. Images were scanned with an Agilent microarray scanner (G2565CA). Agilent Feature Extraction (v10.7) was used to analyze the hybrid image. Agilent GeneSpring GX software was used to normalize raw microarray data and analyze differentially expressed genes. Threshold values of ≥2 absolute fold change (FC) and a Benjamini-Hochberg corrected $P \leq 0.05$ were used to identify differentially expressed genes.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) of miRNA

Four differentially expressed miRNAs, including rno-miR-132-3p, rno-miR-335, rno-miR-466b-2-3p and rno-let-7c-1-3p, were selected to validate the accuracy of microarray results using the SYBR green fluorescent method. The polymerase chain reaction PCR was repeated three times for each sample and U6 was used as an internal control for PCR. The sequences of the primers for real-time qPCR are shown in Table 1. The relative quantity of each microRNA was calculated using the comparative CT (CT) method, as follows: RQ = TS2^-CT/CS2^-CT.

2.6. Bioinformatics analysis

The 12 most popular databases, namely miRWalk, DIANA-microTv4.0, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, PicTar2, PITA, RNA22v2, RNAhybrid2.1 and Targetscan6.2 were used to predicted the target genes of differentially expressed miRNAs. Only those identified in at least five databases were regarded as target genes of differentially expressed miRNA. Gene ontology (GO) was used for functional annotation analysis of predicted genes and Kyoto Encyclopedia of Genes and Genomes (KEGG) database analysis was used to identify the enriched pathways involved. The Top 30 significant enriched terms were used as the cut-off criterion.

2.7. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (IBM Corp; Armonk, NY, USA). Comparisons between groups were performed using the Student's *t*-test. A P-value < 0.05 was considered statistically significant.