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Inhibition of development of experimental abdominal aortic aneurysm by c-jun N-terminal protein kinase inhibitor combined with lysyl oxidase gene modified smooth muscle progenitor cells

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ABSTRACT

Chronic inflammation, imbalance between the extracellular matrix synthesis and degradation, and loss of vascular smooth muscle cells (SMCs) contribute to the development of abdominal aortic aneurysm (AAA). The purpose of this study was to investigate the effect of the therapy with periaortic incubation of c-Jun N-terminal protein kinase inhibitor SP600125 infused from an osmotic pump and subadventitial injection of lysyl oxidase (LOX) gene modified autologous smooth muscle progenitor cells (SPCs) on treatment of AAA in a rabbit model. Obvious dilation of the abdominal aorta in the control group was caused by periaortic incubation of calcium chloride and elastase. But the progression of aortic dilation was significantly decreased after the treatment with SP600125 and LOX gene modified SPCs compared to the treatment with phosphate-buffered saline. This therapy could inhibit matrix metalloproteinases expression, enhance elastin synthesis, improve preservation of elastic laminar integrity, benefit SPCs survival and restore SMCs population. It seemed that this method might provide a novel therapeutic strategy to treat AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is associated with impaired arterial wall integrity, leading to abnormal dilatation and eventual fatal rupture. After initial diagnosis, AAA patients (AAA diameter $\geq$ 2.5 cm) are monitored for an increase in aortic diameter. Surgery aimed at preventing death from enlarged or ruptured AAA is recommended when the diameter reaches $\geq$ 5.5 cm. Treatment options are particularly limited for patients with small or moderate AAA. This group accounts for the largest percentage of all AAA patients. And this number is likely to increase dramatically with the advent of "blanket" screening of asymptomatic subjects with imaging examination. Therefore, novel treatments aimed at inhibiting the progression of AAA immediately after diagnosis would be extremely beneficial.

Development of AAA is a complex remodeling process of the aortic wall as a result of chronic inflammation, imbalance between the extracellular matrix (ECM) synthesis and degradation, and loss of vascular smooth muscle cells (SMCs) (Freestone et al., 1995; Thompson et al., 1997, 2002; Guo et al., 2006; Shimizu et al., 2006). Recently, Yoshimura et al. (2005) reported that SP600125 could block AAA progression and stimulate aneurysm regression by systemic pharmacological inhibition of c-Jun N-terminal protein kinase (JNK), an intracellular signaling switch that controls matrix metalloproteinases (MMPs) production. Through this mechanism, it could inhibit aortic inflammation and degradation of ECM. From this research, we hypothesized that whether the method of periaortic incubation of JNK inhibitor SP600125 infused from an osmotic pump could get the same effect. At the same time, it was considered that smooth muscle progenitor cells (SPCs) as a source of SMCs and lysyl oxidase (LOX) as an important protease of ECM synthesis could be used to restore SMCs population and ECM synthesis (Foubert et al., 2008; Daniel and Sedding, 2011). So the purpose of this study was to investigate the effect of the therapy with periaortic incubation of SP600125 infused from an osmotic pump and subadventitial injection of LOX gene modified autologous SPCs on treatment of AAA in a rabbit model.

2. Materials and methods

All the experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. A prior approval

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was granted by the Animal Ethics Committee of NanChang University.

2.1. Construction and production of recombinant adeno-associated virus vector encoding LOX

Human peripheral blood derived SPCs were collected in lysis buffer. Total RNA was isolated using a kit (RNeasy; Qiagen) according to the manufacturer's description. First-strand complementary DNA (cDNA) was synthesized with 0.2–1 μg of total RNA in 20 μl buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl2) containing 1 μM oligo(dT) or random hexamer, 0.5 mM dNTP, 10 mM DTT, 40 U RNase inhibitor, 200 U reverse transcriptase (Superscript II; Life Technologies). Based on the manufacturer's recommendation, the reaction was incubated at 42 °C for 1 h and heat inactivated at 70 °C for 15 min, and then the cDNA was pooled. Polymerase chain reaction (PCR) was performed with 1 μl pooled cDNA in 50 μl buffer containing 5 U DNA polymerase (Stratagene), 0.2 mM dNTP, 0.5 μM primers. Primers for LOX were selected based on GenBank data (NM_002317.3). The primer sequences were as follows: forward primer: 5′-ATGCGCTTCGCCTGGACC-3′; reverse primer: 5′-CTAATACGGTGAAATTGTGCAGCCT-3′. The reaction was cycled as follows: 3 min at 94 °C; 35 cycles of 40 s at 94 °C, 50 s at 58 °C and 90 s at 72 °C; and a final extension of 5 min at 72 °C. PCR products were run on a 1.5% agarose gel, purified (Gel Purification Kit; Qiagen), and cloned into a vector (PGEM-T Easy; Promega) to get the vector PGEM-T Easy-LOX according to the instructions in the kit manual. Then LOX gene was excised from PGEM-T Easy-LOX using EcoRI/SpeI and cloned into the EcoRI/XbaI sites of pAAV-MCS (stratagene), thus resulting in pAAV-MCS-LOX which was under the control of the cytomegalovirus promoter and followed by human growth hormone polyA. Recombinant adeno-associated virus vectors were generated by triple transfection. The vector was purified by iodixanol gradient. The vector particle titer was determined by quantitative DNA dot-blot hybridization of the DNase I-treated vectors.

2.2. SPCs culture and transduction

Rabbit (or human) mononuclear cells (MNCs) were isolated from peripheral blood by density-gradient centrifugation, plated on fibronectin-coated (Sigma) culture dishes and maintained in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with platelet-derived growth factor (50 ng/ml, Sigma) and basic fibroblast growth factor (10 ng/ml, Sigma). Under daily observation, the first medium change was done about 6 days after plating and nonadherent cells were removed. Thereafter, medium was changed every 3 days. To confirm SPCs phenotype, immunofluorescence analysis was performed with monoclonal antibodies (McAbs) for CD31 (Dako), α smooth muscle actin (α-SMA, Dako), smooth muscle myosin heavy chain (MHC, Dako), and calponin (Dako). Bindings of primary McAbs to cells were detected with CY3-conjugated secondary antibody. Nuclear was stained with Hoechst 33342 (Sigma).

After reaching 80% confluence, rabbit SPCs were transduced with AAV-LOX at multiplicity of infection 1.0×106 v.g./cell in EBM-2 medium without heparin and fetal bovine serum for 6 h. Then the medium was replaced, 72 h later the expression of transgene was detected using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry methods. Total RNA was extracted from transduced SPCs and cDNA was prepared from total RNA adjusted by Trizol reagent (Invitrogen). Reverse transcription was performed with 1 μg of total RNA. PCR primers were as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-GGGAAACTGTGGCGTGAT-3′ and 5′-AAGGTGGAGGAGTGGGTG-3′ (308 bp), LOX 5′-CGGATACGGCACTGGCTACTT-3′ and 5′-GTCCCTTGGTTTTTCACTCTT-3′ (219 bp). Transduced SPCs were fixed in 4% formaldehyde, the expression of human LOX protein was detected by immunocytochemical method with McAb for human LOX (Abcam). Bindings of primary McAbs to cells were detected with biotinylated secondary antibody (Vector Laboratories). The immunocomplexes were visualized with ABC reagent (Santa Cruz) and counterstained with hematoxylin. Non-transduced SPCs served as control.

2.3. Animal studies

Male Japanese white rabbits weighting 2.0–2.5 Kg were anesthetized, and the infrarenal abdominal aorta was exposed by an extra-peritoneal approach. A 10-mm-long segment of abdominal aorta was isolated below the renal artery without dissection of the lumbar arteries. Abdominal aortic dilatation was induced by incubation of the abdominal aorta with 0.75 mol/l $CaCl_2$ for 30 min, then with type I porcine pancreatic elastase (10 U/ml) for 30 min.

Four weeks after the surgery, these rabbits were randomly divided into four groups ($n=8$, each group) and anesthetized again. In group SP600125, dilated abdominal aorta was treated with periaortic incubation of SP600125 infused from an osmotic pump; in group SPC, it was treated with subadventitial injection of AAV-LOX transduced autologous SPCs; in group SP600125/SPC, it was treated with SP600125 and AAV-LOX transduced SPCs; in group control, it was treated with phosphate-buffered saline (PBS).

Autologous peripheral blood SPCs were transduced by AAV-LOX the day before the treatment. To track the transplanted SPCs, they were labeled with CM-Dil (Molecular Probes). In brief, before cellular transplantation, SPCs in suspension were washed with PBS and incubated with CM-Dil at a concentration of 2.5 μg/ml for 5 min at 37 °C and 15 min at 4 °C. After two washes in PBS, the cells were resuspended in PBS. The suspension of AAV-LOX transduced SPCs (0.2 ml, 1×10^7) was subadventitially injected into four different sites of dilated abdominal aorta using a 30-gauge needle.

A PE-10 tube was secured to the anterior aortic surface of the abdominal aorta with a 1-mm thick sheet of circumferential periaortic porous polyvinyl alcohol foam (10-mm-long). The foam was positioned to capture SP600125 infused through the PE-10 tube and to maintain relatively high periaortic concentration gradients. The other end of the PE-10 tube was tunneled into a lateral subcutaneous pocket through the abdominal wall. A 2 ml osmotic pump (Model 2 ml; Azlet) was secured in the pocket and connected to the tubing. The osmotic pumps were preloaded with either SP600125 (30 mg/kg) dissolved in saline containing 2% dimethylsulfoxide or saline alone.

Ultrasonography was used to assess dilatation of the abdominal aorta. The aortic size was measured before each operation.

2.4. Measurement of aortic diameter by ultrasonography

Ultrasonography was utilized to demonstrate dilatation of the abdominal aorta. A cardiovascular ultrasound system (Acuson) and a linear transducer (15 MHz) were used to image the abdominal aorta noninvasively in anesthetized rabbits. Rabbits were scanned transversely to obtain images for the measurement of the luminal diameter of the aneurysm at the segment with maximum diameter. The aortic size was measured before and 4 weeks after AAA model making, and 4 weeks after treatment.

2.5. Histological and Immunohistochemical analysis

Animals were killed at 4 weeks after treatment. Aortic tissue cross-sections (6 μm) were stained with hematoxylin and eosin (HE), elastic van Gieson (EVG) stain and Masson's trichrome stain