VASCULAR SMOOTH MUSCLE CELLS PHENOTYPIC MODULATION BY HYPERGLYCEMIA: AN UPDATE ON UNDERLYING MECHANISMS

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The last years accumulated substantial progress in identification of the disturbed intracellular pathways underlying vascular complications associated with Diabetes mellitus, anticipating their target in new therapies intended amelioration or even reversal of dysfunctions, for the ultimate benefit of the diabetic patient. This review up-dates the latest achievements in understanding the complex relationship between high glucose concentration and the phenotypic change of medial artery smooth muscle cells. We present evidence on their phenotypic switching from the differentiated contractile phenotype to de-differentiated biosynthetic, migratory, proliferative, inflammatory, and osteoblast-like phenotypes, outlining the recently identified molecules behind vascular smooth muscle cells remarkable plasticity. The results originating from our laboratory complete this review. As conclusion, the immediate study directions emerged are included; these involve the further understanding of the signaling cascades intervening in the regulation of smooth muscle cells phenotypic changes, the use of integrative genomics approaches, and of vascular smooth muscle cells-restricted microRNAs as efficient mechanisms to fine-tune cardiovascular homeostasis.

Key words: Phenotype; Biosynthetic; Migratory; Proliferative; Inflammatory; Osteoblast-like.

INTRODUCTION

Under physiological conditions, vascular smooth muscle cells (VSMCs) display a differentiated, contractile phenotype that sustains the structural and functional integrity of the vascular wall. This phenotype is a quiescent state of growth, where contractile genes encode expression of distinct proteins, e.g. smooth muscle α-actin (α-SM actin), smooth muscle myosin heavy chain (SMMHC), transgelin, and smoothelin[1]. The recent data documented several factors that contribute to the maintenance of VSMCs contractile phenotype, such as blood flow, intracellular molecules, matrix factors, the non-coding microRNAs (miR), and the transcription factors. Reportedly, the laminar shear stress exerts protective effects on VSMCs homeostatic phenotype through the activation of PPAR- α/δ by the endothelial cells (ECs)-released PGI_2[2]. Within the cell cytoplasm, Smooth Muscle 22 α (SM22 α) interacts and co-localize with F-actin, facilitates the assembly of actin filaments into bundles, and thus participates in the maintenance of the actin cytoskeleton[3], while the multifunctional LDL receptor-related protein (LRP1) functions as a gatekeeper of VSMCs differentiation[4]. Among the miR, premiR-145 or adenovirus expressing miR-145 (Ad-miR-145) up-regulates the expression of VSMCs differentiation marker genes, such α-SM actin, calponin, and SMMHC[5]. Furthermore, normal expression of mouse miR-143/145 cluster is required for the contractile phenotype of murine VSMCs [6]. The four-and-a-half LIM domain protein 2 (FHL2) was found to be essential for the vasomotor tone, regulating contractile genes expression in VSMCs[7]. Among the transcription factors, the cyclic adenosine monophosphate response element

binding protein (CREB) is important for maintenance of VSMCs quiescence, differentiation, and survival[8]. From the extracellular matrix of the healthy arterial wall, collagen type IV stimulates the binding of Serum Response Factor (SRF) transcription factor to CArG boxes in the promoters of smooth muscle actin and SMMHC, and also the expression of myocardin, a critical SRF co-activator required to drive VSMCs contractile gene expression [9].

At sites of physical injury, as well as under the insult of local or systemic pathological molecules such as high glucose (HG) concentration, reactive oxygen species (ROS), various growth factors and extracellular matrix components, the differentiated VSMCs undergo an unique process known as "phenotypic modulation/switching," transitioning from the quiescent, contractile phenotype to a spectrum of new phenotypes. Phenotypic modulation is coupled with down-regulation of differentiation marker genes, and up-regulation of genes responsive for cells biosynthetic, migratory, proliferative, inflammatory, or osteoblast-like profiles. The integrative genomics approaches are ongoing in order to understand the complex interactions between the transcription factors mediating VSMCs phenotypic plasticity in response to various insults; thus, the Down Syndrome Candidate Region 1 (DSCR1/RCAN1/ MCIP1) was identified as a novel calcineurin/ nuclear factor of activated T-cell (NFAT)-dependent injury-responsive gene[10]. VSMCs differentiation and the de-differentiation programs are also prone to proteolytic regulatory mechanism exerted by membrane-anchored matrix metalloproteinase MT1 on the multifunctional LRP1[4]. Important for explaining the higher incidence of vascular complications in immunosuppressed post-transplant subjects are the results that show the direct effects of cyclosporine-A (used clinically to combat organ graft rejection in transplant subjects) on the vascular wall: it up-regulates expression of Krüppel like factor-4 and promotes phenotypic modulation of VSMCs [11]. Understanding the intimate mechanism of VSMCs phenotypic modulation is essential, taking into account its two-sided effects: benefic, facilitating angiogenesis and blood vessel repair after injury, and harmful, playing a critical role in the pathogenesis of major human diseases including atherosclerosis, intimal hyperplasia, restenosis, post-transplant arteriopathy, and pulmonary hypertension [12, 13].

THE BIOSYNTHETIC PHENOTYPE OF VSMCs

Located beneath ECs layer, VSMCs encounter the diabetes-associated HG concentration either directly (after injury removal of ECs), or indirectly (while meeting the glucose permeated or transported through ECs layer). Both circumstances up-regulate the expression of genes responsive for cells biosynthetic phenotype, while down-regulating contractile genes expression[6]. The morphologic evidence for this new phenotype is the replacement of the contractile elements specific for the quiescent VSMCs by biosynthetic organelles, such as Golgi complex and rough endoplasmic reticulum (rER). Such change takes place both in vitro and in vivo. Thus, human aortic SMCs cultured in medium supplemented with 25mM D-glucose showed a gradual enrichment in rER, along with elevation of ERK1/2 activation, and of non-receptor PTP1B expression[14,15]. The enhanced activity of the secretory pathway in VSMCs exposed in vitro to HG concentration is illustrated also by the augmented expression of P-type Ca^{2+} pump, Ca^{2+}-ATPase 1 (SPCA1 or ATP2C1) located in the Golgi apparatus[16]. Intracellularly, diabetic hyperglycemia increases oxidative stress, activates the NF-κB signaling pathway[17], and enhances the expression of signaling proteins such as $G_q/11\alpha$ and phospholipase C-β (PLC-β) that mediate signaling attributed to angiotensin II type 1 receptor antagonists, and to the endothelin (ET_A, and ET_B) receptors [18].

In diabetic animal models, VSMCs biosynthetic phenotype was evident in various vascular territories such as the aorta and renal artery [19], the femoral vein[20], and the mesenteric resistance arterioles (Fig. 1a). Comparison of saphenous vein SMCs from normal and Type 2 diabetes patients showed that the latter possess a distinct morphology with a predominant rhomboid phenotype, disrupted F-actin cytoskeleton, disorganized α-SM actin network, and increased focal adhesion formation [21]. More recently, in vivo and in vitro models for the de-differentiated VSMCs synthetic phenotype were reported, such as the JCR rat, a model mimicking human metabolic syndrome, and cultured JCR aortic SMCs [22].

THE MIGRATORY AND PROLIFERATIVE PHENOTYPES OF VSMCs

Besides the HG-associated biosynthetic phenotype, media layer SMCs acquire increased

migratory and proliferative potential, migrate into the intima layer where over-proliferate and synthesize new extracellular matrix. This re-position of SMCs

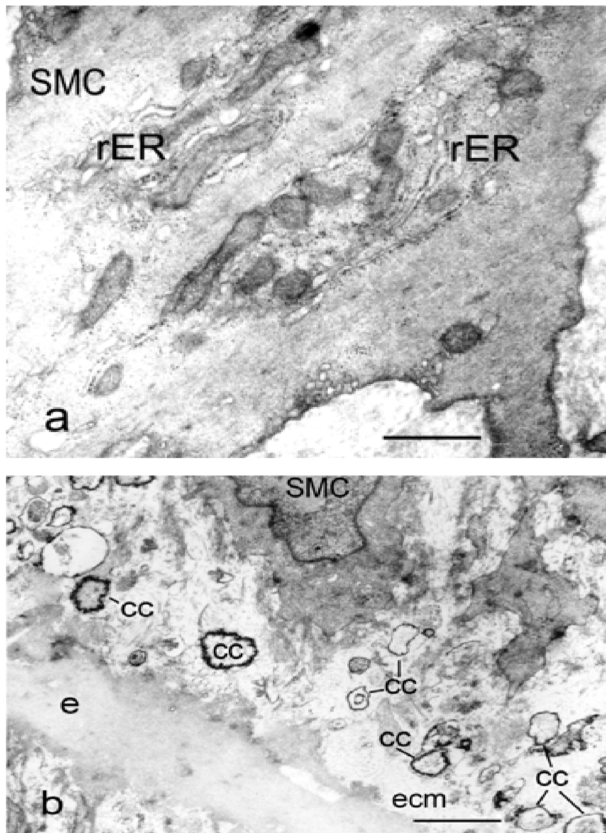

Fig. 1. Electron micrographs of the smooth muscle cells (SMCs) layer in a vascular fragment:

a. mesenteric resistance artery of a 6 months diabetic Golden Syrian hamster. Note the abundance in rough endoplasmic reticulum (rER) that validates cell's biosynthetic phenotype; **b.** the expanded extracellular matrix (ecm) produced by the SMCs layer of the aortic arch of a 6 weeks streptozotocin-injected mouse. Note the numerous calcification centers (cc) distributed along the elastic lamina (e).
Bars: **a.** 0.382 μm, **b.** 0.265 μm.

within the vascular wall promotes blood vessel repair, as well as the pathologic wall remodeling[23]. Reports indicate that the transcription factor ETS1 represses contractile genes transcription (such as α-SM actin, SMMHC and SM22α), while the sustained moesin expression may be necessary to promote the characteristic migratory behavior of synthetic VSMCs[10]. Another stimulator of VSMCs migration is Angiotensin II; its effect involves two pathways: the p38 MAPK-activated c-Src (with contribution of spleen tyrosine kinase, Syk) and the epidermal growth factor receptor transactivation (with contribution of ERK1/2 and partly of p38 MAPK)[24]. From the strategies to inhibit VSMCs migration we quote here inhibition of cells Nox1 by enhanced heme oxygenase-1 and carbon monoxide[25] and the phosphorylation at tyrosine Y168 residue in FAK-related non-kinase [23].

Morphologicaly, VSMCs proliferative phenotype is assessed by the presence of centriols within cytoplasm; these organelles intervene in cells mitosis and were clearly visible at the renal artery SMCs of double transgenic Type 1 diabetic mice[19]. At the jonctional level, the enhanced VSMCs proliferation is associated with reduced expression of connexin 43[26].

A functional link between tetraspanin CD9, integrin α5β1, PI3-K/Akt activity, and enhanced VSMCs migratory and proliferative phenotypes has been demonstrated [27]. Several promoters of VSMCs proliferative phenotype were identified so far, such as HG (that stimulate G1 to S phase progression of cell cycle), and activation of platelet-derived growth factor signaling (that transcriptionally induces expression of microRNA-221; the latter down-regulates the targets p27Kip1 and c-Kit, inhibiting VSMCs-specific contractile gene transcription) [28]. Moreover, Ras activation function as a primary regulator of VSMCs proliferation and migration [29, 30].

With the aim to restrain HG-induced VSMCs over-proliferation, the latest strategies target cell cycle progression by inactivation of p21-activated protein kinase [31]. Reportedly, 3-deazaadenosine (c3Ado) inhibits increased carboxyl methylation of viral protooncogene Ras required for its translocation, membrane association and activation, preventing early cell cycle entry and proliferation of VSMCs [29, 30]. Limitation of proliferation and migration of VSMCs can be accomplished also by elevation of the nuclear hormone receptor coactivator peroxisome proliferator-activated (PPAR)-γ coactivator-1(PGC-1) levels [32], by increasing cGMP-dependent protein kinase (PKG) activity [33], while Rho protein exchange factor Vav3 was considered as a new potential therapeutic target towards limiting vascular proliferative diseases [34]. Other recent reports highlight pharmacological targeting of small molecules, such as the non-coding miR and protooncogenes. Thus, since miR-145 expression is down-regulated in de-differentiated VSMCs (as manifest in culture, in neointima and in atherosclerotic arteries) its restoration via 2'OMe-miR-145 proved to be efficient in inhibiting neointimal growth [5, 35].

THE ADHESIVE AND INFLAMMATORY PHENOTYPE OF VSMCs

Several inductors of inflammation were identified in VSMCs, such as: HG concentration [36], the HG-associated Advanced Glycation End products (AGEs), ligands for the AGEs receptors (RAGE), inactivation of the tumor suppressor Phosphatase and TENsin homolog (PTEN) [37, 38]; moreover, the extracellular matrix collagen type I was found to stimulate the transcription factor NFAT, and to enhance the expression of the inflammatory vascular cell adhesion molecule (VCAM)-1[9].

These dedifferentiated VSMCs phenotypes are characterized by upregulation of adhesion-, inflammation- and survival-related genes, responsible for the increased production of cell adhesion molecules and chemokines[9, 10, 36, 37]. The mechanistic insight beyond the enhanced inflammatory genes expression in VSMCs comprises the involvement of chromatin nucleosomal histones. Thus, in VSMCs exposed to HG as well as in diabetic condition SUV39H1 is down-regulated and the inflammatory genes expressed; it is emphasized that SUV39H1 mediated aberrant methylation of histone H3 (trimethylated at lysine 9, H3K9me3) plays an important role in the negative regulation of inflammatory genes expression in VSMCs[36]. The same laboratory pointed also towards the enhanced levels of histone H3 dimethylated at lysine 4, H3K4me2 and to the reduced lysine-specific demethylase1 (LSD1) occupancy at the promoters of the inflammatory genes, that could lead to increased inflammatory gene expression; conversely, overexpression of the latter inhibited the expression of inflammatory genes in VSMCs of diabetic db/db mice[37].

THE OSTEOBLAST-LIKE PHENOTYPE OF VSMCs

This phenotypic change is a key event driving arterial wall calcification, a complex and dynamic process commonly associated with diabetes mellitus, atherosclerosis, renal failure, as well as to ageing. The life-threatening consequences of arterial wall calcification are related to the diminishment of wall elasticity with the subsequent impairment of blood flow hemodynamics. Recent reports identified specific molecules that orchestrate VSMCs modification to osteoblast-like cells, and the operation of particular intracellular signaling pathways that are to be exploited further as targets to inhibit the calcification process.

The osteoblast-like VSMCs are enriched in osteochondrogenic bone-related molecules, such as core binding factor α-1, osteocalcin, osteopontin, osteoprotegerin, Runx2 protein, bone sialoprotein, bone morphogenetic protein, alkaline phosphatase and type I collagen[39-43].

Within the arterial wall, two populations of SMCs are subjected to osteoblastic differentiation: (i) the SMCs migrated to the neointima of atheromatous plaques, resulting in atherosclerotic plaque calcification associated with the risk of myocardial infarction, and (ii) the VSMCs resident in the tunica media, resulting in medial artery calcification (known also as Mönckeberg's medial calcific sclerosis). The intimal atherosclerotic plaque calcification is a distinct topic. This review is focused on medial calcification that can occur independently of intimal atherosclerotic lesions, and causes vascular stiffness, increased pulse wave velocity, and finally cardiac dysfunction and ischemia[44]. Our laboratory provided evidence that diabetes-associated medial calcification occurs in different vascular territories, such as follows: (i) in the aortic arch of streptozotocin-injected mice, where small but numerous calcification centers with the characteristic profile of hydroxyapatite were present along the elastic lamellae (Fig. 1b), (ii) in the thoracic aorta of hyperlipidemic-diabetic Golden Syrian hamster, where large calcification centers (~ 250nm in diameter) nucleate extensive calcium deposition around dying or dead SMCs, while smaller centers (~ 100 nm in diameter) were formed around membranous vesicles within the enlarged extracellular matrix that separate adjacent SMCs[14], and (iii) in a vascular bed basically involved in blood pressure regulation, such as the small caliber mesenteric resistance arterioles (internal diameter up to 300μm) of streptozotocin-diabetic or of hyperlipemic-diabetic animals, where the calcification centers nucleated around the membrane material within the expanded extracellular matrix secreted by media layer SMCs; in time, the calcification centers increased both in number and in size, and form large deposits that eventually compromise arteriolar distension [45]. The predominant mineral in diabetic arterial medial calcification is hydroxyapatite [39].

The oxidants, the components of the local environment, nuclear factors, vitamin D_3 plus nicotine, inorganic phosphate, calcium, and even

serum have been recently identified to induce medial artery calcification. Formed at an accelerated rate in diabetes, AGEs induced calcification of VSMCs through the AGE-receptor (RAGE)/p38 mitogen-activated protein kinase (MAPK) signaling pathway[46]. Contribution of RAGE pathway to osteoblastic differentiation of VSMCs under the insult of AGEs accumulated in diabetes was also reported [43]. Moreover, the advanced oxidation protein products (novel markers of oxidant-mediated protein damage) significantly decreased smooth muscle α-actin expression in human aortic SMCs, and induced vascular calcification by promoting cells osteoblastic trans-differentiation via oxidative stress and ERK pathway [47]. Other significant contributors to medial calcification in diabetes are the changes in osteopontin expression and the elastin degradation by matrix metalloproteinase-9[39]. Among the nuclear factors, it was established that RANKL binds to RANK, activates the alternative NF-κB pathway, and increases BMP4 production and VSMCs calcification [48]. In rats, vascular calcification was induced by vitamin D_3 plus nicotine treatment for 4 weeks [49]. Reports show that human aortic SMCs undergo mineralization in response to elevated levels of inorganic phosphate (Pi) in an active and well-regulated process, involving increased activity of alkaline phosphatase and augmented expression of core binding factor α-1 (a bone-specific transcription factor) with the subsequent induction of osteocalcin [41]. In rat aorta exposed in vitro to high Pi concentration, apoptosis and subsequent osteoblastic differentiation of media VSMCs occurred as a result of enhanced Pi uptake through the type III sodium-dependent phosphate co-transporter (Pit-1)[50]. Moreover, serum exposure of devitalized rat aorta resulted in calcification of the elastic lamellae of the media; this process was attributed to the presence in serum of a potent calcification factor arising from sites of normal bone mineralization and released into general circulation[51]. It was also demonstrated that diabetic serum induced calcification of VSMCs, and that this process was inhibited by blocking RAGE [46]. Along with the above inducers, the genetic deficiency in calcification inhibitors, such as matrix G1a protein, osteoprotegerin, PC-1 nucleotide triphosphate pyrophosphohydrolase were reported to produce extensive medial artery calcification[52].

For diabetic patients assistance it is important to prevent VSMCs calcification, and to reduce the associated cardiovascular risk. A strategy to this goal is provided by the physiological condition in which medial artery SMCs produce a number of inhibitors of calcification, preserving the normal elastic properties of the vascular wall; targeting the local and circulating inhibitors of calcification, as well as the factors that may ameliorate VSMCs apoptosis will potentially block and ultimately regress VSMCs calcification [53]. Another approach takes advantage of identified molecules/pathways underlying VSMCs calcification. Thus, interruption of the AGE-RAGE interaction might prevent diabetic vascular calcification [43]. In animal models, strategies to prevent or reduce arterial calcification include normalization of serum phosphorus levels (by using phosphate binders or low phosphate diets), correction of the secondary hyperparathyroidism (by calcimimetics and vitamin D analogues), and inhibition of bone turnover using bisphosphonates, osteoprotegerin, and bone morphogenic protein 7 [40]. Recently, heme oxygenase 1 (HO-1)/ferritin was reported to prevent osteoblastic differentiation of human aortic SMCs under the insult of elevated Pi by a mechanism involving the ferroxidase activity of ferritin[41]. In contradistinction to the inductor effects of Pi, extracellular matrix pyrophosphate (PPi) blocks hydroxypapatite formation, and consequently is a potent inhibitor of pathological vascular calcification[54]. PPi is hydrolysed by the nonspecific alkaline phosphatase secreted by osteogenic cells in matrix vesicles; therefore, the conditions that reduce extracellular PPi levels will alter vascular calcification[39]. A PPi analogue is phosphonoformate that also prevented calcification in VSMCs [55]. Reportedly, lanthanum acetate inhibited vascular calcification induced by vitamin D_3 plus nicotine in rats [49]. Other reports suggested that adiponectin plays a protective role against arterial calcification, since it inhibits the osteoblastic differentiation of VSMCs via AdipoR1/p38 signaling pathway [42].

In chronic kidney disease, as a result of the metabolic insults of diabetes, dyslipidemia, oxidative stress, uremia, and hyperphosphatemia, osteoblast-like cells are formed in the vessel wall with contribution of important transcription factors such as Msx 2, osterix, and RUNX2 [56]. In this pathology, hyperphosphataemia induces apoptosis and VSMCs calcification through inhibition of the cell survival pathway gas6/Axl/PI3K/Akt [57]. A particular concern is related to hemodialysis patients where diabetes is strongly associated with the prevalence of calcifications, particularly in

small caliber arteries[58]; the associated arterial stiffening and calcification were related to a deficiency in 25- hydroxyvitamin D3 serum levels, higher brain natriuretic peptide values, and increased pulse pressure [59].

CONCLUSION: WHERE TO GO FROM HERE?

There are several issues that deserve further translational investigation to prevent and treat diabetic VSMCs phenotypic switching; among these we quote the followings: (i) identification of specific regulatory targets for VSMC proliferation[60], (ii) determination of the degree to which GTPases possessing the CAAX sequence are inhibited by c3Ado, as well as the bioavailability of c3Ado, and its efficacy in humans[29], (iii) defining the tyrosine kinase(s) responsible for FRNK phosphorylation, and evaluation of its/their role in limiting the processes of vasculogenesis and neointimal development[23], (iv) studies on the new generation therapeutics that specifically target PTEN to inhibit VSMC phenotypic modulation, proliferation, and inflammation with important clinical implications in the treatment of restenosis[38], and (v) studies on drugs interfering with cell cycle progression in VSMC, as promising candidates for an antirestenotic therapy[61]. Beside these, two new directions in elucidating the molecular mechanisms of vascular injury and VSMCs phenotypic modulation emerge from the recently reported data: the use of integrative genomics approaches[10], and of VSMC-restricted microRNAs, as efficient mechanisms to fine-tune cardiovascular homeostasis and the response of the vessel wall to injury; it is appreciated that the latter will open new avenues for investigation and potentially implement future therapies for vascular diseases [12]. Although vasculature calcification still remains a difficult tissue to target and currently there are no effective treatments in general use, the basic research perspective includes amelioration of endoplasmic reticulum stress, understanding the roles of oxidative stress and inflammation on the fate of VSMCs and their function, and interruption of the AGE-RAGE interaction, as a promising target for therapeutic intervention to reduce VSMCs calcification process [43, 56, 62].

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