의학석사 학위논문

In vitro differentiation of muscle-derived stem cells and their potential role in attenuation of abdominal aortic aneurysm formation

근육줄기세포의 생체 외 분화 유도 및 복부 대동맥류 형성 억제에 대한 연구

2013년 2월

서울대학교 대학원

임상의과학과

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February 2013

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이 논문을 의학석사 학위논문으로 제출함
2012년 10월

서울대학교 대학원
임상의과학과
박 형 섭

박형섭의 의학석사 학위논문을 인준함
2012년 12월

위 원 장 _____ (인)
부위원장 _____ (인)
위 원 _____ (인)

In vitro differentiation of muscle-derived stem cells and their potential role in attenuation of abdominal aortic aneurysm formation

by

Hyung Sub Park

A thesis submitted to the Department of Clinical Medical Sciences, Graduate School in partial fulfillment of the requirements for the Degree of Master of Science in Clinical Medical Sciences at Seoul National University College of Medicine

December 2012

Approved by Thesis Committee:

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논문제목 : **In vitro differentiation of muscle-derived stem cells and their potential role in attenuation of abdominal aortic aneurysm formation**

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제 출 일 : 2012 년 12 월 30 일

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ABSTRACT

Introduction: Abdominal aortic aneurysms (AAA) are a growing problem worldwide, yet there is no known medical therapy. The pathogenesis involves degradation of the elastic lamina by two combined mechanisms: increased degradation of elastin by matrix metalloproteinases (MMP) and decreased formation of elastin due to apoptosis of vascular smooth muscle cells (VSMC). In this study, we set out to examine the ability of muscle-derived stem cells (MDSC) to differentiate to VSMCs in vitro, and their potential role in the attenuation of AAA formation by inhibition of these pathogenetic mechanisms.

Methods: Muscle-derived stem cells were isolated from murine skeletal muscles using a modified preplate technique. These cells were stimulated with PDGF-BB in vitro for differentiation to VSMC-like progenitor cells (VSMC-PC) and were subsequently implanted into elastase-induced AAAs in rats. After 6 weeks, the aortas were harvested and the formation of AAA was investigated. Immunohistochemical staining, RT-PCR, western blot and gel zymography for MMPs were performed and compared to a control group.

Results: Isolated MDSCs showed characteristic expression of markers Sca-1 and CD34. When stimulated in vitro with PDGF-BB, the cells showed expression of α-SMA, a specific marker for smooth muscle cells. In vivo studies of elastase-perfused AAAs showed that the cell therapy group had

decreased rate of aneurysm formation compared to control (83% vs. 50%), and MMP expressions at the genetic, protein and enzymatic levels were significantly decreased in the cell therapy group. Furthermore, direct implantation of VSMC-PCs in the intima of harvested aortas was visualized under immunofluorescent staining, suggesting that these cells were responsible for the inhibition of MMPs and consequent attenuation of AAA formation.

Conclusions: These results show a promising role of stem cell therapy for the treatment of AAAs, and with further studies, may be able to reach clinical significance.

Keywords: Muscle stem cells, vascular smooth muscle cells, progenitor cells, abdominal aortic aneurysm, metalloproteinases

Student Number: 2011-21987

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Introduction

Stem cells have been an active source of research during the last decade because of their applicability in regenerative medicine, especially for treatment of intractable diseases. There are two characteristics of stem cells which are of importance when considering their use for therapeutic means: the ability for multilineage differentiation and self-renewal. However, certain factors hinder the clinical application of these cells. Firstly, the source of the cells may be difficult to obtain or may have ethical issues. Secondly, culturing the cells to remain in their undifferentiated state with their self-renewal ability for long periods of time may be difficult. Thirdly, differentiating the cells to only the lineage of interest and retaining these properties in vivo may not always be possible, and formation of other lineage cells may lead to carcinogenesis.

Muscle-derived stem cells (MDSC) are adult stem cells residing in skeletal muscles. They are multipotential cells, which are known to differentiate into different lineage cells, although their differentiation potential is not as strong as embryonic cells (1). They were first reported to increase muscle regeneration in dystrophin-deficient mice (2) and bone regeneration in skull-deficient mice (3), and since then, they have been an active source of research. The source of these cells is the muscle, which is an abundant and readily available source and can be used in an autogenous fashion, limiting possible rejection after implantation. Furthermore, there are no ethical issues as with embryonic stem cells. MDSCs can also be kept in culture and replicated for

long periods of time without losing its stem cell characteristics or developing features of malignant transformation (4).

Abdominal aortic aneurysm (AAA) is a chronic degenerative disease primarily affecting the elderly males, with an incidence of up to 12.5% in men aged 75 or higher (5). This incidence is increasing as the population life-span increases, and ruptured AAA is currently the 13th leading cause of death in the US. The mainstay of therapy is endovascular or surgical treatment of the aneurysm when the diameter is more than 5 cm. Unfortunately there is no proven medical therapy to treat or halt the progression of AAA, and for aneurysms that do not meet the treatment criteria, watchful waiting is the only option. Many studies have reported possible medical therapies or targets to retard AAA growth but none of them have reached widespread clinical use (6, 7).

The pathogenesis of AAA is very complex, but there is increasing evidence that the degradation of the media layer by means of a proteolytic process is the basic underlying pathophysiologic mechanism. During this process, there is a decrease in elastin content with expansion of the aortic wall, leading to a compensatory increase in collagen synthesis and subsequent remodeling of the aortic wall (8). The main proteolytic enzyme involved is metalloproteinase (MMP), which is directly involved in the degradation of elastin in the aortic wall (9, 10). Many studies have also shown that the decrease in elastin can be attributed to senescence and apoptosis of vascular smooth muscle cells (VSMC), which are involved in the production of elastin (11, 12).

In this study, muscle-derived stem cells (MDSC) were differentiated into

VSMC-like progenitor cells (VSMC-PC) in vitro and were incubated in elastase-induced abdominal aortic aneurysms in rats in an attempt to replenish the elastin content of the aortic wall and stop the progression of AAA formation.

Materials and Methods

Isolation and characterization of MDSCs

Under the approval of the Institutional Animal Care and Use Committee (IACUC) at Seoul National University Bundang Hospital for the whole study, the gastrocnemius muscles from 4 wk old male Spraque-Dawley (S-D) rats were removed and treated sequentially with serum free medium containing 0.2% collagenase II (Sigma-Aldrich, USA) at 37℃ for 1 hr, 3% dispase (Gibco BRL, USA) for 30 min and 0.125% trypsin-EDTA/PBS (Gibco BRL) for 10 min. After enzymatic dissociation, the cells were passed through a 70 ㎛ stainer and cultured in a proliferation medium, consisting of low-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 10% horse serum (Gibco BRL, USA) and 1% penicillin-streptomycin (Sigma, USA). Cells were then sequentially plated using the modified version of the preplate technique (2) to isolate MDSCs from the different populations based on their differences in adhesive properties on collagen-coated flasks. After 6 passages, the cells were plated in collagen-coated dishes and cultured for 1 wk. The remnant cells were our source of MDSC, which were then sorted with MiniMACS (Miltenyi Biotec Inc, USA) to isolate cells with CD34 (+) features. Isolated MDSCs (1-5 x10^4) were cultured in immunocytochemistry (ICC) plate slides (Nalge Nunc, USA) and

blocked with 5% blocking solution (Dako, Denmark). The primary antibodies for CD34 (1:50), Sca-1 (1:50), CD45 (1:50) and desmin (1:50 Abcam, UK) were diluted and incubated with MDSCs at 4℃ overnight. The cells were rinsed with PBS, incubated with their respective secondary antibodies (1:100) conjugated with fluorescein isothiocyanate (FITC) at 4℃ for 3 hr, and mounted with DAPI solution (Vector, USA).

Differentiation of MDSC

The cells were then transfected with lentivirus (Macrogen, Korea) tagged with Green Fluorescent Protein (GFP), by adding 1 mL of lentivirus into 6-well plates containing MDSCs (at 70-90% confluence) and 4-8 ㎍ of polybrene (Sigma, USA). The whole mixture was incubated overnight in a 37 ℃ and 5% CO_2 atmosphere and the cells were moved to a new culture medium after 24 hr. These cells were differentiated by stimulation with 20 ng/㎖ of PDGF-BB (Upstate, USA) for 2-3 d and changed to new culture medium. PDGF-BB was added every time the culture medium was changed and cells were allowed to differentiate for 7-10 d.

Abdominal aortic aneurysm model

An abdominal aortic aneurysm model was created in S-D rats by making a

midline incision under inhalation anesthesia with isoflurane. The infrarenal aorta was exposed and cross-clamped proximally and distally. An aortotomy was made just above the distal clamp site and a silastic tube connected to a syringe was inserted in the cranial direction. The aorta was clamped just above the aortotomy site and 0.1 mL of type I elastase was injected for incubation during 30 min. In the cell therapy group, 1 x 10^7 VSMC-like progenitor cells were incubated for another 30 min, while in the control group, the same amount of saline was used. After incubation, the aortotomy site was closed and clamps were removed for restoration of flow in the aorta. A total of 12 rats were used in our study, 6 in each group.

Immunohistochemical staining, zymography, western blot and RT-PCR

The aortas were harvested at 6 wk and the diameter of the aneurysms and the adjacent normal aorta were measured. Positive aneurysm formation was defined as an enlargement of the aorta by more than 2 times compared to the adjacent normal aorta, in order to compensate for possible measurement errors when using the traditional 1.5 times criteria. The harvested tissues were tested for metalloproteinases (MMP-2 and MMP-9).

Immunohistochemical staining was performed by immediate fixation of AAA specimens with 4% formalin for 24 hr and subsequent embedding in paraffin. Sections were stained by incubation of primary antibodies MMP-2 and MMP-9 (1:100, Abcam, UK) overnight, and respective secondary antibodies for 30

min. Immunoreactivity was visualized by incubation with 3,3-diaminobenzidine (DAB) substrate, counterstained with hematoxylin, mounted and visualized under microscopy,

Gel zymography was performed by homogenizing aortic tissues with lysis buffer consisting of 0.25% Triton X-100 and 0.1M Tris-HCl (pH 8.1) and 15㎕ of the supernatant together with Tris-Glycine SDS sample buffer 2X (Sigma-Aldrich, USA) were loaded onto 10% Zymogram gelatin gel (Invitrogen, USA). Electrophoresis was performed according to the manufacturer's instructions and densitometric analysis of the bands was performed with a computer-assisted bioimage analyzer (EXT-20MX, Vilber Lourmat, France).

Western blotting was performed by homogenizing aortic tissues with PRO-PREP Protein Extraction kit (Intron Biotechnology, USA). The protein concentration was determined using the Bradford method. A Laemmli 2X sample buffer (Sigma-Aldrich, USA) was mixed with the same amount of protein and heated for 5 min, after which SDS-PAGE electrophoresis was performed. Proteins were transferred to a nitrocellulose membrane filter at 100V for 1 hr, blocked with 5% nonfat dry milk in TBS, incubated overnight with primary antibodies MMP-2 and MMP-9 (1:1000, Abcam, UK) and incubated for 1 hr with anti-rabbit IgG, HRP-linked antibody. For analysis of the bands, the enhanced chemiluminescence kit (Amersham, USA) was used and densitometric analysis performed with the Chemiluminescent Image Analysis System (Bio-rad, USA).

RT-PCR was performed for MMP-2 and MMP-9 using the same methodology mentioned previously. Total RNA was extracted using the RNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. Quantification of the extracted RNA was done at 260 nm and 280 nm wavelengths using the Smartspec Plus Spectrophotometer (Bio-rad, USA). Reverse transcription was performed by sequentially mixing 2 μg of total RNA with an oligonucleotide deoxythymidine primer, 5X reaction buffer, 1mM dNTP (Bioline, UK), 20U of RNase inhibitor and reverse transcriptase Bioscript (Bioline, UK). The resulting cDNA was mixed with the respective forward and reverse primers (Table 1) and 10X PCR buffer (MangoMix, Bioline, UK). PCR amplification was performed with PTC-200 PCR Thermo cycler (MJ Research, USA) using the following cycling conditions: 94℃ for 2 min (initial denaturation); 94℃ for 30 s, 53-55℃ for 45 s, 72℃ for 30 s (35cycles); and 72℃ for 10 min (final extension). PCR products were subjected to electrophoresis in 1-2% agarose gel containing ethidium bromide.

Table 1. Primers and PCR characteristics (MMP)

Gene	Accession number	Sequence of forward and reverse primers	Temp (℃)	Size (bp)
MMP-2	NM_031054.2	5'- AGCTCCCGGAAAAGATTGAT-3'	53.5	240
		5'-TCCAGTTAAAGGCAGCGTCT-3'	53.6	
MMP-9	NM_031055.1	5'-TTCGACGCTGACAAGTG-3'	53.9	223
		5'-AGGGGAGTCCTCGTGGTAGT-3'	54.1	
GAPDH	NM_177008.4	5'- TGCCACTCAGAAGACTGT-3'	55	198
		5'-GCATGTCAGATCCACAATGG-3'	55	

Statistical analysis

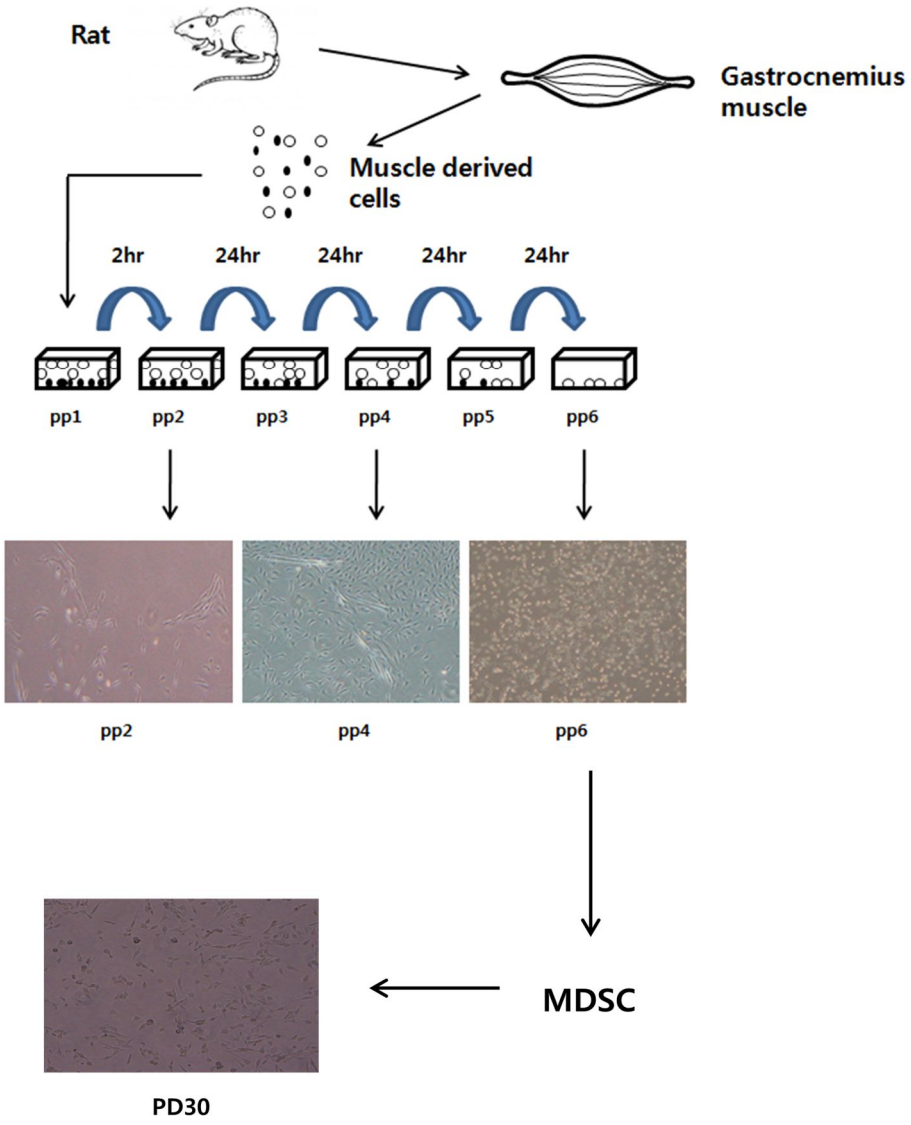
All data are presented as mean and standard error of the mean. Comparisons between two groups were performed using the independent variables Student's t-test. SPSS release 18.0 (SPSS Inc, USA) was used for analysis and differences were considered statistically significant when $P<0.05$.

Results

Isolation of MDSC

Cells obtained from skeletal muscles of S-D rats were mainly composed of myoblasts, fibroblasts and adipocytes, and these cells were separated to obtain a high yield of MDSC. This was done using the preplate technique, which separates different types of cells based on their adhesion properties. During the earlier preplates, fibroblasts and myoblasts adhered to the collagen-coated flasks, thus MDSCs remained in the supernatant. Serial preplating caused adherence of the remaining cells, including satellite cells, with the last preplate (pp6) containing a relatively homogenous population of MDSCs (Fig 1). When the different preplates were viewed under microscopy, the earlier preplates contained spindle-shaped cells (fibroblastic), while the later preplates contained morphologically round and smooth-bordered cells (stem cells). This population was further cultured to obtain a higher yield. We were able to verify that these cells could be cultured up to 30 population doublings without showing features of morphological cell senescence.

Figure 1. Modified preplate technique for isolation of MDSC

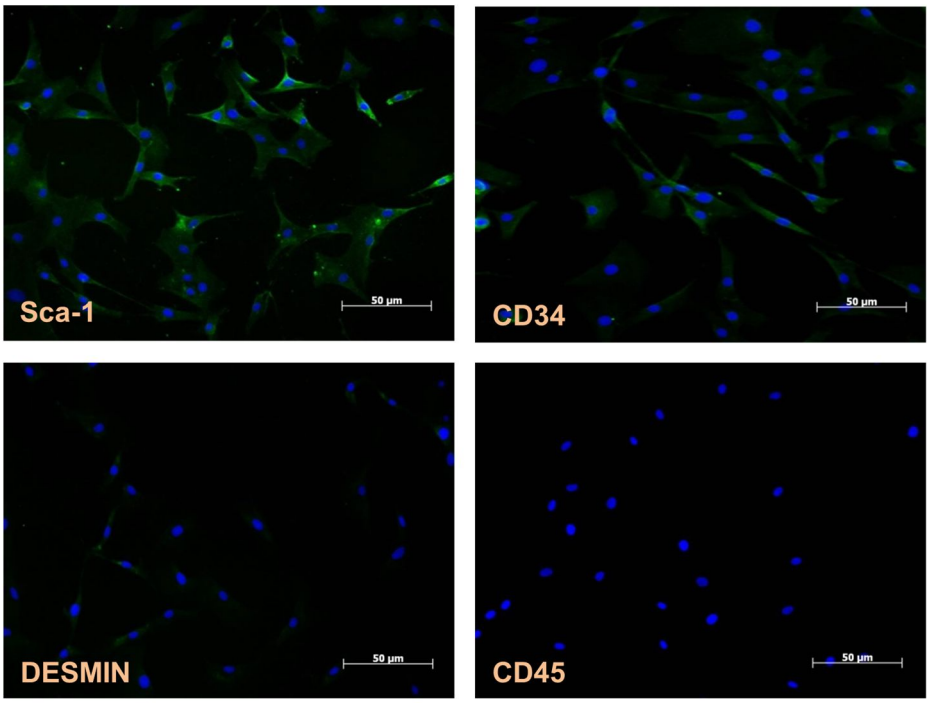


Characteristics of MDSC

Immunocytochemical analysis to verify the properties of MDSCs (pp6) showed a high expression of surface markers Sca-1 and CD34, as shown by the green fluorescence over the blue stained nuclei (DAPI) (Fig 2A). When the expressions were analyzed, Sca-1 and CD34 showed an expression rate of 90% and 70% respectively, which was significantly higher than earlier preplates (pp2). Expression of desmin was indeterminate (30-40%) although it showed a higher expression than early preplate cells while CD45 was negative (data not shown).

Figure 2. Immunocytochemical analysis of markers in MDSC

Immunocytochemical staining of MDSCs showing positive expression of Sca-1 and CD34, while desmin and CD45 expression is not visualized. (200x magnification)

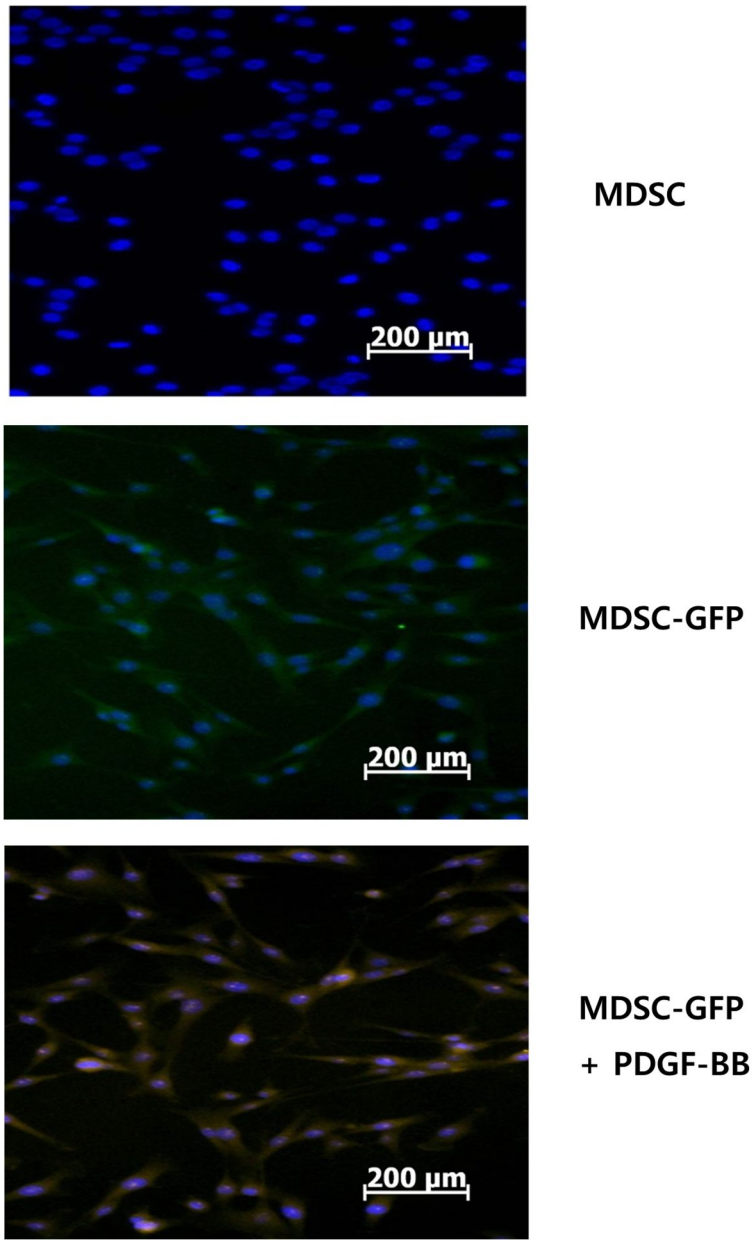


Differentiation to VSMC lineage

Differentiation of MDSCs was performed after transfection of the cells with lentivirus tagged with GFP. Immunocytochemical analysis showed a characteristic green color, demonstrating that the cells were successfully tagged with GFP. When GFP-tagged MDSCs were cultured with PDGF-BB, the resulting cells showed a characteristic yellow color under ICC stain, which was a result of merging of the green GFP with a red color representing α-SMA, a characteristic marker for smooth muscle cells (Fig 3). Also differentiated cells showed morphologically spindle-shaped cells, resembling smooth muscle cells, as opposed to the round shaped native MDSCs,

Figure 3. In vitro differentiation of MDSC with PDGF-BB

Immunocytochemical staining of MDSCs tagged with GFP (green) and after stimulation with PDGF-BB to show expression of α-smooth muscle actin (red) (100x magnification)

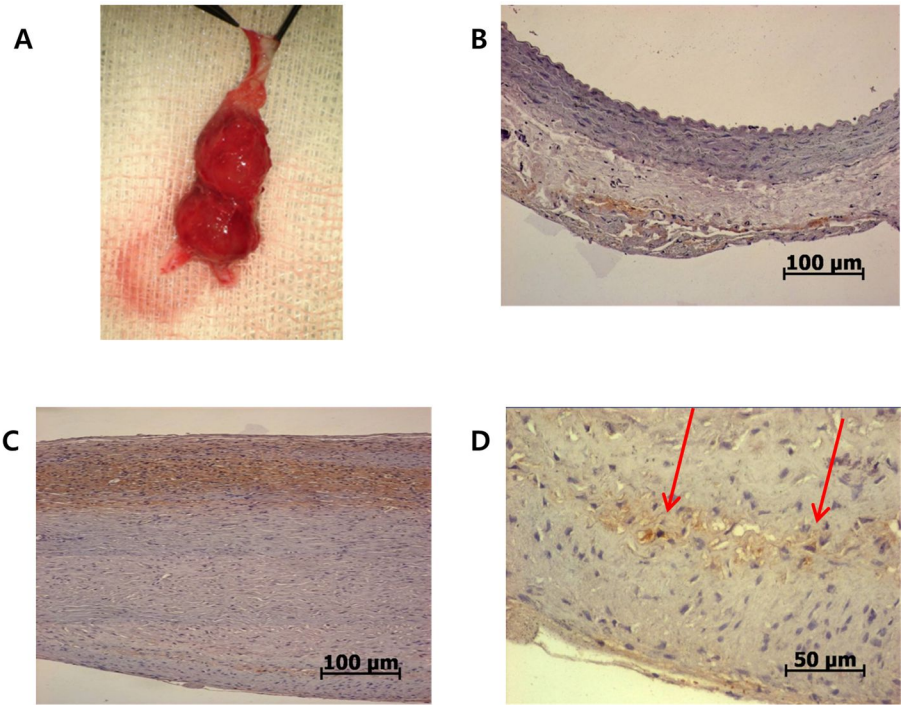


Attenuation of aneurysm formation in S-D rats

Aneurysms created in rats with elastase showed characteristic enlargement of the aorta (Fig 4A). IHC staining with MMP-9 showed an overall higher expression of MMP-9 in the aneurysmal wall compared to control (Fig 4B-D). Aneurysm formation was found in 5 out of 6 rats (83%) in the control group, while only 3 rats out of 6 (50%) had aneurysm formation in the cell therapy group.

Figure 4. Immunohistochemical staining of harvested AAA

(A) Representative infrarenal AAA from elastase perfused rats. (B) Immunohistochemical staining for MMP-9 in (B) normal aorta and (C) aneurysmal tissue (200x magnification). (D) Higher amplification of aneurysmal tissue reveals positive expression of MMP-9 stained cells (arrows) (400x magnification).

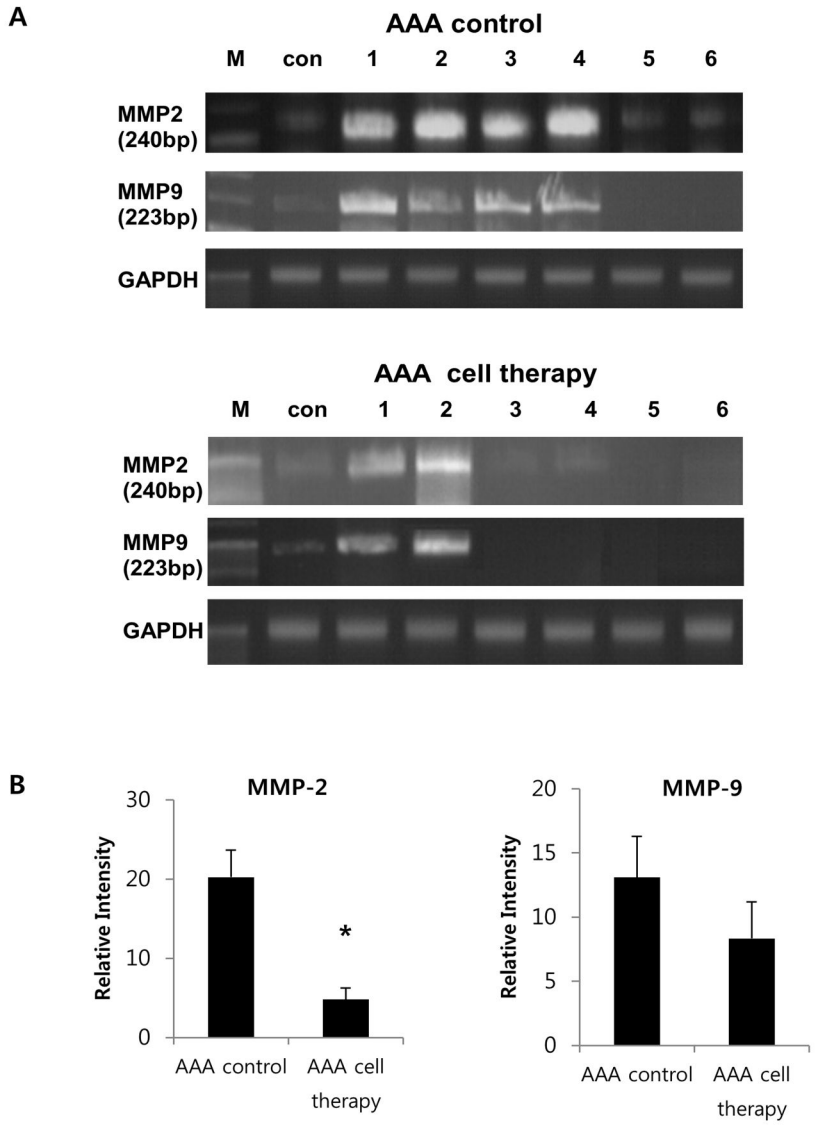


Decreased genetic expression of MMP

Harvested tissues from both cell therapy and control groups were used to perform RT-PCR, Western blot and gel zymography. RT-PCR for MMP-2 and MMP-9 mRNA expression was done for both groups and the relative intensities of the bands were represented graphically (Fig 5). The results show that there was a significantly lower expression of MMP-2 in the cell therapy group compared to control ($P<0.05$). There was also a tendency for lower expression of MMP-9 in the cell therapy group, without statistical significance.

Figure 5. RT-PCR of harvested AAA for MMP

(A) RT-PCR analysis for MMP-2 and MMP-9 in cell therapy and control groups (B) Quantification of gene expression normalized against GAPDH for MMP-2 and MMP-9. Cell therapy group has significantly lower expression of MMP-2 compared to control. * P<0.05

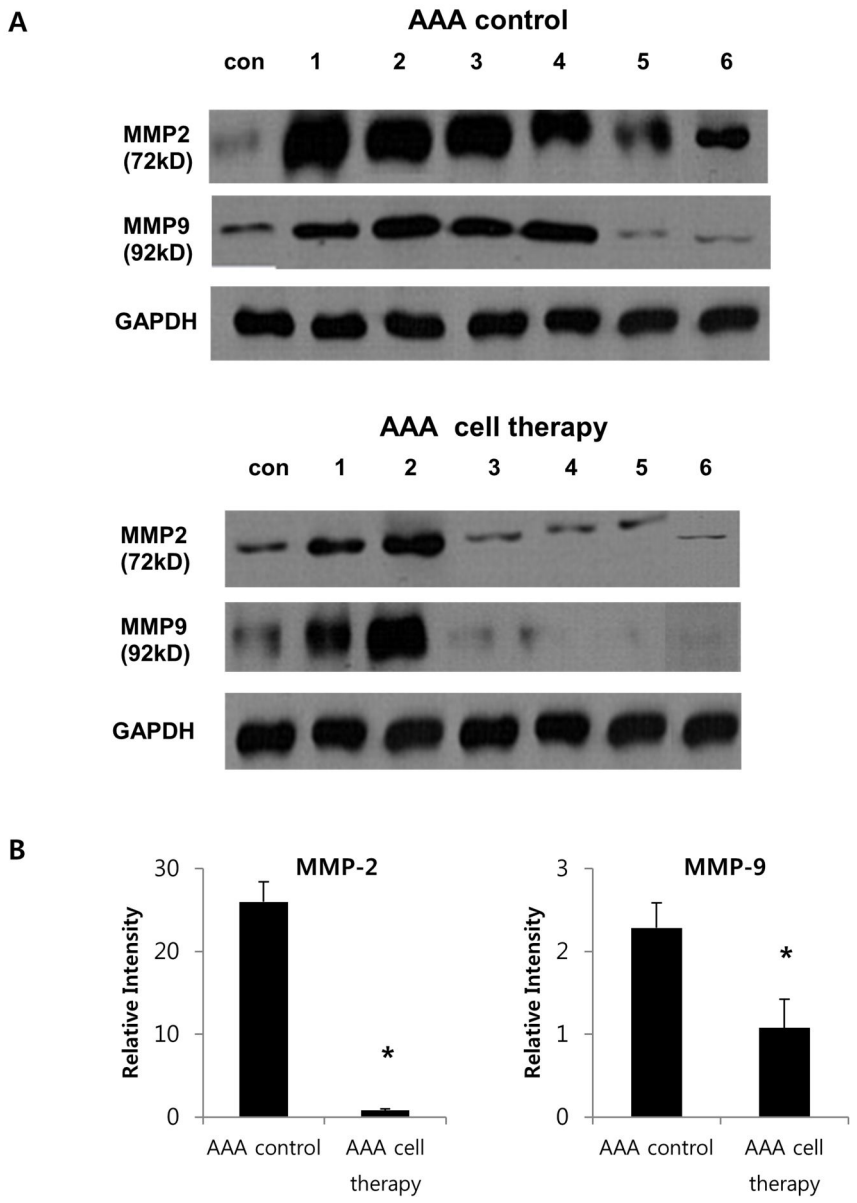


Decreased MMP content

The expression of MMP-2 and MMP-9 were also investigated at the protein level by Western blot (Fig 6A). Both MMP-2 and MMP-9 protein content was lower in the cell therapy group compared to control, which was statistically significant ($P<0.05$) (Fig 6B).

Figure 6. Western blot of harvested AAA for MMP

(A) Western blot of MMP-2 and MMP-9 for cell therapy and control groups. (B) Quantification of protein content normalized against GAPDH shows significantly lower expression on both MMP-2 and MMP-9 in the cell therapy group compared to control. * P<0.05.

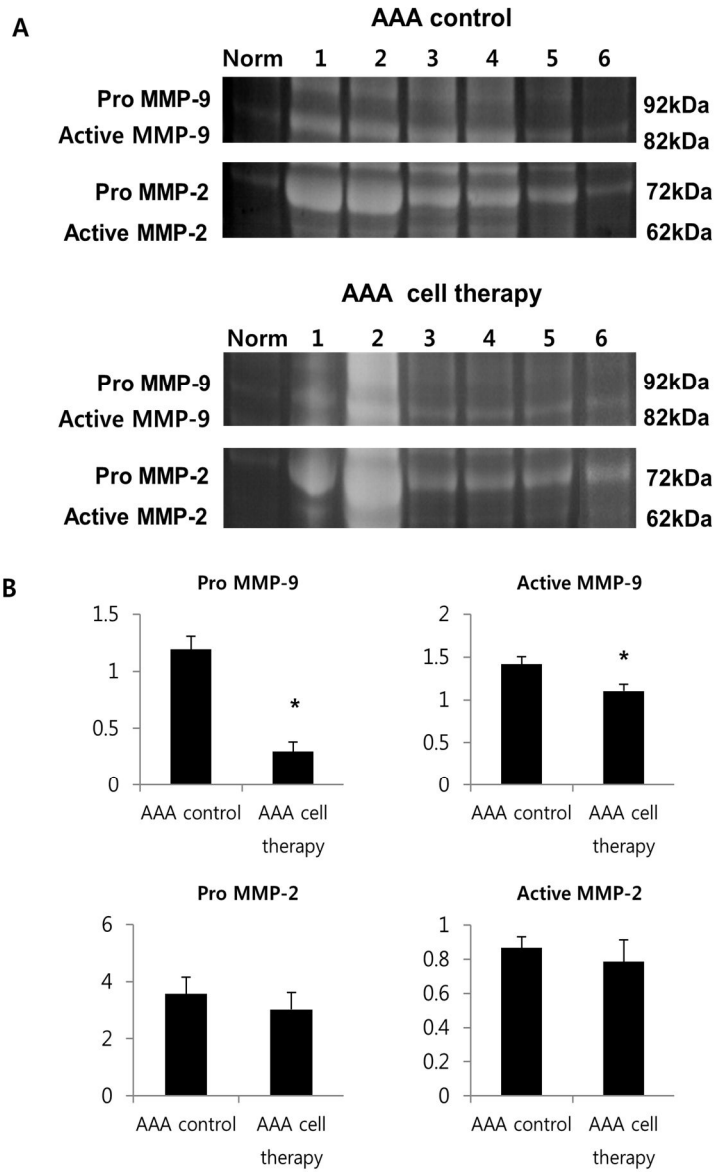


Decreased MMP activity

Gel zymography was performed to demonstrate the enzymatic activity of MMPs, and in both groups characteristic bands were present for pro and active forms of MMP-2 and MMP-9 (Fig 7A). The cell therapy group had a significantly lower activity of pro MMP-9 and active MMP-9 compared to control, and there was a tendency for lower activity of pro and active MMP-2, but without statistical significance (Fig 7B).

Figure 7. Gel zymography of harvested AAA for MMP

(A) Gel zymography shows representative bands for MMP-2 and MMP-9 in both cell therapy and control groups. Norm represents normal aorta. (B) Pro and active MMP-2 and MMP-9 activity normalized against GAPDH. Cell therapy group has significantly lower expression of both pro and active MMP-9 compared to control. * P<0.05.

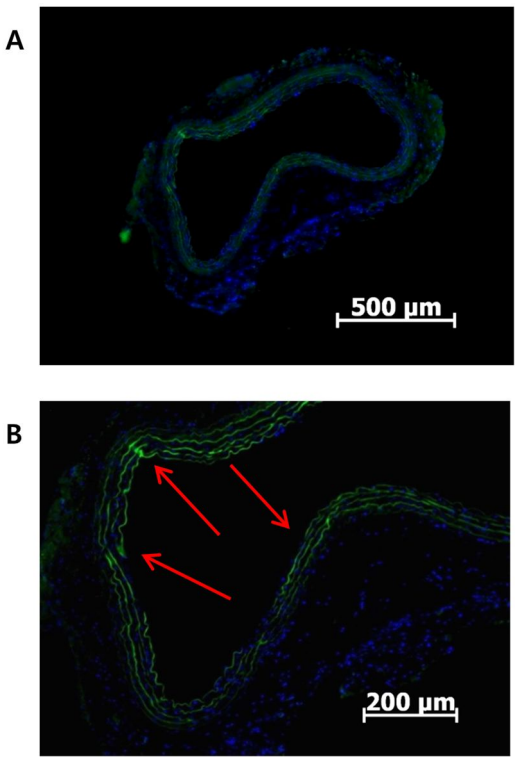


Direct implantation of VSMC-PC in the intima layer of harvested AAA

In the cell therapy group, harvested aneurysms showed positive expression of GFP, which suggests that GFP tagged VSMC-PCs may have had an effect in the overall smaller rate of aneurysm formation in this group (Fig 8).

Figure 8. Immunofluorescent GFP staining of harvested AAA

(A) Immunofluorescent staining of aneurysmal tissue shows implantation of GFP-tagged VSMC-PC in the intima layer (50x magnification). (B) Higher magnification shows characteristic green stained areas (arrows) demonstrating direct VSMC-PC implantation (100x magnification).



Discussion

This study shows that MDSCs isolated from skeletal muscles of rats exhibited VSMC-like properties when stimulated with PDGF-BB for differentiation in vitro. These VSMC-PCs significantly decreased expression of MMPs, especially MMP-2 and MMP-9, at the enzymatic, genetic and protein levels, and were able to attenuate formation of elastase-induced murine AAA. Successful implantation of these cells was observed in the specimens of AAA.

Several studies have demonstrated different marker expressions for MDSCs. There is currently no standard marker to define MDSCs, although Sca-1 is known to be the most consistent marker (1). Reports on CD34 and desmin have shown variable results (13, 14) while others have reported expression of CD13, CD10 and CD56 on these cells (15). In any case, all these studies have shown negative expression of hematopoietic markers, including CD45.

We have previously been able to differentiate MDSCs in vitro into endothelial lineage cells using shear stress and VEGF. These differentiated cells showed positive expression of endothelial markers von Willebrand factor (vWF), VE-cadherin, Flk-1 and CD31, and were able to produce capillary-like tubules in Matrigel assay. When tested in vivo, these cells were able to increase angiogenesis in a murine ischemic hindlimb model, and also attenuate intimal hyperplasia in a murine carotid injury model. In this study, a similar strategy was used where we stimulated MDSCs in vitro with PDGF-BB. PDGF is known to have effects on VSMCs by inducing proliferation, migration and contraction (16, 17) and stem cells have been previously differentiated to

VSMCs by stimulation with PDGF (18-20). Such differentiated MDSCs showed positive α-SMA expression, a specific marker for SMC. Therefore PDGF-BB stimulation of MDSCs was able to drive these multipotent cells to differentiate towards the SMC lineage, thus showing features of VSMC progenitors (VSMC-PC).

Many studies have tried to investigate the role of VSMC progenitors in vascular diseases, especially atherosclerosis, and there is increasing evidence that these cells have a causal effect in the pathogenesis of atherosclerosis, which is contrary to the traditional theory that these cells have a protective effect in vascular diseases (21). The underlying mechanism involves differentiation of stem cells and VSMC progenitor cells into SMCs in the intima layer leading to neointimal hyperplasia and consequent atherosclerosis (22, 23). In the case of AAA, the main etiology is known to be a degenerative/atherosclerotic process, but many studies have highlighted the degenerative process, supported by the role of elevated MMPs. Some degree of atherosclerosis is present in AAA, but there is evidence that this may be a secondary phenomenon to the degenerative process (24). As mentioned previously, this degenerative process involves degradation of elastin in the media layer by increased MMPs and apoptosis of VSMCs. Therefore VSMCs may play an important role and subsequently led to our theory that VSMC-PCs may have inhibitory effects on AAA formation and growth. Indeed, our in vivo studies with VSMC-PCs demonstrated attenuation of AAA formation with suppression of MMP expression. The mechanism related to these findings is unknown, but we were able to show implantation of exogenously

administered VSMC-PCs in the intima layer. Implanted VSMC-PCs probably had some influence, but cannot solely explain for the observed findings. We suggest that administered VSMC-PCs exerted their effects in a paracrine manner via multiple factors such as cytokine or growth factors, depending on the microenvironmental condition. This microenvironment together with the associated immunomodulatory and homing functions by paracrine factors are known to be fundamental for differentiation of stem cells to desired lineages for specific functions (25, 26). In our study, this microenvironment led to the repair of injured aorta, probably by replenishment of apoptotic VSMCs and suppression of MMPs. Under other microenvironmental conditions, it may lead to formation of intimal hyperplasia, as is the case with atherosclerosis.

Recently, a few studies have reported the possible use of stem cell therapy for the attenuation of AAA in animal models (27, 28). Mesenchymal stem cells (MSC) from the bone marrow were used in these studies, with successful attenuation of murine AAA formation through elastin preservation, associated suppression of MMPs and inhibition of inflammatory cytokines, especially IL-17. In another study, the successful implantation of MSCs was demonstrated in a porcine AAA model (29). In our study we used MDSCs, which are known for their high regeneration capacity, their ability to differentiate to multilineage cells, and their self-renewal capabilities. It has been demonstrated that MDSCs can be differentiated into myogenic, osteogenic chondrogenic, adipogenic, endothelial, neuronal and hematopoietic lineages (30-35). MDSCs also maintain their stem cell characteristics in vitro and in vivo even after 200 population doublings, demonstrating a high

regeneration and self-renewal capacity (4). Above all, muscle tissue is the most abundant tissue in the body, which can be easily and safely obtained, making it an attractive source compared to MSCs (36).

Our study has the advantage of stimulating MDSCs in vitro, since we provided the drive for differentiation in a controlled ex vivo environment, which may be more suited to survive the initial environment and contribute to the regeneration process (37). Additionally it is one of the few studies showing the potential role of stem cell based therapies in AAA, and the first to use MDSCs, which is an attractive source. Although this study is limited by the small numbers and the limited use of markers and parameters, the results are very promising. It is also in trend with the recent popularity of stem cell research and therapy and with further studies, it may have potential translational relevance.

References

1. Jankowski RJ, Deasy BM, Huard J. Muscle-derived stem cells. Gene therapy. 2002;9(10):642-7.

2. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R, et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol. 2002;157(5):851-64.

3. Lee JY, Qu-Petersen Z, Cao B, Kimura S, Jankowski R, Cummins J, et al. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. The Journal of cell biology. 2000;150(5):1085-100.

4. Deasy BM, Gharaibeh BM, Pollett JB, Jones MM, Lucas MA, Kanda Y, et al. Long-term self-renewal of postnatal muscle-derived stem cells. Molecular biology of the cell. 2005;16(7):3323-33.

5. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation. 2010;121(7):e46-e215.

6. Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, Furutani A, et al. Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. Nature medicine. 2005;11(12):1330-8.

7. Liu J, Xiong W, Baca-Regen L, Nagase H, Baxter BT. Mechanism of inhibition of matrix metalloproteinase-2 expression by doxycycline in human aortic smooth muscle cells. J Vasc Surg. 2003;38(6):1376-83.

8. Choke E, Cockerill G, Wilson WR, Sayed S, Dawson J, Loftus I, et

al. A review of biological factors implicated in abdominal aortic aneurysm rupture. European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery. 2005;30(3):227-44.

9. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circulation research. 2002;90(3):251-62.

10. Tamarina NA, McMillan WD, Shively VP, Pearce WH. Expression of matrix metalloproteinases and their inhibitors in aneurysms and normal aorta. Surgery. 1997;122(2):264-71; discussion 71-2.

11. Rowe VL, Stevens SL, Reddick TT, Freeman MB, Donnell R, Carroll RC, et al. Vascular smooth muscle cell apoptosis in aneurysmal, occlusive, and normal human aortas. J Vasc Surg. 2000;31(3):567-76.

12. Allaire E, Muscatelli-Groux B, Mandet C, Guinault AM, Bruneval P, Desgranges P, et al. Paracrine effect of vascular smooth muscle cells in the prevention of aortic aneurysm formation. J Vasc Surg. 2002;36(5):1018-26.

13. Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature. 1999;401(6751):390-4.

14. Torrente Y, Tremblay JP, Pisati F, Belicchi M, Rossi B, Sironi M, et al. Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. The Journal of cell biology. 2001;152(2):335-48.

15. Young HE, Steele TA, Bray RA, Detmer K, Blake LW, Lucas PW, et al. Human pluripotent and progenitor cells display cell surface cluster

differentiation markers CD10, CD13, CD56, and MHC class-I. Proc Soc Exp Biol Med. 1999;221(1):63-71.

16. Hughes AD, Clunn GF, Refson J, Demoliou-Mason C. Platelet-derived growth factor (PDGF): actions and mechanisms in vascular smooth muscle. General pharmacology. 1996;27(7):1079-89.

17. Inui H, Kitami Y, Tani M, Kondo T, Inagami T. Differences in signal transduction between platelet-derived growth factor (PDGF) alpha and beta receptors in vascular smooth muscle cells. PDGF-BB is a potent mitogen, but PDGF-AA promotes only protein synthesis without activation of DNA synthesis. The Journal of biological chemistry. 1994;269(48):30546-52.

18. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, et al. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest. 2004;113(9):1258-65.

19. Margariti A, Xiao Q, Zampetaki A, Zhang Z, Li H, Martin D, et al. Splicing of HDAC7 modulates the SRF-myocardin complex during stem-cell differentiation towards smooth muscle cells. Journal of cell science. 2009;122(Pt 4):460-70.

20. Miyata T, Iizasa H, Sai Y, Fujii J, Terasaki T, Nakashima E. Platelet-derived growth factor-BB (PDGF-BB) induces differentiation of bone marrow endothelial progenitor cell-derived cell line TR-BME2 into mural cells, and changes the phenotype. Journal of cellular physiology. 2005;204(3):948-55.

21. Margariti A, Zeng L, Xu Q. Stem cells, vascular smooth muscle cells and atherosclerosis. Histology and histopathology. 2006;21(9):979-85.

22. Han CI, Campbell GR, Campbell JH. Circulating bone marrow cells

can contribute to neointimal formation. Journal of vascular research. 2001;38(2):113-9.

23. Hillebrands JL, Klatter FA, van den Hurk BM, Popa ER, Nieuwenhuis P, Rozing J. Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. J Clin Invest. 2001;107(11):1411-22.

24. Xu C, Zarins CK, Glagov S. Aneurysmal and occlusive atherosclerosis of the human abdominal aorta. J Vasc Surg. 2001;33(1):91-6.

25. Gharaibeh B, Lavasani M, Cummins JH, Huard J. Terminal differentiation is not a major determinant for the success of stem cell therapy - cross-talk between muscle-derived stem cells and host cells. Stem cell research & therapy. 2011;2(4):31.

26. Satija NK, Singh VK, Verma YK, Gupta P, Sharma S, Afrin F, et al. Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. Journal of cellular and molecular medicine. 2009;13(11-12):4385-402.

27. Hashizume R, Yamawaki-Ogata A, Ueda Y, Wagner WR, Narita Y. Mesenchymal stem cells attenuate angiotensin II-induced aortic aneurysm growth in apolipoprotein E-deficient mice. J Vasc Surg. 2011;54(6):1743-52.

28. Sharma AK, Lu G, Jester A, Johnston WF, Zhao Y, Hajzus VA, et al. Experimental Abdominal Aortic Aneurysm Formation Is Mediated by IL-17 and Attenuated by Mesenchymal Stem Cell Treatment. Circulation. 2012;126(11 Suppl 1):S38-45.

29. Turnbull IC, Hadri L, Rapti K, Sadek M, Liang L, Shin HJ, et al.

Aortic implantation of mesenchymal stem cells after aneurysm injury in a porcine model. The Journal of surgical research. 2011;170(1):e179-88.

30. Tamaki T, Akatsuka A, Ando K, Nakamura Y, Matsuzawa H, Hotta T, et al. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. The Journal of cell biology. 2002;157(4):571-7.

31. Bosch P, Musgrave D, Ghivizzani S, Latterman C, Day CS, Huard J. The efficiency of muscle-derived cell-mediated bone formation. Cell transplantation. 2000;9(4):463-70.

32. Lu SH, Yang AH, Wei CF, Chiang HS, Chancellor MB. Multi-potent differentiation of human purified muscle-derived cells: potential for tissue regeneration. BJU international. 2010;105(8):1174-80..

33. De Angelis L, Berghella L, Coletta M, Lattanzi L, Zanchi M, Cusella-De Angelis MG, et al. Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. The Journal of cell biology. 1999;147(4):869-78.

34. Alessandri G, Pagano S, Bez A, Benetti A, Pozzi S, Iannolo G, et al. Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages. Lancet. 2004;364(9448):1872-83.

35. Jackson KA, Mi T, Goodell MA. Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(25):14482-6.

36. Wu X, Wang S, Chen B, An X. Muscle-derived stem cells: isolation, characterization, differentiation, and application in cell and gene therapy. Cell

and tissue research. 2010;340(3):549-67.

37. Deasy BM, Jankowski RJ, Huard J. Muscle-derived stem cells: characterization and potential for cell-mediated therapy. Blood cells, molecules & diseases. 2001;27(5):924-33.

국 문 초 록

서론: 복부대동맥류(AAA)는 전세계적으로 유병률이 높은 질병이나 현재 수술이 아닌 약물 치료법은 알려진 것이 없다. 이 질병은 대동맥의 탄성판이 분해되면서 발생하는데, 이는 기질 금속 단백질 분해효소의 증가로 인한 엘라스틴의 분해와 혈관 평활근세포의 사멸로 인한 엘라스틴 생성 감소에 의해 일어난다. 본 연구에서는 생체 외에서 근육줄기세포를 혈관 평활근세포로의 분화 유도하고 이를 이용한 복부대동맥류의 형성 억제 가능성을 확인하고자 하였다.

방법: 랫트의 골격근으로부터 modified preplate technique를 이용하여 근육줄기세포를 분리하였다. 생체 외에서 PDGF-BB를 이용하여 근육줄기세포를 혈관 평활근세포 유사 전구세포로 분화 유도하였으며, 이 세포들을 랫트의 복부대동맥류 모델에 주입하였다. 6주 후 랫트의 대동맥을 채취하여 복부대동맥류의 형성 여부를 확인하였고, 기질 금속 단백질 분해효소에 대한 면역조직화학 염색, RT-PCR, Western blot 및 gel zymography를 시행하여 대조군과 비교하였다.

결과: 분리된 근육줄기세포는 Sca-1 및 CD34와 같은 표지자의 양성 발현을 보였다. 생체 외에서 PDGF-BB에 의해 분화 유도된 세포들은 평활근세포의 특이적 표지자인 α-SMA의 양성 발현을 보였다. 엘라스테이즈 유도 복부대동맥류 모델에서 세포 주입군이 대조군에 비하여 더 낮은 복부대동맥류 형성률을 보였으며 (83% 대 50%), 세포 주입군에서 기질 금속 단백질 분해효소의 유전 발현, 단백질 형성 및 효소 활성이 유의하게 감소되어 있었다. 또한, 채취된 대동맥의 형광 면역 염색에서 내막에 혈관 평활근세포 유사 전구세포의 이식이 관찰되어, 이러한 전구세포에 의해 기질 금속 단백질 분해효소의 억제 및 이로 인한 복부대동맥류 형성의 감소 효과가 나타난 것으로 판단하였다.

결론: 본 연구를 통해 복부대동맥류의 줄기세포 치료의 가능성을 확인하였으며, 추가 연구를 시행한다면 추후 임상적으로도 적용이 가능할 것으로 판단된다.

주요어: 근육줄기세포, 혈관 평활근세포, 전구세포, 복부 대동맥류, 기질 금속 단백질 분해효소

학 번: 2011-21987