Role of Myoendothelial Gap Junctions in the Regulation of Human Coronary Artery Smooth Muscle Cell Differentiation by Laminar Shear Stress

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Key Words
Human coronary artery smooth muscle cells • Human coronary artery endothelial cells • Co-culture • Shear stress • Myoendothelial gap junction • Connexin

Abstract
Background/Aims: Smooth muscle cells may dedifferentiate into the synthetic phenotype and promote atherosclerosis. Here, we explored the role of myoendothelial gap junctions in phenotypic switching of human coronary artery smooth muscle cells (HCASMCs) co-cultured with human coronary artery endothelial cells (HCAECs) exposed to shear stress. Methods: HCASMCs and HCAECs were seeded on opposite sides of Transwell inserts, and HCAECs were exposed to laminar shear stress of 12 dyn/cm² or 5 dyn/cm². The myoendothelial gap junctions were evaluated by using a multi-photon microscope. Results: In co-culture with HCAECs, HCASMCs exhibited a contractile phenotype, and maintained the expression of differentiation markers MHC and H1-calponin. HCASMCs and HCAECs formed functional intercellular junctions, as evidenced by colocalization of connexin(Cx)40 and Cx43 on cellular projections inside the Transwell membrane and biocytin transfer from HCAECs to HCASMCs. Cx40 siRNA and 18-α-GA attenuated protein expression of MHC and H1-calponin in HCASMCs. Shear stress of 5 dyn/cm² increased Cx43 and decreased Cx40 expression in HCAECs, and partly inhibited biocytin transfer from HCAECs to HCASMCs, which could be completely blocked by Cx43 siRNA or restored by Cx40 DNA transfected into HCAECs. The exposure of HCAECs to shear stress of 5 dyn/cm² promoted HCASMC phenotypic switching, manifested by morphological changes, decrease in MHC and H1-calponin expression, and increase in platelet-derived growth factor (PDGF)-BB release, which was partly rescued by Cx43 siRNA or Cx40 DNA or PDGF receptor signaling inhibitor. Conclusions: The exposure of HCAECs to shear stress of 5 dyn/cm² caused the dysfunction of Cx40/Cx43 heterotypic myoendothelial gap junctions, which may be replaced by homotypic Cx43/Cx43 channels, and induced HCASMC transition

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to the synthetic phenotype associated with the activation of PDGF receptor signaling, which may contribute to shear stress-associated arteriosclerosis.

Introduction

Vasculature is composed of highly differentiated smooth muscle cells (SMCs) residing in the arterial tunica media layer, where SMCs perform contractile function and regulate vascular tone and luminal diameter to maintain an appropriate blood pressure and flow distribution. A number of proteins have been identified as markers of SMC differentiated contractile phenotype, including α-smooth muscle actin (α-SMA), myosin heavy chain (MHC), H1-calponin, SM22α, and smoothelin; among them, H1-calponin and MHC expression is considered the most specific indicator of differentiated SMCs [1, 2]. However, fully differentiated SMCs could undergo phenotypic switching to synthetic SMCs characterized with different morphology, proliferation and migration, and reduced expression of differentiation markers, and are associated with vascular remodeling and atherosclerosis [2].

Shear stress created by blood flow has emerged as an essential factor in atherogenesis. Laminar shear stress is considered atheroprotective at the physiological levels (10-20 dyn/cm²), while at the low levels (0-4 dyn/cm²), it may promote atherosclerosis by inducing SMCs proliferation [3], migration [4], and protein synthesis [5, 6]. SMCs are directly exposed to hemodynamic shear stress after the injury of vascular endothelial cell (EC) layer covering the luminal surface of blood vessels. In addition, recent studies using EC-SMC co-culture models revealed that low shear stress applied to intact endothelium could also indirectly influence the behavior of neighboring SMCs. Thus, in a rat aorta culture model, the exposure of endothelium to shear stress (5 dyn/cm²) downregulated RhoGDIα and induced SMC migration and apoptosis [7]. In vitro, laminar shear stress (5 dyn/cm²) applied to vascular ECs stimulated the secretion of endothelial IGF-1 and induced a loss of SMC contractile phenotype [8]. These findings suggest that in EC-SMC co-cultures, shear stress is a regulator of SMC phenotypic switching; however, the underlying mechanisms are not fully understood.

Myoendothelial gap junctions are intercellular channels that directly link ECs and SMCs through the internal elastic lamina [9]. Endothelial factors such as endothelium-derived hyperpolarizing factor [10] and secondary messengers such as calcium and IP3 [11] could signal directly across the myoendothelial gap junction to regulate smooth muscle cell contraction. Myoendothelial gap junctions can be mimicked in vitro by co-culturing ECs and SMCs on the opposite sides of a porous membrane; this model have been widely used to study the mechanisms of cell-cell interaction in the vasculature. Thus, it has been reported that in human coronary artery SMCs (HCASMCs) co-cultured with human coronary ECs (HCAECs), differentiation markers were significantly induced, indicating a regulatory role of EC-SMC intercellular contacts in SMC phenotypic switching [12]. Furthermore, in a static ECs-SMCs co-culture model using Transwell inserts, myoendothelial gap junctions have been shown to modulate the differentiation of pulmonary arterial SMCs [13, 14]. Although these findings suggest a regulatory role of myoendothelial gap junctions in SMC differentiation, their involvement in the regulation of SMC phenotypic transition by shear stress is still unclear.

Myoendothelial gap junctions are composed of connexins, a family of structurally related transmembrane proteins. Connexins Cx43, 40, and 37 were identified in vascular bridges between endothelium and SMCs [15], where they demonstrated heterogeneous expression and phosphorylation [16, 17]. Cx40 is abundant in ECs, while Cx43 is highly expressed in SMCs and some ECs except coronary cells [18], and has been shown to form functional myoendothelial gap junctions in vitro [9]. In addition, endothelial Cx43 and Cx40, but not Cx37, demonstrated mechanosensitivity to shear stress [19, 20]; thus, Cx43 distribution on the endothelial surface was changed in response to shear stress [21]. A recent study revealed that shear stress at low levels (4 dyn/cm²), but not at high levels (12
dyn/cm²), upregulated Cx43 expression in human umbilical vein ECs (HUVECs) co-cultured with HUVSMCs, and induced HUVEC inflammatory phenotype [22]. Overall, these findings suggest that the exposure to shear stress of ECs co-cultured with SMCs may regulate SMC phenotype switching, and the formation of myoendothelial gap junctions may play a pivotal role in the process.

In the present study, HCAECs were co-cultured with HCASMCs on the opposite sides of a porous membrane. HCAECs were exposed to high or low shear stress, and co-cultures were evaluated for the formation of myoendothelial gap junctions and its role in SMC phenotype switching.

**Materials and Methods**

**Cell culture and shear stress application**

HCAECs (catalog. no. FC-0032) and HCASMCs (catalog. no. FC-0031) were purchased from Lifeline Cell Technology (Frederick, MD, USA). HCAECs and HCASMCs were cultured in LL-0003 and LL-0014 media (both from Lifeline Cell Technology) at 37°C in a humidified 5% CO₂ incubator, and used after 3-4 passages. For contact co-culture experiments, HCAECs were seeded on the bottom side of 6-well Transwell insert membranes (catalog no. CLS3450-24EA; Sigma, St. Louis, MO, USA) at the concentration of 5 × 10⁵ cells/membrane for 2 h to allow cell attachment. Then, HCASMCs were seeded on the top side of the membrane for 24 h to allow cell attachment. Then, HCASMCs were seeded on the top side of the membrane at the same concentration [9].

After 48 h of static co-culture with HCAECs, HCAECs were exposed to laminar shear stress for 24 h using a previously described co-culture flow system [23], which could generate the diverse levels of shear stress (5 or 12 dyn/cm²), and provided on-line microscopic cell visualization during the experiment. The shear stress (τ) was calculated as 6 μQ/wh²; where μ is the viscosity of the perfusate and Q is the flow rate. w (≈ 38mm) and h (≈ 0.25mm) are the height and width of the flow chamber, respectively. μ = 0.007 dyn*s/cm² is the fluid viscosity for the medium at 37°C. In the preliminary experiments, at 24 hours after the exposure of shear stress of 5 dyn/cm², the contractile markers expression was decreased significantly in HCASMCs co-cultured with HCAECs (data not shown). Thus, shear stresses (5 or 12 dyn/cm²) were loaded to HCAECs for 24 hours in the following experiments.

**Immunocytochemistry**

HCASMCs cultured on membranes with or without stress were rinsed with PBS, fixed for 15 min at room temperature, blocked for 2 h in PBS containing 5% normal goat serum and 2% BSA, and incubated with monoclonal antibodies (diluted 1:1000) against α-SMA (catalog no. CBL171; Millipore Corporation, Billerica, MA, USA) or anti-SM22α (catalog no. SAB1400272; Sigma) at 4°C overnight. Cells were rinsed five times in PBS and incubated with Alexa Fluor 488-conjugated (green) or Alexa Fluor 594-conjugated (red) goat anti-mouse antibody (diluted 1:500) for 1 h at room temperature.

**Cell-cell contact detection**

To detect physical contacts between HCAECs and HCASMCs before and after shear stress, Transwell membranes were examined by microscopy. Confocal images were obtained by scanning through the membranes from HCAEC to HCASMC monolayers five times in 2.5-μm increments as described previously [9].

The membranes with HCAECs and HCASMCs were rinsed with cold PBS, fixed in 4% paraformaldehyde for 10 min, and cells were incubated with cold solutions of lipophilic dyes FM 4-64 FX (5 μg/mL, red, catalog no. T3166; Invitrogen, Grand Island, NY) and FM 1-43 FX (5 μg/mL, green, catalog no. T35355; Invitrogen), respectively, for 10 min as described previously [14]. The membranes were rinsed with PBS, immersed in 30% sucrose overnight, frozen in cryoembedding medium (catalog no. 14-373-65; Thermo Fisher Scientific, Rochester, NY, USA), and cut into 10-μm transverse sections. Direct cell-cell contacts in the membrane pores were observed using a Zeiss LSM510 Meta microscope (Carl Zeiss AG, Germany) equipped with a Plan-Apochromat 63× 1.4 NA oil objective for imaging fixed cells in mounting media. FM 4-64 FX-labeled HCAECs and FM1-43 FX-labeled HCASMCs were observed at the excitation/emission wavelengths of 588 nm/734 nm and 510 nm/626 nm, respectively.
The expression of Cx40 in HCAECs and Cx43 in HCASMCs was examined by immunocytochemistry as described above using monoclonal antibodies against Cx40 and Cx43 (catalog no. sc-365107 and sc-271837, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Dye transfer**

HCAECs were loaded with lucifer yellow biocytin (5 mg/mL, catalog no. L-6950; Thermo Fisher Scientific), using a pinocytotic uptake method (catalog no. H14402; Invitrogen, Grand Island, NY). After 20 min, cells on the insert were fixed in 4% paraformaldehyde for 10 min, and the dye uptake in HCAECs and HCASMCs was observed as yellow fluorescence at the excitation/emission wavelengths of 428 nm/536 nm as described previously [24], and assessed semi-quantitatively using the ImageJ software (http://rsbweb.nih.gov/ij/) [25].

**Inhibition of gap junctions**

HCAECs were treated with 50 μM 18-α-glycyrrhetinic acid (18-α-GA) (catalog no. G 8503; Sigma) dissolved in 0.5% DMSO for 24 h to block the formation of gap junctions. Cells treated with DMSO were used as control.

To assess the effect of connexin expression on the formation of myoendothelial gap junctions and HCASMC phenotypic switching, HCAECs were transfected with Cx40 and Cx43 siRNA (catalog no. sc-29276 and sc-43078, respectively; Santa Cruz Biotechnology) or Cx40 DNA (catalog no. sc-401031-ACT; Santa Cruz Biotechnology) according to the manufacturer’s instructions and co-cultured in contact with HCASMCs.

**Western blotting**

Proteins were extracted as described previously [26], separated by 10% SDS-PAGE, electrophoretically transferred to PVDF membranes, and probed with the following primary antibodies: anti-MYH15 (to detection MHC; catalog no. sc-103055), anti-Cx40 (catalog no. sc-365107), anti-Cx43 (catalog no. sc-271837), and anti-GAPDH (catalog no. sc-365062) (all 1:500 dilution; Santa Cruz Biotechnology), and anti-H1-calponin (catalog no. C2687; Sigma). The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution) at room temperature for 1 hour, and the signals were visualized using an enhanced chemiluminescence substrate (Santa Cruz Biotechnology).

**Inactivation of platelet-derived growth factor (PDGF) receptor signaling**

After 24-h shear stress, HCASMC incubation medium in the upper chamber was collected, and used to measure PDGF-BB concentration by ELISA (catalog no. RAB0397; Sigma). AG-17 (catalog no. 658425; Merck Millipore, Shanghai, China; 100 nM), a PDGF receptor tyrosine kinase inhibitor, was used to block PDGF receptor signaling.

**Statistical analysis**

The data were expressed as the mean ± SD, and analyzed by ANOVA followed by least significant difference (LSD) t-test for post-hoc comparison using the SPSS 13.0 software. P values less than 0.05 were considered statistically significant.

**Results**

**HCASMCs co-cultured with HCAECs show a contractile-like phenotype**

Protein expression levels of MHC and H1-calponin, the markers of SMC contractile phenotype, were markedly decreased in HCASMCs co-cultured with HCASMCs at 48 hours and could be barely detected at 72 hours (Fig. 1A). However, such a protein expression profile was reversed in HCASMCs co-cultured with HCAECs at 48 or 72 hours (Fig. 1B). Furthermore, HCASMCs applied to both sides of the membrane for 48 h exhibited a rhomboid shape, diffuse actin staining (Fig. 1C), and a significant loss of SM22α protein expression compared to the initial level (Fig. 1E), which is typical for dedifferentiated SMCs. In contrast, HCASMCs co-cultured in contact with HCAECs demonstrated a spindle shape with actin filaments arranged in parallel bundles characteristic for differentiated cells (Fig. 1D), and maintained the initial
level of SM22α expression (Fig. 1F). Thus, before receiving the exposure of shear stresses, HCAECs were co-cultured with HCASMs for 48 hours for the examination of myoendothelial gap junctions in the following experiments.

Co-cultured HCASMCs and HCAECs form functional myoendothelial gap junctions

HCASMCs and HCAECs seeded on the top and bottom sides of Transwell membranes, respectively (Fig. 2A), extended processes into membrane pores to make close physical contact (Fig. 2B). To confirm the formation of intercellular contacts, the cell membranes of HCASMCs and HCAECs were labeled with lipophilic fluorescent dyes (HCASMCs, FM 1-43FX, green, and HCAECs, FM 4-64, red). The image shows that merged fluorescence signal (yellow) was detectable at a distance of 5 μm from both cell monolayers (Fig. 2B). Furthermore, HCASMCs and HCAECs extended cellular processes toward each other, as indicated by merged fluorescence (yellow) within the Transwell membrane (Fig. 2C&D).

The formation of projections between HCASMCs and HCAECs was further confirmed by the transfer of biocytin (yellow fluorescence) from HCAECs (Fig. 2E) to HCASMCs on the opposite side of the insert membrane (Fig. 2F). In addition, the expression of connexins Cx40 in HCAECs and Cx43 in HCASMCs was colocalized in intercellular projections formed within the Transwell insert membrane, as evidenced by yellow fluorescence (Fig. 2G&H).

Myoendothelial gap junctions are involved in HCASMC differentiation

The formation of myoendothelial gap junctions was further examined by knocking down Cx40 expression in HCAECs by specific siRNA (Fig. 3A). The results indicate that biocytin transfer from HCAECs to HCASMCs was inhibited by the downregulation of Cx40 levels (Fig. 3B&C), as well as by the treatment with 18-α-GA, a gap junction inhibitor (Fig. 3D&E). Furthermore, Cx40 siRNA attenuated protein expression of MHC and H1-calponin in HCASMCs, while 18-α-GA downregulated H1-calponin (Fig. 3F).
Shear stress of 5 dyn/cm² applied to HCAECs impairs the formation of myoendothelial gap junctions

The exposure of HCAECs to shear stress of 12 dyn/cm² did not affect biocytin transfer from HCAECs to HCASMCs compared to the static co-culture model (Fig. 4A&B), while shear stress of 5 dyn/cm² partially inhibited intercellular biocytin transfer (Fig. 4C). However, if HCAECs were transfected with Cx43 siRNA not Cx40 DNA, and exposed to low shear stress, biocytin transfer from HCAECs to HCASMCs was completely blocked (Fig. 4D&E&F).

Cx43 expression was markedly augmented in HCAECs exposed to low shear stress compared to the cells in static co-culture with HCASMCs or subjected to shear stress of 12 dyn/cm² (Fig. 4G). However, in Cx40 DNA-transfected HCAECs, shear stress of 5 dyn/cm² did not increase Cx43 expression (Fig. 4G). At the same time, Cx40, which is highly expressed...
in untreated HCAECs, was significantly decreased by the exposure to shear stress of 5 dyn/cm². Interestingly, in Cx43 siRNA-transfected HCAECs, shear stress of 5 dyn/cm² slightly increased Cx40 expression; however, the difference was not statistically significant (Fig. 4G).

Shear stress of 5 dyn/cm² applied to HCAECs induces HCASMC dedifferentiation

Immunofluorescence staining showed that when HCAECs were subjected to shear stress of 12 dyn/cm², Cx43 was observed evenly distributed in HCASMC monolayers, and sporadically in HCAEC monolayers (Fig. 5A). However, the exposure to shear stress of 5 dyn/cm² increased Cx43 expression in HCAECs and was even detected within the Transwell membrane (Fig. 5B).

In the static co-culture with HCAECs, HCASMCs were randomly aligned and intracellular actin filaments were organized in parallel bundles, as evidenced by immunofluorescence staining for α-SMA (Fig. 5C). When HCAECs were exposed to shear stress of 12 dyn/cm², HCASMCs were aligned in the flow direction (Fig. 5D); however, when HCAECs were subjected to shear stress of 5 dyn/cm², HCASMCs were arranged randomly and exhibited a typical
rhomboid shape and diffuse actin staining characteristic for dedifferentiated SMCs (Fig. 5E). However, Cx43-specific siRNA (but not control siRNA) or Cx40 DNA transfected into HCAECs partially reversed the effect of low shear stress by restoring spindle-like morphology in HCASMCs (Fig. 5F&G&H).

Furthermore, the expression of MHC and H1-calponin in HCASMCs was downregulated by the exposure of HCAECs to shear stress of 5 dyn/cm$^2$ compared to static or shear stress of 12 dyn/cm$^2$. However, the levels of these two proteins in HCASMCs were restored by transfection of HCAECs with Cx43 siRNA or Cx40 DNA (Fig. 5I).

**PDGF signaling is involved in the shear stress-induced dedifferentiation of HCASMCs co-cultured with HCAECs.**

Figure 6A shows that there was no difference in PDGF release between HCASMCs co-cultured with HCAECs in static conditions and under shear stress (12 dyn/cm$^2$). However, PDGF levels in HCASMC culture supernatant were significantly increased after HCAEC exposure to shear stress of 5 dyn/cm$^2$, while Cx43 downregulation by specific siRNA or Cx40 upregulation by specific plasmid DNA reversed the effect (Fig. 6A).

Shear stress of 5 dyn/cm$^2$ attenuated MHC and H1-calponin levels in HCASMCs compared to physiological stress conditions, but their expression was rescued by the pretreatment of HCASMCs with AG-17, an inhibitor of PDGF receptor signaling (Fig. 6B), suggesting that the PDGF pathway mediates the effects of shear stress on HCASMCs.
Discussion

It is known that resident medial SMCs are not terminally differentiated and can undergo phenotypic switching and proliferate and migrate into the intima, where they play an important role in the development of atherosclerotic lesions. SMC proliferation and migration can be induced by the exposure of vascular endothelium to low shear stress, which is considered atherogenic; however, the underlying mechanisms are not fully understood. In the present study, we observed that HCAECS and HCASMCs co-cultured on the opposite sides of a porous membrane formed heterotypic Cx40/Cx43 myoendothelial gap junctions, which were responsible for the contractile phenotype of HCASMCs. However, the exposure of HCAECS to shear stress of 5 dyn/cm² caused the dysfunction of Cx40/Cx43 heterotypic myoendothelial gap junctions, which may be replaced by homotypic Cx43/Cx43 gap junctions, and induced HCASMC phenotypic transition associated with the activation of PDGF receptor signaling.
In vivo, dedifferentiated SMCs that underwent phenotypic transition contribute to arteriosclerosis. In vitro, SMCs co-cultured with ECs in the medium without serum and growth factors could maintain contractile characteristics [27]. However, they would undergo phenotypic changes if cultured alone in conditioned medium [14], which is consistent with the current findings of diffuse cytoplasmic actin staining in HCASMCs cultured alone in full medium.

When co-cultured with HCAECs, HCASMCs demonstrated the contractile phenotype and maintained the expression of MHC and H1-calponin, the markers of SMCs differentiation, suggesting that HCAECs could inhibit HCASMC transition to the synthetic phenotype. Our observations are in agreement with previous findings that in co-culture, ECs inhibited SMC proliferation, migration, and collagen synthesis, and supported SMC contractile phenotype [28-30]. Furthermore, it has been shown that in co-culture, HCAECs could increase the expression of SM-22α and calponin in HCASMCs [12], suggesting a regulatory role of intercellular contacts with HCAECs in HCASMC differentiation. Moreover, when co-cultured on the opposite sides of Transwell membranes, ECs and SMCs were shown to form myoendothelial gap junctions [9, 28], through which ECs could directly communicate with SMCs and regulate their functional activity [31]. Thus, Cx43-containing myoendothelial gap junctions have been shown to participate in the regulation of pulmonary artery SMC differentiation [14]. In this study, HCAECs and HCASMCs extended their cellular processes toward each other through a porous substrate and established intercellular contacts similar to those observed between mouse ECs and SMCs in co-culture [9]. Furthermore, biocytin loaded in HCAECs was transferred to HCASMCs, indicating the formation of functional myoendothelial gap junctions between HCAECs and HCASMCs.
Myoendothelial gap junctions can be formed by different connexin proteins. In coronary endothelium, Cx40 is abundant, while Cx37 is expressed heterogeneously and Cx43 weakly [18, 24]. In the present study, biocytin transfer from HCAECs to HCASMCs was inhibited by Cx40 siRNA, suggesting that the myoendothelial gap junctions between HCAECs and HCASMCs contained Cx40. Given that HCASMCs are rich in Cx43 [17], the current findings suggest that HCAECs and HCASMCs could form heterotypic Cx40/Cx43 myoendothelial gap junctions similar to those observed in vitro for SMCs co-cultured with insulinoma cells [32] and neuroblastoma cells [33].

To investigate the role of myoendothelial gap junctions in HCASMC differentiation, Cx40 expression in HCAECs was knocked down by siRNA, which inhibited intercellular dye transfer from HCAECs to HCASMCs and significantly decreased MHC and H1-calponin levels in HCASMCs. Given that similar effects were caused by 18-α-GA, a gap junction blocker, these results suggest that the formation of Cx40/Cx43 myoendothelial gap junctions between HCAECs and HCASMCs prevents phenotypic switching of HCASMCs.

The expression of connexins can depend on the physiological state, including hibernation, arousal, level of hormone and mechanical environments [34-36]. Several studies demonstrated that Cx43 was increased in ECs cultured alone by high laminar shear stress (15 dyn/cm²) compared to static conditions [19, 37]. However, in the present study we found that shear stress of 5 dyn/cm² not 12 dyn/cm² increased the level of Cx43 compared to static conditions in HCAECs co-cultured with HCASMCs, supported by the findings that shear stress of 4 dyn/cm² induced a higher Cx43 expression in ECs co-cultured with SMCs compared to ECs cultured alone [22]. In case of Cx40, a previous study showed that its endothelial protein expression was elevated markedly by shear stress from 6-10 dyn/cm², while higher shear stress did not affect the expression of Cx40 in HUVECs cultured alone compared to static conditions [20]. However, an earlier study showed that shear stress of 15 dyn/cm² downregulated the level of Cx40 expression in rat aortic HUVECs co-cultured with HUVMCs compared to static conditions [38]. These discrepancies might be explained by different cellular culture models used in the studies and suggested that SMCs might affect the mechanical response of ECs to shear stress.

It should be noted that in the present study, we found that shear stress of 5 dyn/cm² decreased the level of Cx40 in HCAECs co-cultured with HCSMCs, while shear stress of 12 dyn/cm² had no effect, which is not consistent with the findings in the previous study of HUVECs [38]. This discrepancy may be attributed to the difference in the cell lines used, indirectly supported by the difference of HCAECs and HUVECs in the wound closure under low laminar shear stress (3 dyn/cm²) [39]. Furthermore, intercellular communication between HCAECs and HCASMCs (biocytin transfer) was not affected by shear stress at 12 dyn/cm², but inhibited by that at the low level. Taken together, these results suggested that shear stress of 5 dyn/cm² decreased Cx40 expression in HCAECs, which impaired the formation and functional performance of Cx40/Cx43 heterotypic myoendothelial gap junctions between HCAECs and HCASMCs.

The expression ratio of Cx40 to Cx43 could influence the opening and closing of heterotypic gap channels [40]. A recent study showed that induction of Cx40 expression in liver epithelial cell could lead to about 25% reduction of the protein level of Cx43 [41]. Here, we observed that in HCAECs, Cx40 and Cx43, which had high and low expression levels, respectively, were down- and upregulated by shear stress of 5 dyn/cm². To upregulate Cx40 expression in HCAECs by the transfection with Cx40 DNA could reverse the increased Cx43 expression in HCAECs, and could rescue the biocytin transfer from HCAECs to HCASMCs under low shear stress conditions. These findings suggest that the Cx40/Cx43 myoendothelial gap junctions may be regulated by shear stress of 5 dyn/cm² via the Cx40/Cx43 expression ratio. In addition, the exposure of HCAECs to shear stress of 5 dyn/cm² induced HCASMC phenotypic switch characterized by the loss of contractile morphology and decrease in MHC and H1-calponin expression, which was partly rescued by Cx40 augmentation, suggesting that Cx40/Cx43 heterotypic gap junction plays an inhibitory role in the phenotypic switching of HCASMCs co-cultured with HCAECs under low shear stress conditions.
Interestingly, in the present study compared to static conditions, shear stress of 12 dyn/cm² did not change the protein expression of contractile markers in HCASMCs co-cultured with HCAECs for 48 hours, which is inconsistent with the previous findings that shear stress of 12 dyn/cm² augmented the level of MHC and H1-calponin in HUASMCs co-cultured with HUVECs for 24 hours [42]. Other studies showed that SMCs co-cultured with ECs at or more than 48 hours were more prone to the contractile phenotype compared to SMCs cultured alone or with SMCs [43, 44]. However, compared to SMCs cultured alone or co-cultured with SMCs at 24 hours, SMCs co-cultured with ECs at 48 hours exerted a more synthetic phenotype characterized by the “hill and valley” morphology and acceleration in proliferation and protein synthesis, probably due to myoendothelial gap junctions that were not fully formed [28]. Thus, the discrepancy might be attributed to the longer time in co-culture of HCASMCs with HCAECs in this study, which caused a higher basal level of MCH and H1-calponin in HCASMCs before the exposure of HCAECs to shear stress. Then compared to static conditions in this study, the relative elevation in protein expression of MCH and H1-calponin caused by shear stress of 12 dyn/cm² might be too slight to be detected significantly, suggesting that shear stress of 12 dyn/cm² is more prone to prevent HCASMCs from losing the contractile phenotype, which need to be elucidated further in future studies.

It should be noted, however, that shear stress of 5 dyn/cm² did not completely block HCAECs/HCASMCs myoendothelial gap junctions. Cx43 immunofluorescence was mainly detected in HCASMCs, while after shear stress of 5 dyn/cm², it was increased in HCAECs and was even observed inside Transwell membrane pores, suggesting the formation of Cx43 homotypic myoendothelial gap junctions between HCASMCs and HCAECs. In addition, foregoing effects of shear stress of 5 dyn/cm² on the phenotypic switching of HCASMCs was also partly reversed by Cx43 inhibition, suggesting that Cx43/Cx43 homotypic gap junction may play a contributive role in the phenotypic switching of HCASMCs in the present study.

It has been reported that upregulation of Cx40 could reduce the amount of Cx43 in the junctional fraction markedly in cells endogenously expressing Cx43 [41]. Taken together, our findings suggest that HCAEC exposure to shear stress of 5 dyn/cm² prevented the formation of Cx40/Cx43 heterotypic myoendothelial gap junctions between HCAECs and HCASMCs, possibly inducing the assembly of Cx43 homotypic channels, which may facilitate the transition of HCASMCs to the synthetic phenotype. This hypothesis requires further confirmation in future studies.

PDGF is a potent stimulator of SMC phenotypic switching [2]. The injury of the endothelial layer has been shown to induce PDGF synthesis and secretion from co-cultured SMCs, and promote SMC proliferation. In the present study, we observed an increase in PDGF secretion by HCASMCs co-cultured with HCAECs exposed to shear stress of 5 dyn/cm², which was attenuated by Cx43 downregulation in HCAECs, suggesting that PDGF secretion was associated with Cx43-dependent myoendothelial channels. At the same time, Cx40 upregulation in HCAECs also decreased PDGF secretion in HCASMCs, which may be realized indirectly by regulate Cx43 homotypic channels between HCAECs and HCASMCs in this study. However, a contribution of PDGF released from HCAECs after shear stress induction at 5 dyn/cm² could not be excluded. Furthermore, the inhibition of PDGF receptor signaling reversed the downregulation of MHC and H1-calponin levels by shear stress of 5 dyn/cm², suggesting the involvement of PDGF signaling in HCASMC phenotypic switching induced by low shear stress-treated HCAECs.

It should be noted that Cx40 levels in HCAECs were slightly, though insignificantly, increased by Cx43 siRNA. Given that Cx43 downregulation in HCAECs partly reversed HCASMC phenotypic transition, it suggests that the loss of Cx43/Cx40 heterotypic myoendothelial gap junctions induced by shear stress of 5 dyn/cm² may be restored to some degree by inhibiting Cx43 expression in HCAECs. Further experiments are required to support this suggestion.

Besides PDGF, the differentiation of smooth muscle cells could be regulated by several cytokines such as transforming growth factor (TGF)-β1 [14] or inflammatory mediators such as interleukin-1 (IL-1) [45] and salusin-β [46], which were also responsible in ECs to low shear stress [47]. In particular, IL-1 was demonstrated to specifically inhibit heterogenous...
gap junctions [48]. Therefore, whether these factors were involved in the changes of myoendothelial gap junction and phenotypic switching of HCASMCs regulated by shear stress in this study needed to be explored further in the future study.

In vivo, the presence of myoendothelial gap junctions has been demonstrated mainly in resistance arteries, and the frequency of myoendothelial gap junctions increases with decreasing vessel size [49]. Their presence in coronary arteries was suggested by previous studies that SMCs were hyperpolarized by EET-induced endothelial cell hyperpolarization in pig coronary artery [50]. Although the current results suggested that myoendothelial gap channels were changed by laminar shear stress in vitro, it needs to be elucidated whether the organization and distribution of myoendothelial gap junctions are dependent on the mechanical stimulation of shear stress in vivo.

Furthermore, in the coronary arteries, endothelial Cx40 is readily detectable in the proximal parts, whereas endothelial Cx43 is more abundant in the bifurcation region [18] constantly exposed to low and oscillating shear stress and considered atherogenic [51]. Additionally, the shear stress was changed with the size of coronary arteries and cardiac cycle [51, 52]. Thus the levels of laminar shear stress used in this study could not represent the typical shear stress conditions in the coronary systems, although they were found to regulate the myoendothelial gap junctions involved in the phenotypic transition of HCASMCs. Therefore, the effects of other types of shear stress in vivo, especially the oscillating shear stress which is considered more atheroprone, should be explored further in the future study.

In conclusion, the formation of Cx40/Cx43 heterotypic myoendothelial gap junctions between HCAECs and HCASMCs could inhibit phenotypic transition of HCASMCs to the dedifferentiated state. The exposure of HCAECs co-cultured with HCASMCs to laminar shear stress of 5 dyn/cm² could prevent the formation of Cx40/Cx43 heterotypic myoendothelial channels and favor that of Cx43 homotypic channels, which correlated with HCASMC phenotypic switching and PDGF receptor signaling in HCASMCs, suggesting that changes of myoendothelial gap junctions might be implicated in shear stress-associated arteriosclerosis.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81200206 to ZQ Zhang), Science and Technology Commission Foundation of Bashan District in Shanghai (11-E-5 to ZQ Zhang), and Doctoral Fund of the Ministry of Education of China (20110073120096 to ZQ Zhang).

Disclosure Statement

We declare that we have no conflict of interest.

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Zhang et al.: Myoendothelial Gap Junction Regulates Smooth Muscle Cell Differentiation by Laminar Shear Stress


