Modulation of Vascular Smooth Muscle Cell Phenotype by High Mobility Group AT-Hook 1

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ABSTRACT

Objective: The purpose of this study is to examine the effect of high mobility group AT-hook 1 (HMGA1) on the phenotypic change of vascular smooth muscle cells (VSMCs).

Methods: Gene silencing and overexpression of HMGA1 were introduced to evaluate the effect of HMGA1 expression on the phenotypic change of VSMCs. Marker gene expression of VSMCs was measured by promoter assay, quantitative polymerase chain reaction, and western blot analysis. Common left carotid artery ligation model was used to establish in vivo neointima formation.

Results: HMGA1 was expressed strongly in the synthetic type of VSMCs and significantly downregulated during the differentiation of VSMCs. Silencing of HMGA1 in the synthetic type of VSMCs enhanced the expression of contractile marker genes thereby enhanced angiotensin II (Ang II)-dependent contraction, however, significantly suppressed proliferation and migration. Stimulation of contractile VSMCs with platelet-derived growth factor (PDGF) enhanced HMGA1 expression concomitant with the downregulation of marker gene expression which was blocked significantly by the silencing of HMGA1. Silencing of HMGA1 retained the Ang II-dependent contractile function, which was curtailed by PDGF stimulation, however, overexpression of HMGA1 in the contractile type of VSMCs suppressed marker gene expression. Proliferation and migration were enhanced significantly by the overexpression of HMGA1. Furthermore, the Ang II-dependent contraction was reduced significantly by the overexpression of HMGA1. Finally, the expression of HMGA1 was enhanced significantly in the ligated artery, especially in the neointima area.

Conclusion: HMGA1 plays an essential role in the phenotypic modulation of VSMCs. Therefore, paracrine factors such as PDGF may affect vascular remodeling through the regulation of HMGA1.

Keywords: HMGA; Smooth muscle; Phenotype; Cell proliferation; Platelet-derived growth factor

INTRODUCTION

Generally, vascular smooth muscle cells (VSMCs) exist in two phenotypes. In healthy blood vessels, the mature form of VSMCs is the contractile type of VSMCs, which expresses the marker genes involved in the contractile function, such as smooth muscle actin (SMA),
smooth muscle 22 α (SM22α), calponin, and myosin heavy chain 11 (Myh11). The contractile type of VSMCs show halted proliferation and migration activity. Because VSMCs have phenotypic plasticity, unlike cardiac or skeletal muscles, the contractile type of VSMCs can be converted to the type with more proliferation and migration activity, which is also called the synthetic phenotype of VSMCs. The phenotypic conversion of VSMCs is induced by several environmental cues, such as vascular damage, vascular inflammation, aging, and metabolic stresses. Upon vascular damage, platelets aggregate rapidly to form a thrombus and stop bleeding. During this process, aggregated platelets secrete many paracrine factors involved in fibrin coagulation, blood vessel constriction, and tissue repair factors that induce damage-associated molecular patterns (DAMPs). One of these secreted paracrine factors, platelet-derived growth factor (PDGF) plays a role as a tissue repair factor. For example, PDGF facilitates 10T1/2 cells to differentiate into VSMCs. Moreover, PDGF changes the contractile marker gene expression profile, and the intracellular calcium regulation mechanism by PDGF is regulated differentially in the contractile and synthetic types of VSMCs. Finally, expression of the PDGF receptor is upregulated in VSMCs in response to injury, and stimulation of VSMCs with PDGF facilitates the phenotypic changes. Therefore, PDGF appears to play a pivotal role in the regulation of VSMC phenotype during the vascular injury.

High mobility group AT-hook 1 (HMGA1) is a non-histone chromatin protein involved in various inducible gene transcriptions. HMGA1 might regulate the global gene expression pattern by chromatin remodeling because HMGA1 can induce chromatin clustering in vivo. HMGA1 proteins interact physically with various transcription elements and the regulator of the chromatin structure, coordinates their assembly in the gene promoter and enhancer areas, and provides an important function during gene-specific transcription regulation. Therefore, HMGA1 could be necessary for the physiological responses needed to change the global gene expression pattern, e.g., differentiation/dedifferentiation, in this case, the phenotypic modulation of VSMCs.

Several studies assigned the important role of HMGA1 in regulating normal cell proliferation, embryonic cell growth, and cell differentiation. In general, high levels of HMGA1 have been observed in actively growing cells, and low levels of HMGA1 have been shown in terminally differentiated and non-dividing cells. For example, the expression of HMGA1 is high in cells in the early stages of development, cancer cells, and those related to diseases showing abnormal growth. On the other hand, cells in a stable condition with completed growth express low levels of HMGA1. VSMCs retain their two different characteristic stages, one is the actively growing synthetic type, and the other is the non-dividing contractile type. This study tested the hypothesis that HMGA1 plays a key role in the phenotypic modulation of VSMCs as a downstream transcriptional factor of PDGF.

MATERIALS AND METHODS

1. Reagents
Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin (antibiotics) were purchased from Hyclone Laboratories, Inc (Logan, UT, USA). Angiotensin II (Ang II), anti-SMA and anti-calponin antibodies obtained from Sigma-Aldrich (St Louis, MO, USA). The anti-Myh11 antibody was acquired from Proteintech Group Inc. (Burlingame, CA, USA). The anti-SM22α and anti-HMGA1 antibodies were supplied by Abcam (Cambridge, UK). The anti-actin antibody was supplied by MP biomedicals.
4′,6-diamidino-2-phenylindole (DAPI), Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and Cy3-conjugated goat anti-rabbit secondary antibody were bought from Molecular Probes, Inc. (Carlsbad, CA, USA). IRDye700- and IRDye800-conjugated rabbit/mouse secondary antibodies were obtained from Li-COR Bioscience (Lincoln, NE, USA). The promoter region of SM22α, SMA subcloned in the pGL3 vector, was a kind gift from Dr. Gary K. Owens (University of Virginia). All other chemicals were purchased from Sigma-Aldrich unless indicated otherwise.

2. Isolation and phenotypic conversion of VSMCs, and neointima formation

Synthetic type of VSMCs were isolated from 3-week-old male Sprague-Dawley rats as described previously. Briefly, VSMCs were isolated from 3-week-old Sprague-Dawley rats and the surrounding fat and connective tissues were discarded. The vessels were cut longitudinally and fragmented into 3–5 mm lengths, and the explanted lumen was placed side down on 6-well culture dishes. After 7 days of explanting, the tissue fragments were discarded, and the sprouted VSMCs were collected (referred to as P0). The synthetic type of VSMCs was cultured at low density (<20%). To induce phenotypic conversion of VSMCs, the synthetic type of VSMCs (P0) was cultured on laminin-coated plates at high density (up to 100%), and passages between P3 and P4 were defined as the contractile type of VSMCs. Carotid artery ligation-induced neointima formation was performed as described previously. Since our experiments used cells isolated from aortic tissues sex difference would not affect in vitro experimental results. All animal procedures were performed in accordance with the Animal Care Guidelines of the Laboratory Animal Resource Center of Pusan National University School of Medicine after receiving approval of Pusan National University Institutional Animal Care and Use Committee (mouse: PNU-2019-2253, rat: PNU-2019-2254). The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 8023, revised 1978).

3. Cell proliferation assay

To measure cell proliferation, 5×10³ VSMCs were seeded on a 6-well plate and grown for 6 days in a normal culture medium. Cells of 0, 2, 4, and 6 days were fixed and counted. Zero days refers to 3 hours after seeding. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), and the nuclei were stained with DAPI (Sigma-Aldrich). The stained cells were visualized with a fluorescence microscope and random images of three fields were taken at ×10 magnification.

4. Migration assay

Migration of VSMCs were measured as described previously. Briefly, 5×10³ cells were overlaid on a ChemoTx membrane (Neuroprobe Inc., Gaithersburg, MD, USA) and incubated for the indicated times. The membrane was stained with DAPI, and the migrated cells on bottom side were counted under a fluorescence microscope at ×10 magnification.

5. Collagen gel contraction assay

Ang II-induced VSMC contraction was performed as described previously. Briefly, VSMCs were trypsinized, resuspended in serum free DMEM, and diluted to 1×10⁶ cells/mL. The cell suspension was mixed on ice with collagen gel solution (6 mg/mL of collagen type I in 2X PBS pH8.0) to give 5×10⁵ cells/mL and 3 mg/mL of collagen gel solution. One hundred µL of VSMCs-collagen gel solution was added per well to 24-well plates. Where indicated, various inhibitors were added. The plate was incubated at 37°C to allow for gel polymerization. After 1 hour, the gels were floated with serum-free DMEM and Ang II (1 µM) was added to initiate
contraction while capturing images using a digital charge-coupled device camera. Collagen gel contraction was measured as a decrease in the gel area using image software (Image J; National Institutes of Health, Bethesda, MD, USA).

6. Lentiviral knockdown and retroviral overexpression of HMGA1
To silence HMGA1, short hairpin RNA oligonucleotides tagged with a 5′-end AgeI site and a 3′-end EcoRI site were designed for shHMGA1 (forward primer: 5′-CCG GTA AGG GGC AGA CCC AAG AAA CTC TCG AGA GTT TCT TGG TGC TGC CCC TTT TTT TG-3′, reverse primer: 5′-ATT CAA AAA AAG GGG CAG ACCCAA GAA ACT CTC GAG AIT TIT TG TGT CTG CCC CTT A-3′), and subcloned into the AgeI / EcoRI site of a pLKO.1 lentiviral vector. To overexpress HMGA1, FLAG-tagged HMGA1 was subcloned into BglII-EcoRI site of pMIGR2 retroviral vector. Lentiviral knockdown and retroviral overexpression of HMGA1 was performed as described previously.20

7. Promoter, quantitative polymerase chain reaction (qPCR), western blot assay
The promoter activity of SM22α and SMA was measured as described previously.19 To analyze mRNA expression, cDNAs were amplified as manufacturer’s information (Sigma-Aldrich) by using specific primers for HMGA1 (Forward: 5′-GGATGGGACTGAGAAGCGAG-3′; Reverse: 5′-GTAACCTTCCGGCTTGGGC-3′), SM22α (Forward: 5′-AT CCTATGGCGATGAGCCTG-3′; Reverse: 5′-CAGGCTGTTCACCAACTTGTGC-3′), SMA (Forward: 5′-ACCATCGGGAATGACGCTT-3′; Reverse: 5′-CTGTCAGCAATGCCTGGGT T-3′), calponin (Forward: 5′-GCCCGAGGAACTATGTCTTAA-3′; Reverse: 5′-CCGCGGCTGGAGCTTTGGATA-3′), and Myh11 (Forward: 5′-CAGGCTGTTCACCAACTTGTGC-3′; Reverse: 5′-CAGGGAGCAGCTGCAGCC-3′). Expression of glyceraldehyde 3-phosphate dehydrogenase was used as the internal control (Forward, 5′-GTCAGTGGTGGACCTGACCT-3′; Reverse, 5′-TGAGCTTGACAAAGTGGTCG-3′). Expression of the target genes was quantified by real-time qPCR (Roche, Basel, Switzerland). The data were analyzed using the 2^ΔΔCT method. Western blot assay was performed as described previously.20

8. Statistical analysis
For the analysis of mRNA expression, proliferation, migration and contraction, the results are expressed as the means ± standard error of the mean (SEM) of triplicated experiments. For immunohistochemistry, the results are expressed as the means ± SEM of 6 independent experiments. An unpaired Student’s t-test was used to assess the differences between the 2 groups. The p-values <0.05 were considered significant.

RESULTS

1. Differential expression of HMGA1 in the synthetic and contractile type of VSMCs
As shown in Fig. 1A and B, marker proteins for the contractile type of VSMCs were strongly expressed in the contractile type of VSMCs compared to the synthetic type of VSMCs. In addition, the promoter activity of SM22α and SMA was significantly elevated in the contractile type of VSMCs compared to the synthetic type of VSMCs (Fig. 1C). However, the proliferation and migration activities were greater in the synthetic type of VSMCs than in the contractile type of VSMCs (Fig. 1D and E). The synthetic type of VSMCs also showed a weak Ang II-dependent contraction compared to the contractile type of VSMCs (Fig. 1F).
HMGA1 Regulates VSMC Proliferation

**Fig. 1.** Validation of HMGA1 expression in synthetic and contractile VSMCs. (A and B) Marker gene expression was validated by western blot. (C) Promoter activity of SM22α and SMA was measured in the synthetic and contractile of VSMCs as described in “Materials and Methods”. (D and E) Proliferation and migration activity were measured in the synthetic and contractile type of VSMCs. (F) Ang II-dependent contraction of both cell-type was measured as described in “Materials and Methods”. Data are the means ±SEM of 3 independent experiments (n=3 for each experiment). (G and H) The expression of HMGA1 was determined in the synthetic and contractile type of VSMCs. (I) The synthetic type of VSMCs was plated on a laminin-coated dish, and differentiation was induced in “Materials and Methods”. Data are the means ±SEM of 3 independent experiments (n=3 for each experiment). (J and K) The cells were harvested during the indicated time point, and the expression of the marker proteins and HMGA1 was measured by western blot and qPCR analysis. Data are reported as the mean ±SEM of three independent experiments (n=3 for each experiment).

**Fig. 2A and B**, the expression of HMGA1 was relatively higher in the synthetic type of VSMCs. During differentiation of the synthetic type of VSMCs to the contractile type VSMCs (Fig. 1I), the expression of contractile type marker genes, such as SM22α, SMA, calponin, and Myh11 was enhanced significantly, but the expression of HMGA1 was downregulated significantly (Fig. 1J and K).

2. Silencing of HMGA1 in the synthetic type of VSMCs facilitates phenotypic conversion to the contractile type
As shown in Fig. 2A and B, the silencing of HMGA1 induced the expression of the contractile marker genes. In addition, the proliferation and migration activities of the synthetic type of
VSMCs were reduced significantly by the silencing of HMGA1 (Fig. 2C and D). Finally, the silencing of HMGA1 in the synthetic type of VSMCs recapitulated the contractile properties in response to Ang II stimulation (Fig. 2E).

3. Silencing of HMGA1 suppresses PDGF-induced dedifferentiation of the contractile type of VSMCs

As shown in Fig. 3A, stimulation of the contractile type of VSMCs with PDGF induced morphological changes to the synthetic phenotype. On the other hand, PDGF-dependent morphological changes were not observed in the cells silencing HMGA1. As shown in Fig. 3B and C, stimulation of the contractile type of VSMCs with PDGF strongly suppressed the expression of the contractile marker genes, whereas the expression of HMGA1 was enhanced significantly. In addition, the PDGF-dependent downregulation of contractile marker gene expression was blocked by the suppression of HMGA1 induction by introducing shRNA for HMGA1. As shown in Fig. 3D, the Ang II-dependent contractile function was not curtailed by PDGF stimulation in the VSMCs silencing HMGA1.

4. HMGA1 is expressed strongly in the neointima, and the overexpression of HMGA1 in the contractile type of VSMCs recapitulates the synthetic phenotype

The expression of HMGA1 was examined in the highly proliferating neointima lesion because HMGA1 was expressed strongly in the synthetic type of VMSCs. As shown in Fig. 4A and B,
HMGA1 expression was highly elevated in the neointima area. To confirm the effect of HMGA1 expression on the modulation of the VSMCs phenotype, HMGA1 was overexpressed in the contractile type of VSMCs. As shown in Fig. 4C and D, the forced expression of HMGA1 in the contractile type of VSMCs curtailed the expression of the contractile marker genes. In addition, the synthetic properties of VSMCs, such as proliferation and migration, were enhanced significantly by the overexpression of HMGA1 (Fig. 4E and F). On the other hand, Ang II-dependent contractile function was reduced significantly by overexpression of HMGA1 (Fig. 4G).
The contractile type of VSMCs could be converted to the synthetic phenotype often found in unhealthy blood vessels. The synthetic phenotype of VSMCs proliferate and migrate more rapidly than the contractile type of VSMCs and secrete various extracellular matrix proteins, leading to the formation of a fibrous cap during atherosclerosis. Indeed, the marker gene expression was reduced significantly in the synthetic type of VSMCs (Fig. 1A and B), which might be due to an impairment of the promoter activity in the synthetic type of VSMCs.

**DISCUSSION**

The contractile type of VSMCs could be converted to the synthetic phenotype often found in unhealthy blood vessels. The synthetic phenotype of VSMCs proliferate and migrate more rapidly than the contractile type of VSMCs and secrete various extracellular matrix proteins, leading to the formation of a fibrous cap during atherosclerosis. Indeed, the marker gene expression was reduced significantly in the synthetic type of VSMCs (Fig. 1A and B), which might be due to an impairment of the promoter activity in the synthetic type of VSMCs.
The synthetic type of VSMCs grow and migrate faster than the contractile type of VSMCs\(^{21}\); the same results were observed in this study (Fig. 1D and E). Because the synthetic phenotype of VSMCs expresses low levels of contractile marker proteins, which are necessary for contractile function, the contraction would be subtle compared to the contractile type of VSMCs. Indeed, the Ang II-dependent contraction ability was reduced drastically in the synthetic type of VSMCs (Fig. 1F). Currently, it is unclear how the impediment of Ang II-dependent contraction in the synthetic phenotype of VSMCs is acquired but recent evidence suggests that Ang II receptor expression is downregulated in synthetic type of VSMCs.\(^{22}\) It is also possible that insufficient expression of downstream signaling molecules in the synthetic type of VSMCs would account for the impediment of Ang II-dependent contraction.

HMGA1 is expressed strongly in rapidly growing cells. For example, HMGA1 is expressed marginally in adult tissues but enhanced drastically in rapidly growing cells such as cancer cells.\(^{23}\) Moreover, the overexpression of HMGA1 transforms rat fibroblasts.\(^{24}\) In addition, the transgenic mice overexpressing HMGA1 showed hematopoietic malignancies and pituitary adenomas.\(^{25}\) In contrast, mice with homozygote and heterozygote HMGA1 show cardiac hypertrophy, lymphoma suppression, and insulin resistance, respectively.\(^{26,27}\) Therefore, HMGA1 is necessary for the proliferative function of various cell types. Because the synthetic phenotype of VSMCs grows more rapidly than the contractile type VSMCs, it is reasonable to assume that the synthetic type of VSMCs would express more HMGA1. In line with this idea, the synthetic phenotype of VSMCs expressed high levels of HMGA1, which decreased gradually during differentiation of the synthetic type to the contractile type (Fig. 1G-K). In the present study, several lines of evidence support the idea that HMGA1 modulates the phenotypic status of VSMCs directly. First, the silencing of HMGA1 in the rapidly growing synthetic type of VSMCs suppressed the proliferation and migration rate (Fig. 2C and D). Second, the forced expression of HMGA1 in the contractile type of VSMCs regained the proliferation and migration activity but lost the contractile properties (Fig. 4). Third, HMGA1 was expressed strongly in neointima lesions, where the highest population of VSMCs had the synthetic phenotype (Fig. 4). These results also suggest that HMGA1 expression is not just result of phenotypic changes in the VSMCs but also a major leading cause of phenotypic modulation.

In the present study, PDGF-dependent phenotypic modulation is regulated by HMGA1 expression. PDGF is involved in many physiological processes during vascular remodeling.\(^{28}\) Because PDGF is produced mainly by platelets during the vascular damage\(^{29}\) and phenotypic change of VSMCs is observed in vascular remodeling, PDGF may regulate the process involved in the phenotypic modulation of VSMCs. Indeed, it was reported that PDGF exerts the contractile type of VSMCs to induce a phenotypic change to the synthetic phenotype.\(^{20}\) Activation of the mammalian target of rapamycin complex 1 (mTORC1) appears to play a critical role in this process, and recent evidence supports that specificity protein 1 (SPI) may be responsible transcriptional factor in the expression of HMGA1. For example, high glucose enhances expression of HMGA1 which was blocked by phosphatidylinositol 3-kinase (PI3K) inhibitor and silencing of SPI.\(^{20}\) Likewise, it is possible that PDGF induces HMGA1 through the regulation of PI3K/mTORC1/SPI signaling cascade. In the present study, PDGF strongly enhanced the expression of HMGA1 concomitant with the suppression of contractile marker gene expression (Fig. 3). On the other hand, the PDGF-dependent phenotypic change of contractile VSMCs was blocked significantly by suppressing PDGF-dependent induction of HMGA1. These results suggest that HMGA1 is a downstream transcriptional regulator in PDGF-dependent phenotypic change in VSMCs.
Although the precise molecular mechanism that regulates the PDGF-dependent phenotypic change of VSMCs is still ambiguous, methylation/demethylation of the miR195/miR497 promoter may regulate the phenotypic regulation of VSMCs. For example, regulation of HMGA1 mRNA expression by miR195/miR497 affects the inhibitor of DNA binding 3 (Id3) expression, which suppresses muscle gene expression by binding with the myogenic regulatory factors (MRF), thereby maintaining the proliferation properties of myoblasts. Similarly, HMGA1 may regulate the expression of Id3 in VSMCs, thereby maintaining the synthetic phenotype of VSMCs. Therefore, examining the expression of miR195/miR497 during the PDGF-dependent phenotypic modulation of VSMCs would shed light on the mechanistic pathway.

In summary, HMGA1 is expressed strongly in the synthetic type of VSMCs. Suppression of HMGA1 expression in the synthetic phenotype of VSMCs facilitates a phenotypic change into the contractile phenotype. In addition, PDGF induces a phenotypic change in the VSMCs by enhancing the expression of HMGA1. The suppression of HMGA1 induction by PDGF blocks phenotypic change in VSMCs. Moreover, the forced expression of HMGA1 facilitates phenotypic conversion even in the absence of PDGF. Given these results, HMGA1 plays a critical role during vascular remodeling and could be a possible therapeutic target for vascular diseases.

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