Interactive CardioVascular and Thoracic Surgery 21 (2015) 62–70
doi:10.1093/icvts/ivv074 Advance Access publication 31 March 2015

Cite this article as: Mao N, Gu T, Shi E, Zhang G, Yu L, Wang C. Phenotypic switching of vascular smooth muscle cells in animal model of rat thoracic aortic aneurysm. Interact CardioVasc Thorac Surg 2015;21:62–70.

Phenotypic switching of vascular smooth muscle cells in animal model of rat thoracic aortic aneurysm

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Received 30 December 2014; received in revised form 20 February 2015; accepted 4 March 2015

Abstract

OBJECTIVES: To explore if there is phenotypic switching in the vascular smooth muscle cells (vSMCs) of rat thoracic aortic aneurysms and the role it plays in the process of aneurysm formation.

METHODS: Male SD white rats were assigned randomly to the aneurysm group (AG) and control group (CG). The animal aneurysm model was obtained by soaking the peri-adventitia with porcine pancreatic elastase (PPE). The rats in the CG were given saline to provide contrast. A vascular ultrasound was used to monitor the diameter of the aneurysm. Specimens were stained with haematoxylin and eosin (HE), and α-SMA, SM-MHC, matrix metalloproteinase (MMP)-2 and MMP-9 were detected with immunohistochemistry staining. α-SMA, SM-MHC, MMP-2 and MMP-9 were conducted with western blot. vSMCs taken from the descending aorta of both of the CG and AG were separated and cultured until Passage 3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method were used to analyse cell proliferation. Western blot was used to evaluate MMP-2, MMP-9 expression and flow cytometry was employed to assess cell apoptosis.

RESULTS: Vascular ultrasound showed obvious dilatation of soaked descending aorta. HE staining showed thickening of thoracic aorta and disarrangement of cells after soaking with PPE. Immunohistochemistry staining showed high expression of MMP-2 and MMP-9 but low expression of SM-MHC and α-SMA in the AG. Tissue western blot analysis of the AG showed that the protein gray value was high in MMP-2 and MMP-9, but low in α-SMA and SM-MHC, which had statistical differences compared with CG with a *P*-value of <0.05. MTT analysis showed vSMC proliferation activity was higher in the AG than in the CG. Flow cytometry analysis revealed that cell apoptosis between the control and aneurysm groups had significant statistical differences.

CONCLUSIONS: There is vSMC phenotypic switching in animal models as seen through the rat thoracic aortic aneurysms. This may play an important role in the formation of aneurysms. Our findings are relevant to human aneurysms and may be conducive in the research of aortic aneurysm pathology and treatment.

Keywords: Vascular smooth muscle cell • Thoracic aortic aneurysm • Phenotype switch

INTRODUCTION

Thoracic aortic aneurysms occur at a frequency of 5.9 persons per 100 000 per year [1]. The death rate is ∼70% among untreated patients; however, the mechanisms of such aneurysms are still uncertain. Vascular smooth muscle cells (vSMCs) are one of the major components of the aortic wall which can be divided into quiescent contractile and activated synthetic phenotypes. vSMC shows highly differentiated cell functions at maturity; however, the main function is to regulate vascular tension and maintain blood pressure. Phenotypic switching of the vSMC [2] (which was defined as any change in the normal structure or function of the differentiated vSMC) is related to a number of human diseases, especially vascular disease. We want to explore whether this phenomenon occurs in the development of rat thoracic aortic aneurysms and what role it plays in the procedure of aneurysm formation.

METHODS

Animal care

The animal study was approved by our institutional committee for the care and use of laboratory animals. Singly housed animals were exposed to a 12-h day–night cycle in 50% humidity and 708° F temperature controlled rooms, and fed standard chow *ad libitum*.

Rat thoracic aortic aneurysm model

About 8- to 12-week old, male SD white rats, weighing between 150 and 200 g were assigned to the aneurysm group (AG) and control group (CG) randomly ($n = 20$). Animals were anesthetized with pentobarbital (40 mg/kg) intraperitoneally and placed on the

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operating table in a supine position. All the animals received tracheal intubation through tracheotomy and were ventilated using a small animal ventilator. The ventilator parameters have a breath rate of 90/min and pressure support of 0.01 MPa. The rat underwent left thoracotomy through the fifth intercostal space. The descending aorta was dissociated 1 cm away from left subclavian artery for 1 cm. A thin piece of cotton yarn with saline or porcine pancreatic elastase (PPE; 1.5 U/μl) was lightly wrapped around the exposed aorta for 20 min (Fig. 1). After washing with saline, a drainage tube was implanted and the chest was closed with an interrupted 6-0 polypropylene suture. Finally, a syringe was used for the deairing of the thoracic cavity and the rat was weaned of the ventilator. About 100 000 U of penicillin was injected intraperitoneally for infection prevention.

Vascular ultrasound surveillance

All rats were given ultrasound surveillance (Sonoline G50 SIEMENS, North Rhine-Westphalia, Germany) for 7, 14 and 21 days postoperatively. The diameter of the dilated artery was calculated with the formula: $[D(d) - D(p)]/D(p)$, where $D(d)$ = diameter of the dilated artery and $D(p)$ = diameter of the proximal artery. The standard for successful aneurysm formation was $[D(d) - D(p)]/D(p) > 0.5$.

Haematoxylin and eosin staining and immunohistochemistry

Rat aortas were harvested for histological analysis after a left ventricular injection of 4% paraformaldehyde solution in phosphate-buffered solution (PBS). The arteries were harvested and fixed with 10% formalin. Then, the specimens were embedded with paraffin and blocks were sectioned continuously at 1 mm. The region of interest of the thoracic aortic artery underwent haematoxylin and eosin (HE) and immunohistochemistry staining.

The primary antibodies α-SMA (Boster, Wuhan, China), matrix metalloproteinase (MMP)-2 (Wanlei life sciences, Shenyang, China), MMP-9 (Wanlei life sciences, Shenyang, China) and SM-MHC (Boster) were diluted to 100 times using PBS and bathed the specimens overnight. The second antibodies IgG/Biotin goat anti-rabbit (Beyotime, Shanghai, China) and IgG/Biotin goat anti-mouse (Beyotime) were diluted to 200 times using PBS and the specimens were soaked three times for 30 min each cycle. The results were observed and photographed under microscopy with amplification of 40 times.

Western blot

Protein extraction was achieved by placing the specimen into radio immunoprecipitation assay and centrifuging at the rate of 12 000 rpm for 10 mins at 4°C. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was prepared by filling 5% spacer gel of pH 6.8 above 6% and 10% separation gel of pH 8.8. The primary antibodies and second antibodies were diluted and incubated overnight at 4°C and 45 min at 37°C, respectively. After electrogenerated chemiluminescence luminol exposure in the darkroom, the photographic film was scanned and the optical density of each band was analysed by Gel-Pro-Analyze software.

Vascular smooth muscle cell isolation and culture

In parallel, protocol aorta subjected to PPE or saline 14 days postoperatively were isolated, and the intima were scraped off gently with a scalpel blade. The medial layer was dissected from the underlying adventitia, cut into 0.5 mm-long sections, and washed twice with warm PBS. The tissue slices were then pooled, enzymatically digested in Dulbecco's modification of Eagle's medium (DMEM) containing.

125 U/mg collagenase type II and 3 U/mg elastase for 30 min at 37°C. The digestates were then centrifuged (1400 rpm; 5 min), and the pelleted tissue pieces were cultured in T-25 flasks with DMEM containing 10% v/v fatal bovine serum (FBS) for more than

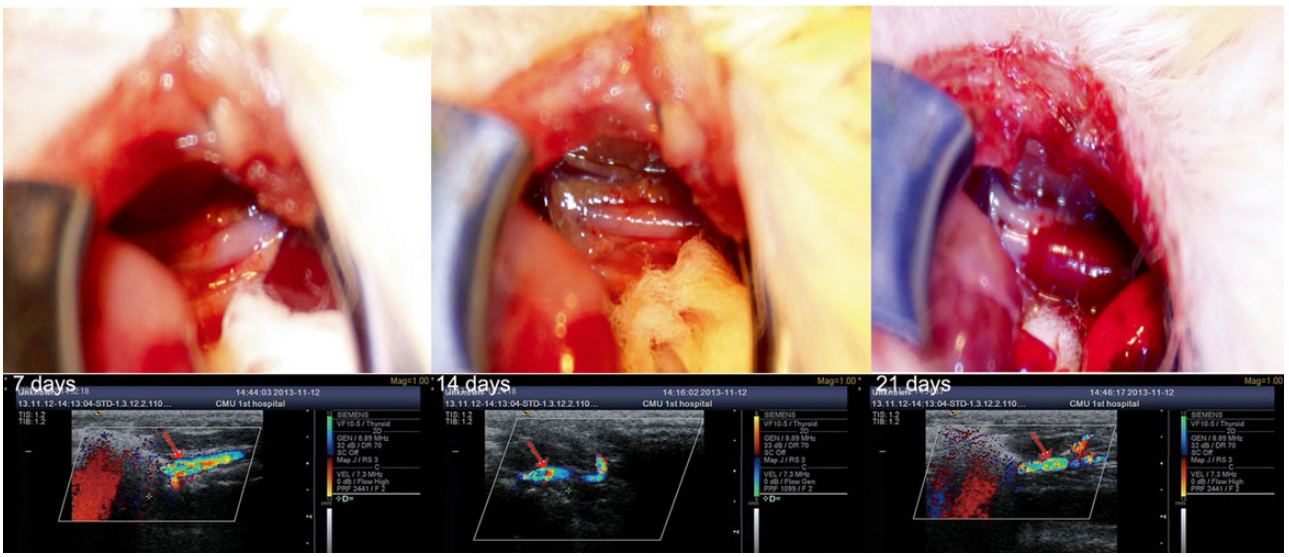

Figure 1: Exposure of descending aorta through the fifth intercostal space. (**A**) Normal aorta; (**B**) aorta soaking with saline; (**C**) aorta soaking with PPE. Vascular ultrasound surveillance of AG 7, 14 and 21 days postoperatively showed dilatation of descending aorta (red arrow). The colourful signal showed the blood flow in aorta. Diameter of the dilated segment was almost double of the proximal normal artery after 14 days. PPE: porcine pancreatic elastase; AG: aneurysm group.

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15 days. Primary aneurysmal vSMCs derived by outgrowth from these tissue explants were cultured for up to 2 weeks, and the cells were passaged when confluence was attained. For culture studies, Passage 3 vSMCs were seeded onto 12-well tissue culture plates at a density of 1×10^5 cells/well and cultured in DMEM medium containing 10% v/v FBS and 1% v/v penstrep.

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Statistical analysis

All the data and results were expressed as mean ± SD. Student–Newman–Keulsa *t*-test was used to compare between groups and a *P*-value of <0.05 was identified as having statistical significance. All the data were analysed by SPSS 19.0.

RESULTS

Vascular ultrasound

The aortas showed immediate dilatation when soaked with porcine pancreatic elastase after 20 min and dilated aortas became stable after 2 weeks (Fig. 1). [*D*(d) – *D*(p)]/*D*(p) variation of AG within 3 weeks detected by vascular ultrasound showed the dilatation rate >0.5 at 1 week, 2 weeks and 3 weeks were 60, 80 and 80%, respectively. The diameter changes between the CG and AG within 3 weeks are shown in Figure 2. The diameters of the CG were 2.2 ± 0.12, 2.2 ± 0.10 and 2.1 ± 0.10 mm, respectively. The diameters of the AG were 3.4 ± 0.13, 3.5 ± 0.16 and 3.5 ± 0.15 mm, respectively.

Tissue analysis

Because we found the aneurysm became stable within 2 weeks, we draw materials at the end of the second week. HE staining showed thickening of thoracic aortic walls, cell hyperplasia and disarrangement of extracellular matrix 14 days after soaking with porcine pancreatic elastase (Fig. 3). Immunohistochemistry showed high expression of MMP-2 and MMP-9, but low expression of α-SMA, SM-MHC in AG (Fig. 4). Results of tissue western blot showed higher MMP-2, MMP-9 expression and lower α-SMA, SM-MHC expression in the AG than the CG. Statistical analysis showed significant statistical differences with a *P*-value of <0.05 (Fig. 5).

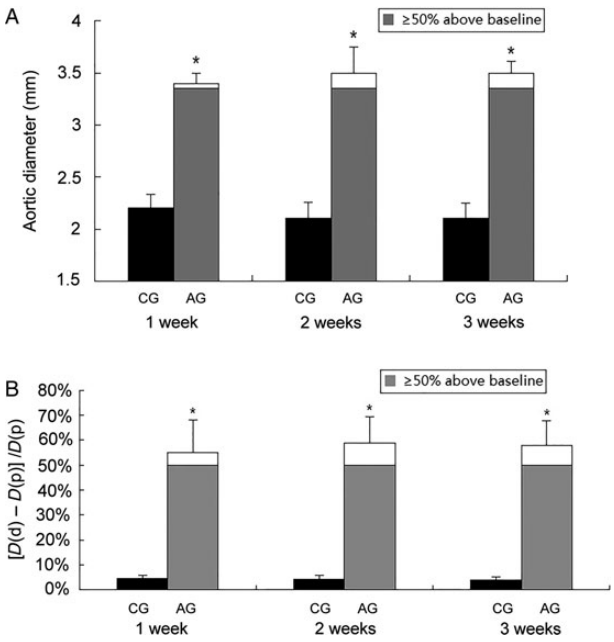

Figure 2: (**A**) Vascular ultrasound surveillance of CG and AG in the 1st, 2nd and 3rd week postoperatively. The diameters of CG were 2.2 ± 0.12, 2.2 ± 0.10 and 2.1 ± 0.10 mm, respectively. The diameters of AG were 3.4 ± 0.13, 3.5 ± 0.16 and 3.5 ± 0.15 mm, respectively. There are significant statistical differences between CG and AG with a *P*-value of <0.05. (**B**) Dilatation ratio of CG and AG in the 1st, 2nd and 3rd week postoperatively. The success rate was 60, 80 and 80% in the 1st, 2nd and 3rd week postoperatively. CG: control group; AG: aneurysm group; *D*(d), diameter of the dilated artery; *D*(p), diameter of the proximal artery (**P* <0.05 vs CG).

Vascular smooth muscle cell analysis

vSMC proliferation activity analysis [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method] showed vSMC proliferation activity in the AG was higher than in the CG with a *P*-value of <0.05 (Fig. 6). MMP-2, MMP-9 expression in cultured vSMC showed higher protein gray value in AG and significant statistical differences with a *P*-value of <0.05 (Fig. 7). Flow cytometry analysis of Passage 3-cultured vSMC showed that the comparison of cell apoptosis between CG and AG had significant statistical differences with a *P*-value <0.01 (Fig. 8).

DISCUSSION

Inflammation response is one of the important component in aortic aneurysm formation. Inflammatory cells which secrete various humoral inflammatory factors (e.g. cytokines, chemokines, leuko-trienes, reactive oxygen species and immunoglobulins) infiltrate the luminal thrombus and all layers of the aneurysm wall. The persistent inflammatory state leads to a proteolytic imbalance that exacerbates matrix degradation in the disease. Schonbeck *et al.* first concluded that aortic aneurysm was a T-helper(Th)2-driven disease which characterized by dominance of Th2-associated cytokines IL-4, IL-5 and IL-10, along with minimal expression of the

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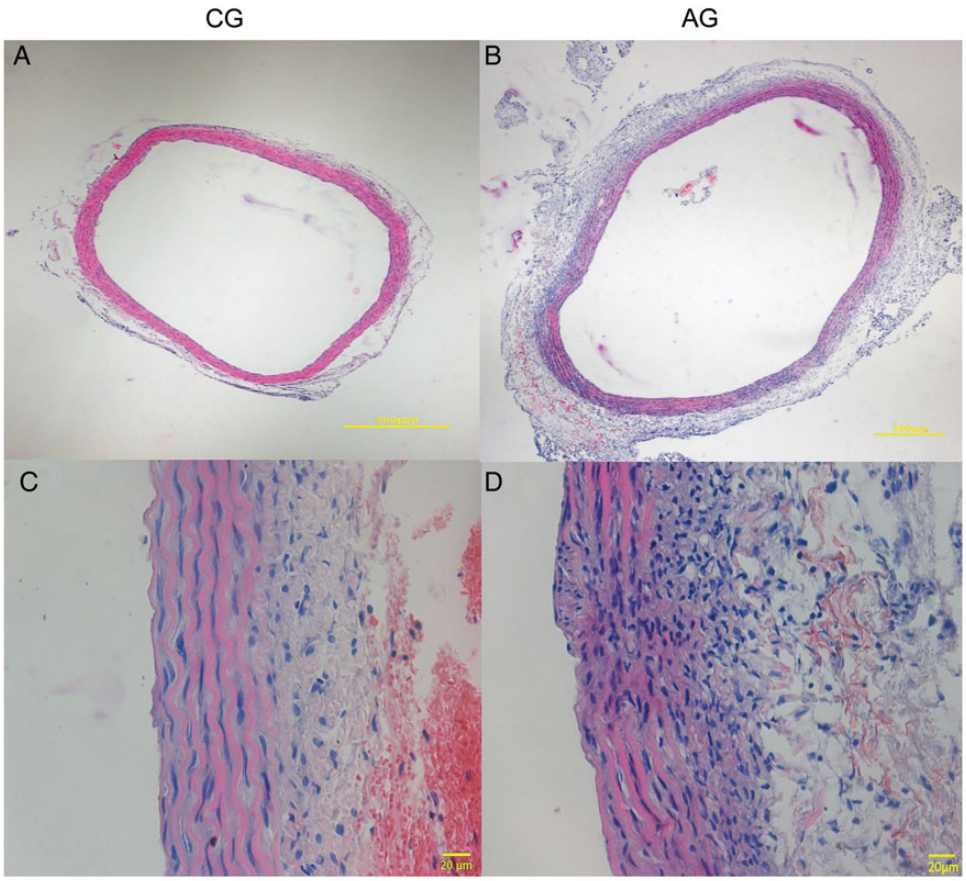

Figure 3: Specimen collected 2 weeks postoperatively. HE staining (×4): (**A**) Normal thoracic aorta of CG; (**B**) Wall thickening of AG 14 days after soaking with PPE. HE staining (×40): (**C**) Thoracic aorta with normal elastin layer; (**D**) Proliferation and migration of intramural cells and disarrangement of extracellular matrix. CG: control group; AG: aneurysm group.

Th1-associated cytokine interferon-γ [3]. However, Xiong *et al.* [4] and Galle *et al.* [5] later reported Th1 polarization in animal and human aortic aneurysms. Afterwards, Lindeman *et al.* performed a comprehensive evaluation of the inflammatory fingerprint of aortic aneurysm and indicated that aneurysm was best described as a general proinflammatory condition with IL-6 and IL-8 hyper-expression [6]. It could be identified that aortic aneurysm which involved many cell factors is driven by inflammatory cascades.

Historically, many methods have been applied to produce animal models of aortic aneurysms. The first animal model was induced by intralumen injection of trypsin in the abdominal aorta. Thereafter, the methods of skiving the aortic media appeared which followed by using a balloon to dilate the aorta. Other methods included insertion of a new blood vessel between normal aorta and suturing a vascular patch on the aorta. All of the above methods are either technically difficult or time-consuming and are mainly used in abdominal aorta. In recent years, periadventitial adhering of $CaCl_2$ was introduced by Ikonomidis, and has become popular as it is simple and practicable [7]. The drawback is the long circle of aneurysm formation which has been reported to be at least 4 weeks. In this research, the rat thoracic aortic aneurysm was induced by periadventitial soaking with porcine pancreatic elastase which showed immediate dilatation after 20 min. The diameter of the aneurysm was monitored through vascular ultrasound and the standard for successful aneurysm formation was $[D(\mathrm{d}) - D(\mathrm{p})]/D(\mathrm{p}) > 0.5$. We found that the aneurysm tended to be stable after 2 weeks and the 2-week success rate was 80%. We have proved that we can obtain an ideal animal aneurysm model through this method rapidly.

The biochemical and pathological changes related to aortic aneurysms have been well characterized. Aneurysms demonstrate arterial dilatation, wall thickening and dramatic reduction in the elastin/collagen ratio [8]. These changes are accompanied by an inflammatory infiltrate [9] and excessive production of matrix metalloproteinases (MMPs) [10], which regulate widespread matrix degradation. The pathology and morphology of our animal model are mostly consistent with human, so it is an ideal model to mimic human aortic aneurysm.

vSMC is a highly differentiated cell at maturity whose main function is to regulate vascular tension and maintain blood pressure. The matured contractile phenotype could switch to the synthetic phenotype when vSMC responds to patho/physiological stimuli. Nowadays, aberrant vSMC plasticity has been implicated in a variety of diseases. For example Riches *et al.* [11] considered that there was an SMC phenotype switch in the saphenous vein with type 2 diabetes mellitus. Poittevin *et al.* found that SMC could be primarily affected genetically or by toxic metabolic molecules. After a stroke, this pathological phenotype had an impact on the incidence, pattern, severity and outcome of the cerebral ischaemic disease [12]. Gomez and Owens conducted a thorough exploration of atherosclerosis in regards to SMC phenotypic switching [13]. Zhang *et al.* also discovered that compared with the contractile SMCs in the unaffected media, those

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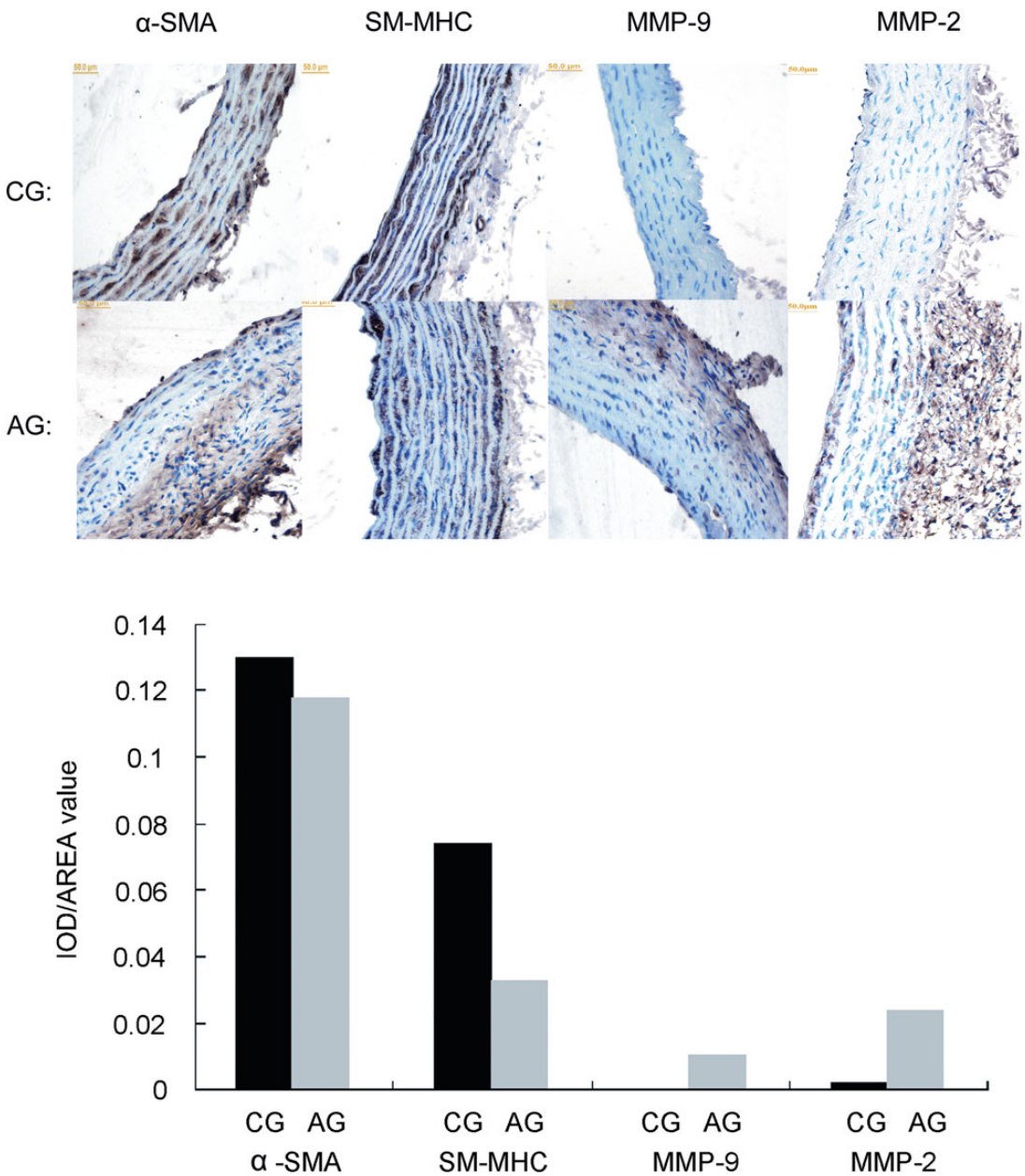

Figure 4: Specimen collected 2 weeks postoperatively. The wall thickness of CG was thinner than AG. α-SMA expression was higher in CG. IOD/area value calculated with Image-Pro Plus software was 0.13 and 0.1181 in CG and AG, respectively. SM-MHC expression was higher in CG. IOD/area value was 0.07395 and 0.03293 in CG and AG, respectively. MMP-9 expression was higher in AG. IOD/area value was 0 and 0.01055 in CG and AG, respectively. MMP-2 expression was higher in AG. IOD/area value was 0.00213 and 0.02374 in CG and AG, respectively. CG: control group; AG: aneurysm group; IOD: integrated optical density.

in the dissected thoracic aortic media manifested phenotypic switching from the contractile to the synthetic type in human subjects [14].

Regulation of phenotypic switching of vSMC is a complex procedure. vSMC phenotype is determined through the integration of numerous environmental cues, including cytokines, cell–cell contact, cell adhesions, extracellular matrix interactions, injury stimuli and mechanical force. In particular, growth factor/cytokine signalling can dramatically affect the differentiation status of vSMCs. Platelet-derived growth factor promotes multiple aspects of the synthetic vSMC phenotype, including reduced expression of contractile genes and increased rate of proliferation and migration. Conversely, transforming growth factor-β and its related family member bone morphogenetic protein 4 reduce vSMC proliferation and migration and promote increased expression of vSMC contractile genes [2]. Several signalling pathways *in vitro* model studies have been demonstrated which could mediate vSMC phenotypic switching, including PDGF-B, PDGFRβ, KLF4 and Sp1. Lately, molecule regulation has been studied comprehensively to investigate the potential mechanisms in which some microRNAs (e.g. microRNA-663 [15], microRNA-145 [16], microRNA-133 [17] etc.) seem to participate in the modulation procedure. Other possible mechanisms that could affect vSMC phenotypic switching include DNA methyltransferase 3A activity found by Jiang *et al.* [18], interaction between interferon regulatory factor 8 and SRF/myocardin

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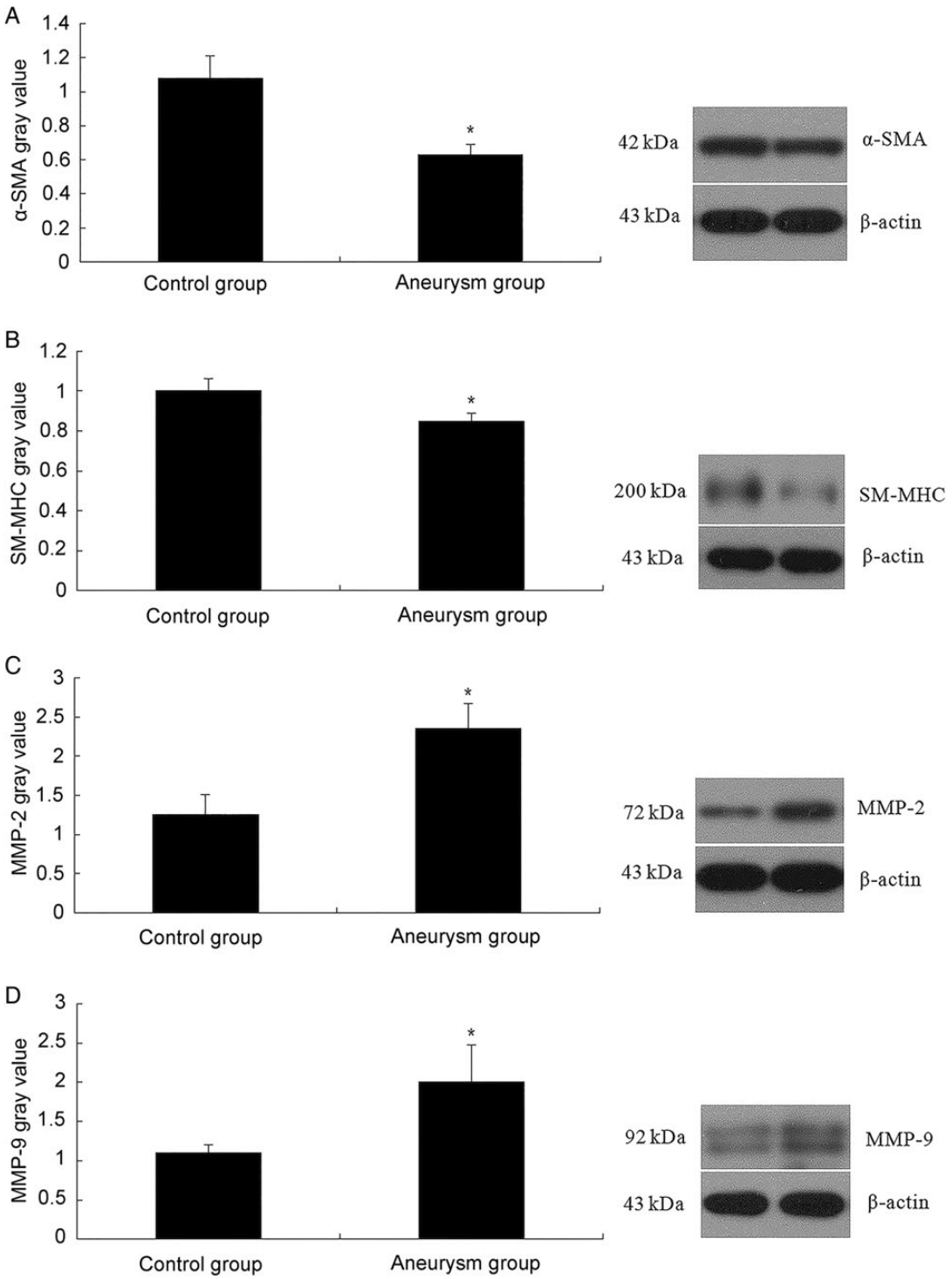

Figure 5: Quantitative analysis of α-SMA, SM-MHC, MMP-2 and MMP-9 using western blot 14 days postoperatively. (**A**) α-SMA protein gray value was higher in CG than AG. (**B**) SM-MHC protein gray value was higher in CG than AG. (**C**) MMP-2 protein gray value was higher in AG than CG. (**D**) Active-MMP-9 protein gray value was higher in AG than CG. The comparation results between AG and CG showed significant statistic differences with a *P*-value of <0.05 (*P <0.05 vs CG). CG: control group; AG: aneurysm group.

complex discovered by Zhang *et al.* [19], and PI3K/Akt demonstrated by Garat *et al.* [20]. vSMC phenotypic switching is a complicated process that can be regulated by a variety of pathways and mechanisms.

Phenotypic switching of the vSMC has been defined as any change in the normal structure or function of the differentiated vSMC. This phenomenon was found in the animal model of rat thoracic aortic aneurysms in our research. Immunohistochemistry staining showed great matrix MMPs expression and was confirmed by a western blot analysis in the AG; however, the expression of α-SMA and SM-MHC which represent contractile phenotypic markers were lower in the AG. The cell culture *in vitro*

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showed higher proliferation activity in the AG than in the CG by MTT method. Flow cytometry analysis of Passage 3-cultured vSMC showed significant differences between the CG and AG as regards to cell apoptosis. Also, vSMC extracted from AG was demonstrated to have higher MMPs expression which further proved phenotypic switching in vSMC. The results of our experiment can also be found in human aneurysms. Airhart *et al.* discovered that MMP-9 increased in cultured abdominal aortic aneurysm SMC, in which they presumed was attributed to a failure of post-transcriptional control of MMP-9 synthesis [21]. Wang *et al.* found that SMC derived from the thoracic aortic dissected media exhibited enhanced proliferation and elevated MMP-2 production [22]. Our research could in some aspect, provide aid in the clinical treatment of human aneurysms.

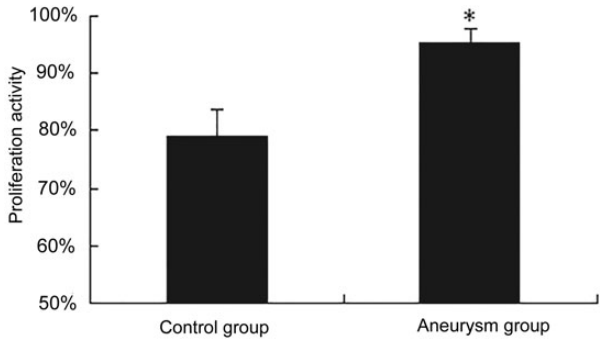

Figure 6: vSMC proliferation activity using MTT method showed that proliferation activity of CG and AG was 78.94 ± 4.69 and 95.42 ± 2.53%, respectively. There was statistic differences between the two groups with a *P*-value of <0.05 (**P* <0.05 vs CG). CG: control group; AG: aneurysm group; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide.

Then we must ask ourselves of the relationship between vSMC phenotypic switching and aortic aneurysms. The pathology of rat thoracic aortic aneurysms in our experiment showed the loss of vSMC in aortic walls and abnormal expression of MMPs which manifested as a low expression of α-SMA, SM-MHC and a high expression of MMP-2, MMP-9. Loss of vSMC results in decrease of vascular elasticity and tension, and leads to vessel dilatation. MMPs are a family of proteolytic enzymes that degrade various components of the extracellular matrix, and thus play an important role in vascular remodelling. Abnormal expression of MMPs can lead to extracellular matrix degradation and medial layer degeneration. It has been reported that the levels of MMP-2 and MMP-9 are elevated during the formation of aortic aneurysms [23], which is in line with our results both *in vivo* and *in vitro*. So we presume that under some circumstances, vSMC phenotype switches in aortic aneurysms could lead to the decrease of vSMC count and the increased synthesis of MMPs. This, in turn causes the loss of vascular elasticity and tension accompanied with degeneration of elastin and collagen. The persistent existence of vSMC phenotype switch could result in the gradual expansion of the aorta.

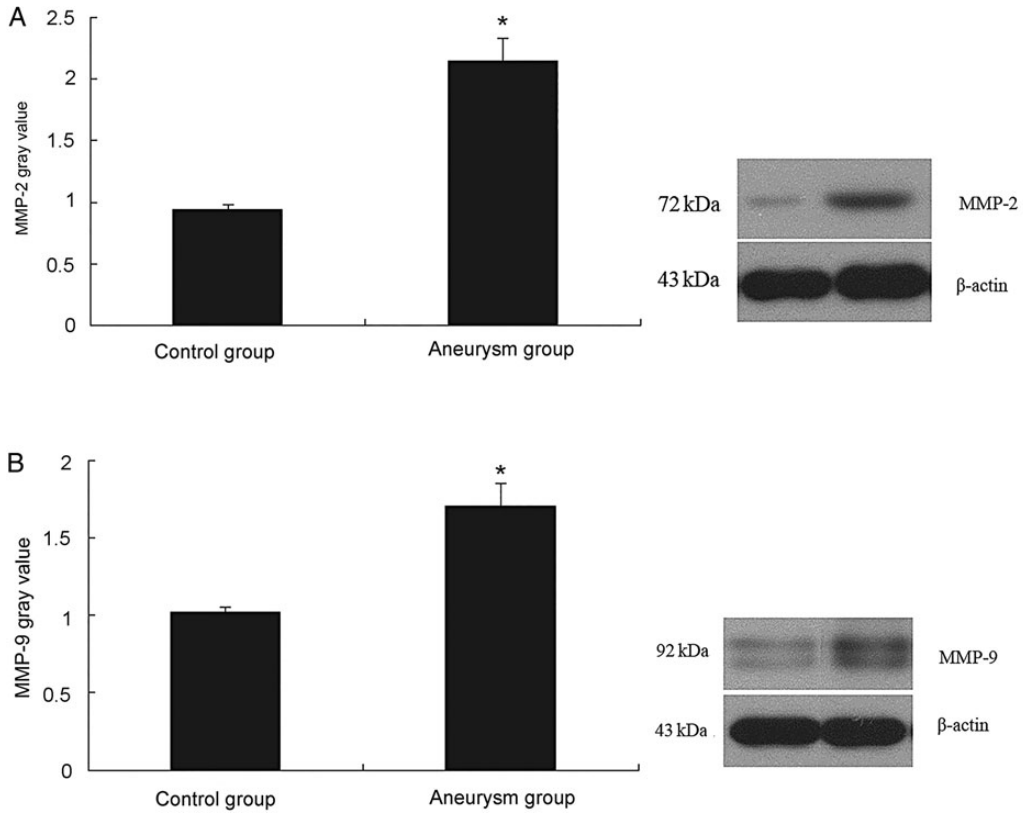

Figure 7: Western blot analysis of Passage 3-cultured vSMC showed the gray value of MMP-2 and MMP-9. (**A**) MMP-2 gray value in AG was higher than CG with significant statistic differences. (**B**) Active-MMP-9 expression in AG was higher than CG with significant statistic differences. CG: control group; AG: aneurysm group (**P* <0.05 vs CG.).

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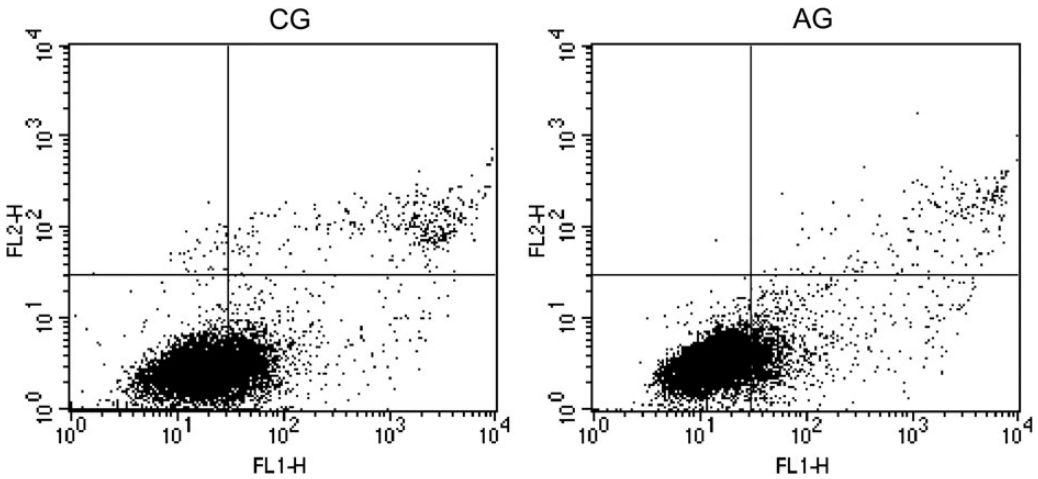

Figure 8: Flow cytometry analysis of Passage 3-cultured vSMC. (**A**) Flow cytometry analysis of CG showed the percentage of necrotic cells (LU), survival cells (LL), late apoptotic cells (RU) and early apoptotic cells (RL) were 0.37 ± 0.06, 72.12 ± 2.91, 3.40 ± 0.33 and 21.12 ± 3.17%, respectively. (**B**) Flow cytometry analysis of AG showed the percentage of necrotic cells (LU), survival cells (LL), late apoptotic cells (RU) and early apoptotic cells (RL) were 0.017 ± 0.0058, 85.88 ± 1.19, 2.05 ± 0.085 and 12.06 ± 1.17%, respectively. There were significant differences as regard to the cell apoptosis with a *P*-value of <0.01. LU: left upper quadrant; LL: left lower quadrant; RU: right upper quadrant; RL: right lower quadrant; CG: control group; AG: aneurysm group.

CONCLUSION

We researched the animal model of rat thoracic aortic aneurysms and found that vSMC phenotypic switching, which is crucial in aortic aneurysm formation, occurred during this procedure. The results of our experiment are applicable to human aneurysms and this research may provide useful recommendations on the aspect of aortic aneurysm pathology and treatment.

LIMITATION

The relationship between inflammatory factors and vSMC apoptosis has been previously indicated in atherosclerosis in which vSMC apoptosis could induce inflammatory infiltrate. Yamanouchi *et al.* [24] demonstrated that blocking apoptosis with a pan-caspase inhibitor protected mice from angiotensin II-induced vascular inflammation and aneurysm expansion which suggested vSMC apoptosis in an aneurysm setting might promote the inflammatory response. In our research, we only gave the phenomenon of vSMC apoptosis *in vitro* but did not evaluate apoptosis *in vivo* which might provide important information about aneurysm. Maybe it will be assessed in our further studies.

Conflict of interest: none declared.

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