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Matrix stiffness determines the phenotype of vascular smooth muscle cell *in vitro* and *in vivo*: Role of DNA methyltransferase 1

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ABSTRACT

Cells perceive the physical cues such as perturbations of extracellular matrix (ECM) stiffness, and translate these stimuli into biochemical signals controlling various aspects of cell behavior, which contribute to the physiological and pathological processes of multiple organs. In this study, we tested the hypothesis that during arterial stiffening, vascular smooth muscle cells (SMCs) sense the increase of ECM stiffness, which modulates the cellular phenotype through the regulation in DNA methyltransferases 1 (DNMT1) expression. Moreover, we hypothesized that the mechanisms involve intrinsic stiffening and deficiency in contractility of vascular SMCs. Substrate stiffening was mimicked *in vitro* with polyacrylamide gels. A contractile-to-synthetic phenotypic transition was induced by substrate stiffening in vascular SMCs through the down-regulation of DNMT1 expression. DNMT1 repression was also observed in the tunica media of mice aortas in an acute aortic injury model and a chronic kidney failure model, as well as in the tunica intima of human carotid arteries with calcified atherosclerotic lesions. DNMT1 inhibition facilitates arterial stiffening *in vivo* and promotes osteogenic transdifferentiation, calcification and cellular stiffening of vascular SMCs *in vitro*. These effects may be attributable, at least in part, to the role of DNMT1 in regulating the promoter activities of Transgelin (SM22α) and α-smooth muscle actin (SMA) and the functional contractility of SMCs. We conclude that DNMT1 is a critical regulator that negatively regulates arterial stiffening via maintaining the contractile phenotype of vascular SMCs. This research may facilitate elucidation of the complex crosstalk between vascular SMCs and their surrounding matrix in healthy and in pathological conditions and provide new insights into the implications for potential targeting of the phenotypic regulatory mechanisms in material-related therapeutic applications.

1. Introduction

Physical properties of the extracellular matrix (ECM) and mechanical forces are integral to tissue homeostasis. Cells have evolved sophisticated systems to perceive both their native microenvironments and the material properties of artificial implants in terms of ECM stiffness, translate these stimuli into biochemical signals controlling various aspects of cell behavior, with the consequent modulation in the physiological and pathological processes of natural organs as well as in biocompatibility of artificial organs [1,2]. Matrix stiffness directs the lineage specification of embryonic and adult stem cells with pluripotency and self-renewing properties. For example, soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle are

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myogenic, and comparatively rigid matrices that mimic bone are osteogenic [3]. Matrix stiffness also acts solely or synergistically (with some biochemical factors) to affect the phenotypic modulation of highly-differentiated/mature cells that retain lineage plasticity and multipotential capability; that is, cell-fate control [4–6]. Vascular smooth muscle cells (SMCs) are able to undergo modulation in their phenotypic continuum, ranging from a differentiated contractile and quiescent state, to a dedifferentiated synthetic, secretory, and proliferative state [7]. These states differ in the expression of vascular SMC-restricted contractile marker genes and synthetic or osteoblastic marker genes, and cell behavior [8]. Several *in vitro* studies have shown that increase of substrate stiffness downregulates the expression of α-smooth muscle actin (SMA) in cultured vascular SMCs [9], promotes SMC migration [10] and proliferation [11], and exacerbates SMC response to platelet-derived growth factor (PDGF) [12]. Such findings implicate that vascular SMC phenotype and behavior could be changed due to increased vessel stiffness during the development of vascular diseases. However, the underlying mechanisms of how matrix stiffness affects SMC phenotype and function are largely elusive.

Arterial stiffening, occurring as a consequence of aging, hypertension, arteriosclerosis, atherosclerosis, diabetes, and chronic kidney failure, is featured with an increase in elastic modulus of the arteries [13–16]. The mean elastic modulus of thoracic aortas from Apolipoprotein E (ApoE)-deficient mice (which are susceptible to atherosclerosis), examined by atomic force microscope, reached up to 15 kPa in comparison with 5 kPa from the age-matched wild-type mice [15]; the elastic modulus of aortas from Lewis polycystic kidney rat, a rodent model of chronic kidney disease, was 56% higher compared with the wild-type Lewis rat [16]. The increasing of arterial stiffness is assumed to be mainly attributed to changes in both the composition and the organization of the ECM, i.e., deposition of excess ECM and calcium phosphate salts, fragmentation and loss of arterial elastin, and crosslinking of adjacent collagen fibers [17,18]. However, arterial stiffness may also increase with changes in phenotype and structural properties of vascular SMC. Emerging evidences have revealed that arterial stiffening is associated with increased vascular SMC proliferation, migration, apoptosis [19], and osteochondrogenic transformation [20], accompanied by changes in intrinsic mechanical properties of the cells [21,22]. An interplay exists between the phenotypic modulation of SMCs and the structure and content of ECM. The key molecules responsible for sensing arterial stiffness and mediating the SMC responses instructed by the cellular microenvironment remain incompletely understood.

Covalent methylation of DNA cytosine occurs almost exclusively in the context of CpG dinucleotides and in most cases promotes transcriptional repression or sometimes results in activation of genes and noncoding genomic regions [23]. The importance of DNA methylation in maintaining normal development and biological functions is reflected by the development of many diseases due to hyper- or hypo-methylation of DNA with improper temporal or spatial regulations [24,25]. Maintenance of methylation patterns during cell replication is mediated by DNA methyltransferase 1 (DNMT1), which catalyzes the transfer of a methyl group from S-adenosyl methionine to hemi-methylated DNA [23]. Aberrant DNMT1expression and DNA methylation have been observed in cultured aortic SMCs in propathogenic conditions. For instance, recent data demonstrated a global DNA hypomethylation and a decrease of DNMT1 expression in aortic SMCs in proliferating and replicative aging [26]. Additional reports documented that treatment of aortic SMCs with 5-Aza-2′-deoxycytidine (5-Aza), a DNA methyltransferase inhibitor, increases the expression of genes related to osteogenesis and facilitates the inorganic phosphorus-induced mineralization of the cells [27]. These findings suggest a contribution of smooth muscle DNMT1 dysfunction to arterial stiffening. We have previously showed that DNMT1 is a mechanosensitive molecule, as evidenced by its shear stress-regulated expression and activation in vascular endothelial cells *in vivo* and *in vitro* [28]. Thus, we wondered whether DNMT1 mediates the regulation of ECM stiffness on SMC phenotype and how DNMT1 is involved in arterial stiffening.

In this study, we elucidated whether ECM stiffness modulates smooth muscle phenotype through DNMT1. Substrate stiffening is mimicked *in vitro* with polyacrylamide (PA) gels in which the concentration of bis-acrylamide crosslinking sets the elasticity, and adhesion is provided by coating the gels with fibronectin. We observed a contractile-to-synthetic phenotypic transition induced by substrate stiffening in vascular SMCs through a down-regulation of DNMT1 expression. By using an acute aortic injury mice model and a chronic kidney failure mice model, both of which show a phenotype of arterial stiffening, we provided the first evidence that DNMT1 is repressed in stiffening arteries. Data of DNMT1 expression in calcified atherosclerotic lesions of human carotid arteries are in line with the results obtained in the mice models. We also showed that DNMT1 inhibition facilitates arterial stiffening *in vivo* and promotes osteogenic transdifferentiation, calcification and cellular stiffening of vascular SMCs *in vitro*. Finally, we suggested a mechanism that DNMT1 responds to and regulates arterial stiffness, at least in part, by increasing the promoter activities of smooth muscle contractile genes, Transgelin (SM22α) and SMA, and by promoting SMC contractility.

2. Materials and methods

2.1. Cell culture

Primary human umbilical artery SMCs (HUASMCs) at passages 3 to 8 were used for all experiments. HUASMCs were maintained in Nutrient Mixture F12 Ham Kaighn's Modification (F12K, Sigma-Aldrich) supplemented with 20% FBS (Gemini) and 10% SMC Growth Medium (Cell Applications). To inhibit DNMT1 expression and activity, the cells were either incubated in 5-aza-2′-deoxycytidine (5-Aza) in culture medium at a concentration of 10 μmoL/L for three days with daily replacement of the medium, or infected with recombinant adenovirus expressing shRNA specifically targeting human DNMT1 genes (ad-shDNMT1) three days before the experiments. To inhibit myosin ATPase and microtubule assembly, cells were incubated respectively with 2,3-Butanedione 2-monoxime (BDM, 10 mmol/L) or nocodazole (5 μmoL/L) in culture media for 5 h after being cultured on gels for 6 h.

2.2. Antibodies, reagents, plasmids and adenovirus

Rabbit polyclonal antibody (pAb) against DNMT1 and rabbit pAb against fibronectin were purchased from ABclonal. Goat pAb against proliferating cell nuclear antigen (PCNA), rabbit pAb against Cyclin A, rabbit pAb against smooth muscle myosin heavy chain (SMMHC), rabbit pAb against SM22α were purchased from Santa Cruz Biotechnology. Rabbit pAb against RunX2 and rabbit pAb against bone morphogenetic protein 2 (BMP2) were from Abcam. Rabbit pAb against GAPDH was from Easybio. Mouse mAb against 5-methlcytosine (5-meC) was from Eurogentec. 5-aza-2′-deoxycytidine was from Aladdin. BDM was obtained from Abcam. Nocodazole was from Sigma. pcDNA3/Myc-DNMT1, in which the full-length cDNA for human DNMT1 was cloned into EcoRI and NotI sites of pcDNA3/Myc, was a gift from Arthur Riggs (Addgene plasmid # 36939). Ad-shDNMT1 and the control adenovirus expressing GFP (ad-GFP) were obtained from Vigene Biosciences.