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Research paper

Identification of transcription factors and gene clusters in rabbit smooth muscle cells during high flow-induced vascular remodeling via microarray

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ABSTRACT

Sustained blood flow, especially high blood flow causes the remodeling of arteries. The molecular mechanism of vascular remodeling has been mainly investigated in cultured cells. However, the in vivo molecular mechanism is poorly understood. In this study, we performed microarray analysis to explore the gene expression profile of smooth muscle cells (SMCs) during vascular remodeling. Transcriptional profiles indicated that 947 genes were differentially expressed in SMCs responding to high flow compared with the sham control, of which 617 genes were up-regulated and 330 genes were down-regulated. Gene ontology analysis revealed the special participation of extracellular matrix related genes during high flow-induced vascular remodeling. KEGG pathway analysis showed the enrichment of metabolism and immune function associated genes in SMCs exposed to high flow. Besides, we also identified 25 differentially expressed transcription factors potentially impacted by hemodynamic insult. Finally, we revealed FOXN4 as a novel transcription factor that could modulate MMP2 and MMP9 transcriptional activity. Collectively, our results revealed major gene clusters and transcription factors in SMCs during vascular remodeling which may provide an insight into the molecular mechanism of vascular remodeling and facilitate the screening of candidate genes for vascular diseases.

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1. Introduction

Sustained hemodynamic stress, especially high flow, can lead to vascular remodeling, during which the vasculature senses the hemodynamic stress and integrates stress signals to increase vascular diameter while only causing very small changes in wall thickness (Hashimoto et al., 2001; Hashimoto et al., 2006). It has been reported that high flow-induced active vascular remodeling participates in many physiological processes, such as blood vessel growth and angiogenesis during development, wound healing and exercise training (Lu et al., 2009). However, destructive vascular remodeling can induce various vascular diseases, such as aneurysms, atherosclerosis and brain arteriovenous malformations (Xu et al., 2000; Shojima et al., 2004; Nakamura et al., 2008).

Many people suffer from subarachnoid hemorrhages as a result of ruptured intracranial aneurysms, which often cause high morbidity and mortality each year (Silver and Vita, 2006). Notably, abnormal vascular remodeling has been identified as one of the key pathologic processes during the development and rupture of intracranial aneurysms (Renna et al., 2013; Penn et al., 2014).The cellular events involved in vascular remodeling during the initiation of intracranial aneurysm have been well characterized in high flow-induced animal models (Shojima et al., 2004; Meng et al., 2007; Infanger et al., 2008). However, the molecular mechanisms that lead to vascular remodeling prior to the initiation of intracranial aneurysm are largely unknown.

Many studies have reported that multiple components contribute to high flow-induced vascular remodeling. Located in the inner part of the vascular wall, endothelial cells rapidly respond to hemodynamic shear stress by altering cell growth, survival and function via the production of cytokines, adhesion molecules and reactive oxygen species (ROS) (Sho et al., 2003; Dorfmuller et al., 2011; Li et al., 2013; Hsieh et al., 2014). Moreover, owing to local or secondary effects, inflammatory

Abbreviations: SMCs, smooth muscle cells; GO, gene ontology; TFs, transcription factors; ROS, reactive oxygen species; IHC, immunohistochemistry; IF, immunofluorescence; IGF1, insulin-like growth factor 1; PRKG1, cGMP-dependent protein kinase 1; PTH1R, parathyroid hormone 1 receptor; FOXN4, forkhead box N4; RYR-2, ryanodine receptor 2; GPX7, glutathione peroxidase 7; CACNB2, voltage-dependent l-type calcium channel subunit beta-2; PTHLH, parathyroid hormone-like hormone; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; COL1A2, collagen I; LUM, lumican; BGN, biglycan; SPP1, secreted phosphoprotein 1; TIMP4, tissue inhibitor of metalloproteinase-4; PITX2, paired-like homeodomain 2; KLF15, Kruppel-like factor 15; OSR1, odd-skipped related transcription factor 1; ECM, extracellular matrix.

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cells (mainly macrophages, neutrophils and lymphocytes) have been shown to infiltrate into remodeling sites (Nuki et al., 2009; Li et al., 2013). Additionally, SMCs have been identified as critical components in the responses of vessels to hemodynamic insults during vascular remodeling (Mandelbaum et al., 2013). However, how these different cell types are distinctively and coordinately regulated is not fully understood.

In this study, we identified differentially expressed genes and special gene clusters in SMCs responding to high flow-induced vascular remodeling. The results of this study will facilitate future investigations into the molecular mechanisms that lead to vascular remodeling and will provide insights into the functions of these genes in vascular diseases.

2. Materials and methods

2.1. Ethics statement

The rabbits used in this study were anesthetized by intravenous administration of 20 mg/kg sodium pentobarbital and euthanized by intravenous administration of 100 mg/kg sodium pentobarbital as previously reported (Dolan et al., 2012). The use of the rabbits and all of the procedures in this study were approved by the Animal Care and Use Committee of the Second Military Medical University.

2.2. Rabbit model of high flow-induced vascular remodeling

Bilateral common carotid arteries of female New Zealand White rabbits were ligated to increase blood flow in their basilar arteries (Gao et al., 2008; Kolega et al., 2011; Dolan et al., 2012). The control group underwent the same procedure to expose the common carotid arteries but without ligation. In both groups, the rabbits were euthanized by intravenous administration of 100 mg/kg sodium pentobarbital on day 5 after ligation or exposure of the bilateral common carotid arteries.

2.3. Detection of blood flow and measurement of basilar artery diameter

The velocity of blood flow in the basilar artery was obtained by using Transcranial Doppler (Philips Medical). The measurement was blinded to the procedures. Digital subtraction angiography (DSA) was performed in a Philips Medical (V3000) angiography suite. A 5-Fr sheath was used through the femoral artery, and the diameters of the basilar arteries were measured by comparison to a reference sphere, which had a 6.23 mm diameter.

2.4. Isolation and purification of SMCs

SMCs were isolated as previously described (Robertson et al., 1993). After euthanization, each whole basilar artery was removed. The arteries were washed in a PBS buffer, cannulated and perfused under constant flow with a cocktail that contained PBS, 0.4 mg/ml elastase (Sigma) and 1 mg/ml collagenase (type IA, Sigma). After an incubation time of 10 min at 37 °C, the arteries were washed with PBS to remove the intima, and the remaining tissue was digested for another 30 min. After incubation, SMCs were released from the arteries and spun down. To exclude potential contamination of endothelial cells and fibroblasts, the released cells were stained with CD31 (Abcam, ab9498) and CD90 (Abcam, ab226) and sorted with a FACSAria II flow cytometer (BD Bioscience) (Aikawa et al., 2002; Kisselbach et al., 2009).

2.5. RNA extraction, purification, amplification and labeling

Total RNA was extracted from the SMCs using a TRIzol Reagent (Cat#15596-018,Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA integration, concentration and quality were checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). Qualified total RNA was further purified using an RNeasy micro kit (Cat#74004, Qiagen, GmbH, Germany) and RNase-Free DNase Set (Cat#79254, Qiagen, GmbH, Germany).

Then, the RNA was amplified and labeled using a low input quick amp labeling kit, one-color (Cat#5190-2305, Agilent Technologies, Santa Clara, CA, USA), following the manufacturer's instructions. Labeled cRNA was purified using an RNeasy mini kit (Cat#74106, Qiagen, GmbH, Germany).

2.6. Hybridization, washing and scanning of microarrays

Each slide was hybridized with 1.65 μg of Cy3-labeled cRNA using a gene expression hybridization kit (Cat#5188-5242, Agilent Technologies, Santa Clara, CA, USA) in a hybridization oven (Cat#G2545A, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. After 17 h of hybridization, the slides were washed in staining dishes (Cat#121, Thermo Shandon, Waltham, MA, US) with a gene expression wash buffer kit (Cat#5188-5327, Agilent Technologies, Santa Clara, CA, USA), following the manufacturer's instructions.

The slides were scanned using an Agilent microarray scanner (Cat#G2565CA, Agilent Technologies, Santa Clara, CA, US) with default settings: dye channel = green, scan resolution = 5 μm, PMT 100%, 10%, and 16 bit. The data were extracted with Feature Extraction software, version 10.7 (Agilent Technologies, Santa Clara, CA, USA).

2.7. Microarray bioinformatics analysis

We conducted three independent experiments using an Agilent rabbit 4 × 44K microarray with two grouped samples. The gene chip tests were performed by professionals at the Shanghai Biotechnology Corporation. The raw data were normalized using a Quantile algorithm contained in Gene Spring Software, version 11.0 (Agilent Technologies, Santa Clara, CA, USA). Normalized data were analyzed using a t-test with unequal variance and no statistical corrections. All data sets related to this study were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE61212.

To identify differentially expressed genes, we performed re-annotation analysis using different expression probes. We used Better Bunny (http://cptweb.cpt.wayne.edu/BB/index.php) to match orthologs from humans with a minimum of 50% identity threshold (Craig et al., 2012). Genes with an arbitrary fold change (FC) >2 and a P value < 0.05 were selected. After detecting the differentially expressed genes, we omitted the transcripts without any annotation in the three data bases (Ensemble, GenBank and RefSeq) (Edgar et al., 2002). Then, we deleted the duplicate transcripts. Finally, the remaining 947 genes were used for further gene function analysis. The genes of rabbit and human orthologs are listed as supporting information in Table S2.

To determine the biological function of these differentially expressed genes, GO (Ashburner et al., 2000) and KEGG (Kanehisa et al., 2012) pathway enrichment analyses were performed using DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009). The significance threshold was set to 0.05 in our enrichment analysis.

2.8. Real-time PCR analysis

Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol and was reverse-transcribed with a SuperScript RT-PCR kit (Invitrogen). A reaction solution was prepared with SYBR® Green Real-time PCR Master Mix, primers, PCR-grade water and template cDNA. The reaction volume was 20 μL. Real-Time PCR was performed using SYBR® Green Real-time PCR Master

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