Dendropanax morbifera Extract Inhibits Intimal Hyperplasia in Balloon-Injured Rat Carotid Arteries by Modulating Phenotypic Changes in Vascular Smooth Muscle Cells

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Abstract – The plant *Dendropanax morbifera* Léveille is effective folk medicines for the treatment of several conditions, such as infectious diseases, skin diseases, and other illnesses. Although the inhibitory effects of *D. morbifera* on the proliferation and migration of vascular smooth muscle cells (VSMCs) have been shown in our previous study, its effects *in vivo* remain to be elucidated. In this study, we aimed to investigate the protective effects of the extracts from *D. morbifera* (EDM) on neointimal hyperplasia of rat carotid artery and explore the underlying mechanisms. We observed that the ratio of intima to media thickness (I/M) was significantly decreased in the EDM-treated groups by ~80% compared to that of the control. The expression of Ki-67 and proliferating cell nuclear antigen was decreased by ~70% in the EDM-treated groups compared to that of the control. In addition, matrix metalloproteinase (MMP)2 and MMP9 significantly reduced in the neointimal layer of the EDM-treated groups. Moreover, the decreased levels of contractile phenotypic markers of VSMCs, such as α-smooth muscle actin, myocardin, and smooth muscle-myosin heavy chain, were successfully restored by EDM treatment. Furthermore, the levels of synthetic phenotypic markers, cellular retinal binding protein 1 and connexin 43 were also restored to normal levels. These results suggest that EDM inhibits vascular neointimal hyperplasia induced by balloon injury in rats via phenotypic modulation of VSMCs. Therefore, EDM may be a potential drug candidate for the prevention of restenosis.

Keywords – *Dendropanax morbifera*, balloon injury, neointimal hyperplasia, vascular smooth muscle cells, phenotypic change

Introduction

Increased proliferation and migration of vascular smooth muscle cells (VSMCs) are important processes involved in the development of atherosclerosis and restenosis after balloon angioplasty.[1-2] Restenosis, a major disadvantage of percutaneous transluminal coronary angioplasty (PTCA), occurs after the proliferation and migration of smooth muscle cells from the media to the intima leading to the secretion of extracellular matrix proteins, which in turn results in neointima formation and luminal narrowing.[3-4]

Indeed, a restenosis rate was reported 19% even after successful balloon angioplasty.[5-6] Moreover, follow-up studies indicated a restenosis rate of roughly 33%.[7] More than 30 years later, despite pharmacological and dynamic developments to reduce the rate of restenosis, it is left still an important problem, particularly in high-risk patient groups. During the neointima formation process, VSMCs undergo a phenotypic change, which is a dedifferentiation from the contractile to synthetic or proliferative phenotype.[8] It is known that α-smooth muscle actin (α-SMA), myocardin (Myocd), smooth muscle-myosin heavy chain (SM-MHC), cellular retinal binding protein 1 (CRBP1), and connexin 43 (Cx43) are the key markers of differentiation and dedifferentiation of VSMCs. Recently, the role of natural products in the prevention of cardiovascular diseases, such as hypertension, myocardial infarction,

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arrhythmia, and atherosclerosis, has been extensively studied because some natural products are more effective and less toxic than standard treatments.[9] It was reported that ginsenoside Rb$_1$ inhibited neointimal hyperplasia in balloon-injured rats through the suppression of phenotypic modulation of VSMCs.[10]

The plant *Dendropanax morbifera* Léveille belonging to the family Araliaceae is distributed throughout the southern part of Korea.[11] It is used for the production of golden varnishes. *D. morbifera* is also an effective folk medicine for the treatment of several conditions, such as infectious diseases, skin diseases, headaches, and other illnesses.[12] Recent studies have shown anticomplement activity that polyacetylene compounds isolated from *D. morbifera*.[12] Thereafter five compounds, dendropanoxide, oleifolioside A and B, α- and β-amyrin have been isolated from *D. Morbifera* and identified anti-diabetic,[13] anti-atherogenic,[14] anti-plasmodial,[15] anti-complement activity,[16] anti-inflammatory and anti-cancer effects.[11,17,18] However, the effects of the extracts from *D. morbifera* (EDM) on cardiovascular injury have not yet been reported. In our previous study, we determined the inhibitory effects of EDM on platelet-derived growth factor (PDGF)-BB-stimulated VSMC proliferation and migration *in vitro*.[19] Furthermore, we identified the reducing effects of EDM on hypoxia/reoxygenation (H/R) injury caused by augmented ROS and concentration of Ca^{2+} in cardiomyocytes.[20] In the present study, we analyzed the effect of EDM on neointima formation in balloon-injured rat carotid arteries *in vivo* to determine the ability of the extract in preventing restenosis. In addition, alterations in the expression levels of phenotypic markers of VSMC differentiation were evaluated.

Experimental

Cell culture and reagents – Rat aortic smooth muscle cells (RAoSMCs) were isolated from 6-week-old Sprague-Dawley rats. The thoracic aortas were removed and freed from connective tissues, and any remaining blood clots were removed. Subsequently, the aorta was severed and transferred into a tube containing a mixture of collagenase type I (1 mg/mL, Sigma, St. Louis, MO, US) and elastase (0.5 mg/mL, Worthington, NJ, US) and incubated for 30 min at 37 °C. The aorta was placed into a 100-mm cell culture dish and the adventitia was stripped with forceps under a binocular microscope. Each piece of aorta was transferred into a tube containing 5 mL of enzyme disso-ciation mixture (containing collagenase and elastase), and the tubes were incubated for 2 h at 37 °C. The dispersion

of the tissue was accomplished by slow pipetting. The suspension was centrifuged (1600 ×g for 5 min), and the obtained pellet was resuspended in DMEM with 10% fetal bovine serum (FBS, WelGENE, KOREA) and centrifuged again (1600 ×g for 5 min). The cells were cultured for up to 10 passages at 37 °C in an incubator with a humidified atmosphere of 95% air and 5% CO_2.

Preparation of *D. morbifera* extract – The plant material, which comprised the leaves and stem of *D. morbifera*, was collected in Jangheung province (batch no. 2014F) in Korea and then authenticated. In this study, we used an aqueous extract (aqueous EDM), because it has been shown to have stronger anti-proliferation or anti-migration activity in VSMCs than extracts obtained using other solvents such as ethanol, methanol, and butanol.[19] Briefly, dried leaves (1.4 g) and stems (21 g) of *D. morbifera* were extracted with distilled water (500 mL) at 60 °C for 24 h. The primary extract was centrifuged to remove the plant material. The solution was filtered under sterile conditions before further use.

Balloon injury model – All animal experiments were conducted in accordance with the International Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Research Committee of the Chosun University School of Medicine (Protocol No. CIACUC2013-A0006). White Sprague-Dawley rats (6- to 7-week-old, body weight 200 ± 50 g) were anesthetized by an intramuscular injection of 20 mg/kg Zoletil 50® (Virbac Corp., Fort Worth, TX, US) and 10 mg/kg of Rompun® (Bayer Corp., Pittsburgh, PA, US). After midline incision of the neck, the left external carotid artery was exposed and then injured by using a 2-F balloon catheter (Edwards Lifesciences Corp., Irvine, CA, US) introduced into the aortic outlet of the common carotid artery, followed by inflation and deflation to expand the artery and denude the endothelium. After this procedure was repeated three or four times, the catheter was removed, the external carotid artery was ligated, and the wound was closed. EDM (90 mg/kg/day) was orally administered for 2 weeks prior to the surgery or for 2 weeks after carotid injury. The bilateral carotid arteries were excised 14 days after surgery. Some parts of the carotid artery were used in western blotting and real-time quantitative polymerase chain reaction (qPCR), while the remaining parts were fixed in 4% paraformaldehyde (PFA). The bilateral carotid arteries were embedded in paraffin for the mor-phometric analysis of neointima formation by hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). The cross-sectional areas of the blood vessel layers, including the lumen area (LA), intimal area (IA),

and medial area (MA) were quantified in at least three different sections (proximal, middle, and distal) by using Image J software (NIH, Bethesda, MD, US).

Morphometric analysis and Immunohistochemistry – For the assessment of proliferation in the vessel wall, the cross-sectional slices were immunohistochemically stained with antibodies against proliferating cell nuclear antigen (PCNA), Ki-67, MMP2, or MMP9. Paraffin-embedded sections were hydrated by incubation with xylene and a series of ethanol solutions (100%, 95%, 80%, and 70%). The sections were incubated with antigen unmasking solution (citrate or Tris-EDTA buffer) at 100 °C for 20 min, washed in water and hydrogen peroxide [H_2O_2 (3%), 5 min], and then treated with standard blocking serum [10% normal horse serum in phosphate-buffered saline (PBS, WelGENE, KOREA)] supplemented with 0.1% Triton X-100 at room temperature for 20 min. The sections were then incubated overnight at 4 °C with monoclonal primary antibodies against the following proteins: PCNA (Cell Signaling, Danvers, MA, US, 1: 1000 dilution); Ki-67 (Thermo, Waltham, MA, US, 1:100 dilution); MMP2 (Abcam, Cambridge, MA, US, 1:500 dilution); and MMP9 (Abcam, Cambridge, MA, US, 1:100 dilution). Subsequently, the sections were washed in PBS, incubated with the appropriate biotinylated secondary antibody solution (1:200 dilution) at room temperature for 1 h, and washed again in PBS. The sections were incubated with VECTASTAIN® ABC Reagent (Vector Laboratories, Burlingame, CA, US), washed in PBS, developed in peroxidase substrate solution (3,3'-diaminobenzidine, DAB), dehydrated in a series of ethanol solutions (70%, 95%, and 100%) and xylene, and then mounted in Malinol mounting medium (MUTO PURE CHEMICALS CO., Tokyo, Japan). The labeled areas were quantified in the neointimal tissue from digital images acquired at 400× magnification.

Immunoblot analysis – The tissues of the carotid artery were washed with ice-cold PBS and dissociated in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. The protein concentrations were determined by using the Bradford protein assay kit (Bio-Rad, Hercules, CA, US), and equal quantities of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, US). After incubation with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5 or 10% skim milk for 1 h at room temperature to prevent non-specific binding, the membrane was incubated overnight with primary antibodies at 4 °C.

The primary antibodies were used at the following dilutions in blocking buffer: MMP2 (Abcam, Cambridge, MA, US, 1:2000 dilution), MMP9 (Abcam, Cambridge, MA, US, 1:500 dilution), β-actin (Sigma, St. Louis, MO, US, 1:5,000), PCNA (Cell Signaling, Danvers, MA, US, 1:2000 dilution), and Ki-67 (Thermo, Waltham, MA, US, 1:2000 dilution). The membrane was washed five times with TBS-T for 5 min and incubated for 1.5 h at room temperature with the appropriate secondary antibodies. After extensive washing, enhanced chemiluminescence reagent (ECL, BIONOTE, Animal Genetics Inc., Tallahassee, FL, US) was applied and the bands were detected.

Real-time qPCR – Total RNA was isolated by using Trizol reagent (QIAGEN, Germantown, MD, US) in accordance with the manufacturer's protocol. Total RNA was subjected to reverse transcription by using HelixCript™ First-Strand cDNA Synthesis Kit (NanoHelix, KOREA). Real-time qPCR with RealHelix™ qPCR kit (NanoHelix, KOREA) was performed by the SYBR Green method using an Applied Rotor-Gene 3000™ (Corbett Research, Sydney, AU). The ratios of the transcript levels of the genes of interest in the experimental and control samples were compared with the ratios of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels in corresponding samples. The primer sequences for qPCR are listed in Table 1.

Statistical analysis – All quantified data from at least triplicate samples were analyzed with SPSS 13.0 software. Data are expressed as mean ± SD. Statistical comparisons between two groups were performed using Student's t-test. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA). A two-tailed P < 0.05 was considered statistically significant.

Result and Discussion

In a previous study, we demonstrated that EDM inhibited VSMC proliferation and migration *in vitro*.[19] To examine whether EDM prevents restenosis, we evaluated the effects of EDM administration on neointima formation in a rat balloon injury model. As shown by H&E staining in Fig. 1A, neointima formation of rat carotid artery was significantly lower in both EDM pre- or post-treated rats than in the untreated controls. The mean intimal area of the sham control was significantly higher than that of the uninjured control, but significantly decreased by 65 and 53% in the pre- and post-treatment groups, respectively. I/M ratios in the pre-treatment and post-treatment groups were 57 and 50% lower, respectively, than the ratio in the positive control (Fig. 1B and 1C). No significant differences

Table 1. The sequence of primers used for real-time quantitative PCR

| Gene	Primer sequence
GAPDH	Sense: 5'-CAGTGCCAGCCTCGTCTCAT-3' Antisense: 5'-TGGTAACCAGGCGTCCGATA-3'
MMP2	Sense: 5'-ACGATGGCAAGGTGTGGTGT-3' Antisense: 5'-CCTTGGTCAGGACAGAAGCC-3'
PCNA	Sense: 5'-TGAAGTTTTCTGCGAGTGGG -3' Antisense: 5'-CAGTGGAGTGGCTTTTGTGAA -3'
α-SMA	Sense: 5'-AACTGGTATTGTGCTGGACTCTGG -3' Antisense: 5'-CACGGACGATCTCACGCTCAG -3'
Myocd	Sense: 5'-CAGAAAGTGACAAGAACCATACAG -3' Antisense: 5'-TGAAGCAGCCGAGCATAGG -3'
SM-MHC	Sense: 5'-ATGGAGACAAATGCTAATCAGCC -3' Antisense: 5'-CAGTTGGACACTATGTCAGGGAAA -3'
CRBP1	Sense: 5'-CACCATGCCTGTGGACTTCAAC -3' Antisense: 5'-TCAGTGTACTTTCTTGAACAC -3'
CX43	Sense: 5'-GGCCTTCCTGCTCATCCA -3' Antisense: 5'-GGGATCTCTCTTGCAGGTGTAGA -3'
PDGF-A	Sense: 5'-CCTGTGCCATCCGCAGGAAGAGA -3' Antisense: 5'-TTGGCCACCTTGACGCTGCGGTG -3'
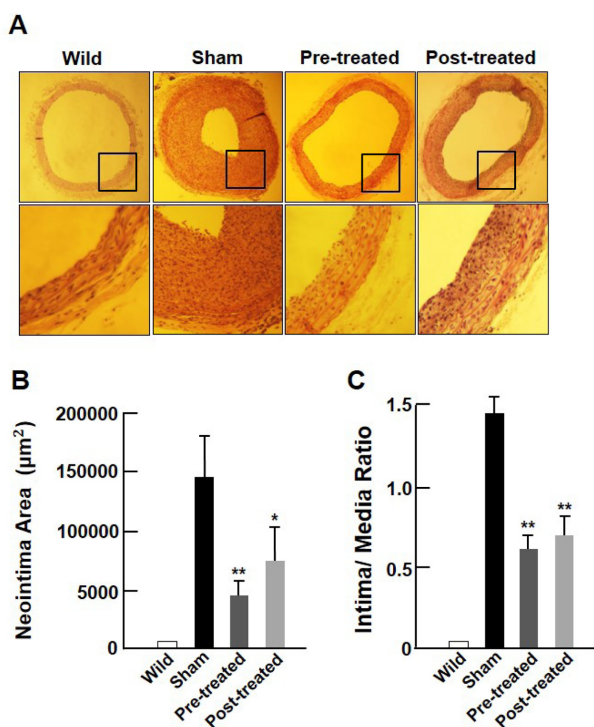
Fig. 1. Effects of EDM on neointimal hyperplasia induced by balloon injury. (A) Representative morphological analysis of neointimal hyperplasia sections (the upper and lower panels are 100× and 400× magnification, respectively) of rat carotid arteries stained with hematoxylin and eosin in the sham control, balloon injury (BI) model, 2 weeks of 90 mg/kg/day EDM pretreatment, and 2 weeks of 90 mg/kg/day post-treatment groups, respectively. (B) Neointimal area and (C) intima/media ratio area computed using ImageJ as the mean ± SD of 6 – 8 rats (*p < 0.05 and **p < 0.01 *vs.* BI model).

were observed in the medial area of all groups.

PCNA is an acidic nuclear protein that increases in the late G1 to S phases, and is well correlated with cell proliferation.[21] Ki-67 is a nuclear protein and a marker of proliferation.[22] We investigated the inhibitory effects of EDM on the proliferative activity of VSMCs in the neointimal region by IHC and immunoblotting. The positive staining of Ki-67 and PCNA was significantly lower in the pre- and post-treatment groups than in the controls (Fig. 2A). PCNA expression was increased by 270% in balloon-injured rat carotid arteries compared to that in the sham control; however, the increased expression of PCNA was restored to normal levels by pre- or post-treatment with EDM (Fig. 2B). No significant changes were observed in the expression of Ki-67 in immunoblot assay (data not shown). In addition, we observed that the levels of phosphorylated ERK increased by balloon injury were restored by EDM treatment (Fig. 2C), indicating that ERK is a key regulator of VSMC proliferation in neointima formation.

There is enough evidence to support the critical nature of degradation of extracellular matrix and basement membrane by proteases such as MMPs for neointima formation initiated by VSMCs.[23,24] In particular, MMP2 and MMP9 are highly expressed in VSMCs than in other cells and tissues. Their mRNA and protein levels are further increased in migratory and proliferative VSMCs.[25] It is well established that MMP2 and MMP9 are closely associated with VSMC migration and consequently
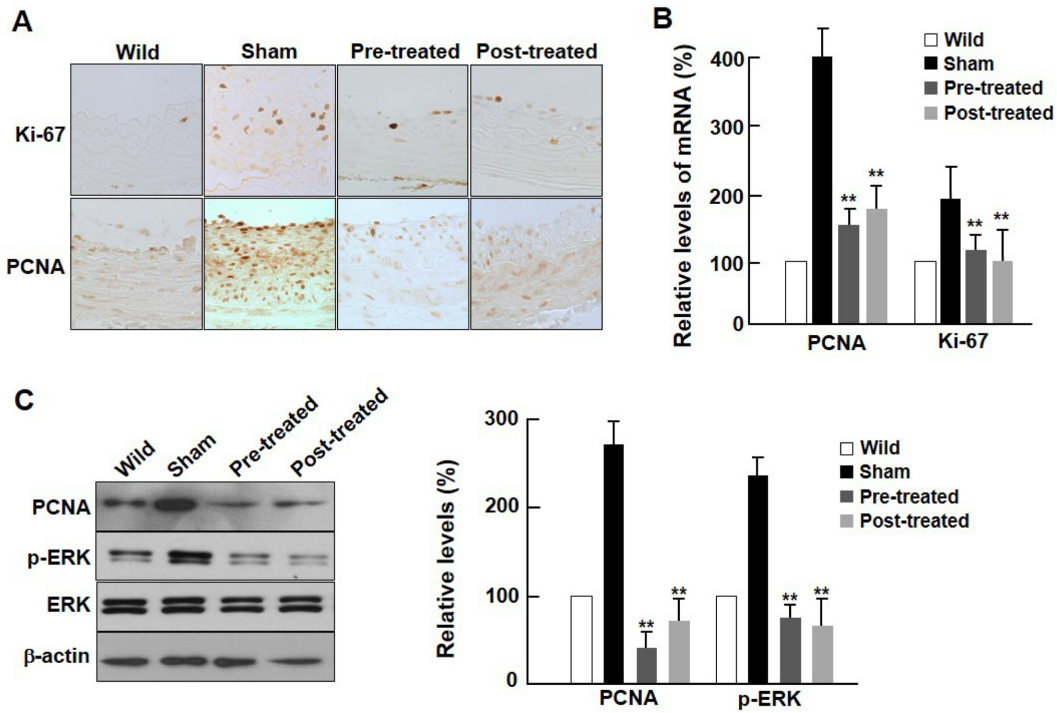
Fig. 2. Effects of EDM on proliferative activity in the neointimal region. (A) Immunohistochemical staining of the balloon-injured carotid artery from the balloon injury (BI) model or EDM-treated groups was conducted using antibodies against Ki-67 or PCNA. (B) Alterations in the mRNA expression of the proliferative genes PCNA and Ki-67. (C) Immunoblotting analysis of PCNA or p-ERK in balloon-injured neointimal tissues. Data are shown as the mean ± SD (**p < 0.01 *vs.* BI model).
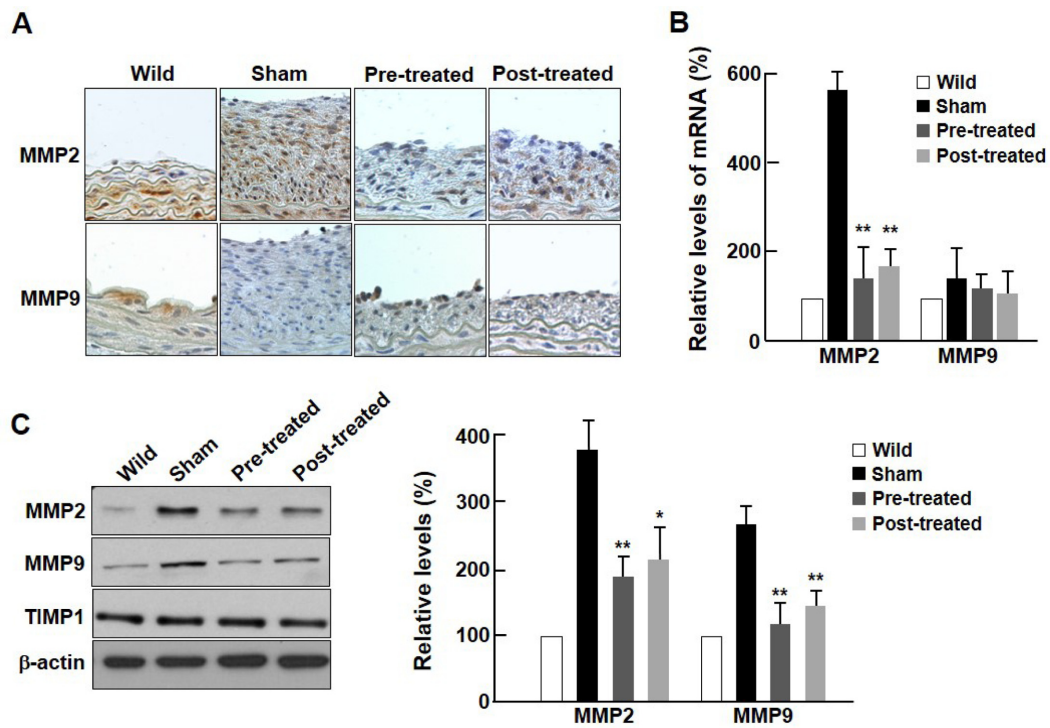
Fig. 3. Effects of EDM on VSMC migration in the neointimal region. (A) Immunohistochemistry of balloon-injured carotid artery from the balloon injury (BI) model or EDM-treated groups using antibodies against MMP2 or MMP9. (B) Alterations in the mRNA expression of MMP2 and MMP9 (C) Immunoblotting analysis of MMP2, MMP9, or TIMP1 in balloon-injured neointimal tissues. Data are shown as the mean ± SD (*p < 0.05 and **p < 0.01 *vs.* BI model).

atherogenesis.[24,26,27] In the present study, we investigated the inhibitory effects of EDM on the migration of VSMCs in the balloon-injured region by IHC. The expression of MMP2 and MMP9 was significantly decreased by pre- or post-treatment with EDM compared to that in the balloon injury model (Fig. 3A). We observed that the mRNA levels of MMP2 were increased by balloon injury (Fig. 3B), which were significantly inhibited by EDM treatment. However, the mRNA levels of MMP9 did not coincide with the protein expression. This discrepancy might result from the effects of EDM on MMP9 processing rather than on the regulation of gene expression. Indeed, the expression of MMP2 and MMP9 was increased by 375 and 280%, respectively, in tissue from balloon-injured rat carotid artery compared to that in the sham control. The increase in MMP2 expression was significantly inhibited by pre- and post-treatment with EDM (50 and 60%, respectively). MMP9 expression was also decreased to a similar extent (Fig. 3C). TIMP1 expression remained unaffected in all the groups, indicating that the effects of EDM were specific for MMP2 and MMP9.

To investigate the mechanism underlying the inhibition of neointimal hyperplasia by EDM, we investigated the altered expression levels of the key proteins associated with VSMC phenotypic modulation (α-SMA, Myocd,

SM-MHC, CRBP1, and Cx43) both *in vitro* and *in vivo*. Initially, we observed that the mRNA expression of contractile markers (α-SMA, Myocd, and SM-MHC) was significantly decreased and that of synthetic markers (CRBP1 and Cx43) was increased in balloon-injured carotid arteries. These alterations were successfully restored by pre- or post-treatment with EDM (Fig. 4A). Consequently, the altered expression levels of phenotypic markers in balloon-injured carotid arteries were successfully restored in EDM-treated groups (Fig. 4B). We analyzed the mRNA expression of key proteins *in vitro* to confirm that these effects are specific to VSMCs. The transient modulation of contractile to synthetic phenotype was induced by PDGF treatment.[28] The mRNA levels of α-SMA, Myocd, and SM-MHC were significantly decreased by 65, 60, and 35%, respectively, and those of CRBP1 and Cx43 were increased by 180 and 230%, respectively (Fig. 4C). Furthermore, we observed that EDM treatment significantly restored the changes in phenotypic markers in VSMCs in a dose-dependent manner, indicating that the protective effects of EDM on neointima formation occurred specifically via phenotypic modulation of VSMCs.

Neointima formation by VSMC migration and proliferation is a critical step in atherosclerosis and restenosis after balloon angioplasty. Our previous study showed that
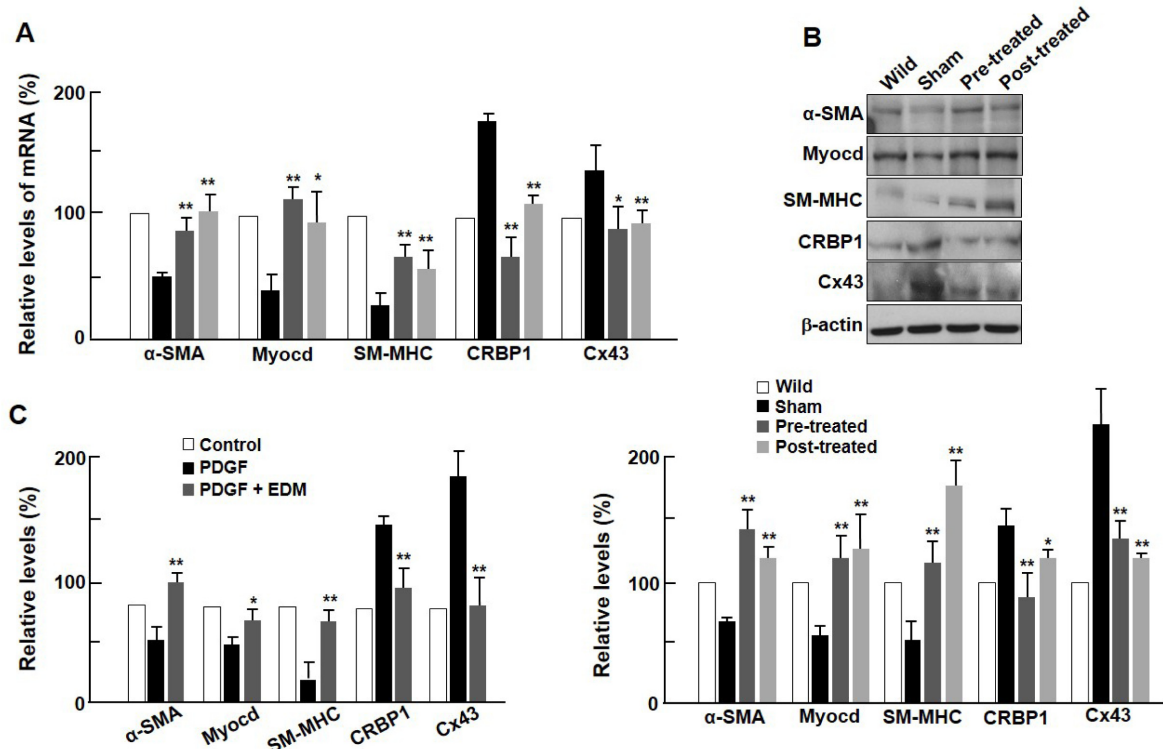
Fig. 4. Altered mRNA (A) or protein (B) expression of phenotypic markers in the neointimal region and VSMCs (C). Data are shown as the mean ± SD (*p < 0.05 and **p < 0.01 *vs.* balloon injury (BI) model or PDGF-treated VSMCs).

EDM exerted inhibitory effects on VSMC migration and proliferation *in vitro*.[19] Owing to their safety, low toxicity, and general acceptance as dietary supplements, natural dietary elements have been investigated in detail for the prevention of several diseases, such as cancer,[29] diabetes, and cardiovascular diseases.[30] In particular, the natural products like resveratrol, allicin, catechin, curcumin, and lycopene prevent and cure intimal hyperplasia by inhibiting abnormal proliferation and migration of smooth muscle cells, leading to decrease the prevalence of restenosis.[31]

Many reports have shown that EDM exerts antiplasmodial, anti-atherogenic, anti-diabetic, and anti-cancer effects. The extracts from the stem of *D. morbifera* facilitated cadmium excretion from the blood and kidneys of cadmium-exposed rats and inhibited cadmium-induced oxidative stress in the brain through increased levels of antioxidants.[32] The ethanol extracts of *D. morbifera* markedly inhibited the growth of human leukemia-U937 cells through the reduction of cell proliferation and induction of apoptosis through the caspase-dependent pathway.[33] It was reported that the essential oil from *D. morbifera* has significant lipid-lowering effects.[14] Our previous study showed that EDM exerted inhibitory effects on VSMC migration and proliferation *in vitro*.[19] However, the effects of EDM on cardiovascular diseases *in vivo* have not yet been reported.

In the present study, we demonstrated that EDM decreased vascular intimal hyperplasia in balloon-injured rat carotid arteries. Histopathological analyses showed that the oral administration of 90 mg/kg/day EDM exerted considerable protection from neointima formation in balloon-injured rats. These effects have been observed in rats treated with EDM for 2 weeks before and after surgery, which suggests that EDM might possess both preventive and therapeutic activities. Furthermore, histological analyses indicated that EDM significantly restored the markers of VSMC proliferation, PCNA and Ki-67, and the migratory proteins MMP2 and MMP9 in balloon-injured carotid arteries, which supported the immunoblotting results. Notably, the increased phosphorylation of ERK in balloon-injured carotid arteries was significantly inhibited by the administration of EDM, suggesting that the effects of EDM on VSMC proliferation might occur through blocking of ERK signaling. We observed that the mRNA levels of PCNA, Ki-67, and MMP2 were consistent with the findings of histological analyses; however, we did not observe a significant change in the mRNA expression of MMP9. This might indicate that the effects of EDM on MMP9 did not occur through the regulation of gene expression, but instead through participation in the conversion process from pro-MMP9 to active MMP9. However, further investigation is needed to determine the detailed underlying mechanisms.

Thus, we demonstrated that EDM inhibited neointima formation through the regulation of proliferation and migration of VSMCs. A low proliferation of VSMCs is maintained in the normal media layers, which are characterized by contractile phenotype with high expression levels of contractile marker proteins, such as α-SMA, SM-MHC, and Myocd, and low expression levels of proliferation marker proteins, such as PCNA and MMPs.[8] Furthermore, synthetic VSMCs, which are characterized by higher expression of CRBP1, Cx43, and PDGF-A than contractile VSMCs, have an important role in the progression of atherosclerosis and restenosis.[2,34] In our study, the elevated mRNA and protein levels of the synthetic markers CRBP1 and Cx43 induced by balloon injury were significantly decreased by EDM administration. In contrast, the expression of the contractile markers, α-SMA, SM-MHC, and Myocd, was significantly increased. In addition, we observed a parallel phenomenon in cultured VSMCs, suggesting that the protective activities of EDM on neointima formation after balloon injury occurred through the phenotypic modulation of VSMCs. Therefore, further investigation will be needed to identify the chemical components of EDM responsible for the phenotypic modulation of VSMCs and to elucidate the detailed mechanisms.

Our results provide conclusive evidence that EDM successfully inhibited neointimal hyperplasia induced by balloon injury. These effects were clearly related to its anti-proliferative and anti-migratory activities, which were exercised through the phenotypic modulation of VSMCs from synthetic to contractile type. Therefore, EDM might be useful in preventing or treating atherosclerosis or restenosis. Further investigations on the chemical component responsible for the effects of EDM, the mechanisms underlying MMP regulation, and the signaling pathways involved in the process of phenotypic modulation of VSMCs are necessary to allow the future use of EDM in the treatment of vascular diseases.

Acknowledgments

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