Vascular smooth muscle cell motility: From migration to invasion

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Over the past decade, extensive research has focused on identifying the molecular mechanisms and signal transduction pathways involved in the modulation of vascular smooth muscle cell phenotypes. In the present review, the characteristics of vascular smooth muscle cell (VSMC) phenotypes as they relate to cell migration are discussed based on insights from recent molecular findings. A central theme is the mechanisms involved in nonpathogenic VSMC migration during tissue repair versus VSMC invasion that leads to the development of vascular diseases. The issue of how various factors that are released locally following tissue injury influence cell migration will also be addressed.

Key Words: *Cell invasion; Cell migration; Phenotypic remodelling; Signalling pathways; Vascular disease*

Smooth muscle cell (SMC) migration is a normal process that occurs during vascular development or for tissue repair in response to vascular injury. At the same time, pathological migration is a major factor in atherogenesis and restenosis. The distinction between physiological and pathological migration has been attributed to a failure to cease migration once tissue repair has been completed. It is now recognized that modulation of vascular SMC (VSMC) phenotypes is the driving force (1-3).

VSMC PHENOTYPES: FROM NORMAL TO PATHOGENIC

VSMCs exist in a diverse range of phenotypes (4-6). In normal mature blood vessels, the predominant phenotype is the quiescent or differentiated VSMC, known as the contractile phenotype, which functions as the regulator of blood vessel diameter (vasodilation and vasoconstriction) and blood flow (7-9). The switch from the contractile phenotype to the synthetic, migratory and proliferative phenotype takes place for tissue repair in response to injury. In this situation (response to injury), VSMC proliferation and migration are normal processes – in fact, they are necessary. The response to injury is multicellular and involves the production of a number of growth factors including epidermal growth factor, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor (PDGF), transforming growth factor, vascular endothelial growth factor (VEGF) and angiotensin II (AngII). In this situation, several cells act in a paracrine manner to activate the healing response of neighbouring cells. This response requires these cells to proliferate and migrate, which are hallmarks of the synthetic phenotype. If migrating/proliferating VSMCs fail to switch back to the contractile phenotype, they induce pathogenic vascular remodelling and generate intimal vascular lesions (7,8,10). The failure to switch to the contractile phenotype may take place due to the presence of excessive mitogens in the cellular microenvironment.

VSMC PHENOTYPIC MODULATION: CONTRIBUTION OF PROLIFERATION AND MIGRATION

The noncontractile/synthetic phenotype of VSMC (also termed dedifferentiated cells) has reduced the expression of protein required for normal regulation of contractile function. The synthetic phenotype of VSMC has an increased capacity to generate extracellular matrix (ECM) proteins (5,11). In other words, the noncontractile phenotype of VSMC does not regulate vascular contraction but instead controls vascular reconstruction (12). The migration and proliferation of VSMC in the synthetic phenotype are, thus, key elements in atherosclerosis and restenosis (13). It has been observed that VSMC remodelling in vivo and in vitro begins with an extracellular stimulus that activates receptors located on the cell surface. These, in turn, transduce the external signal to several pathways, leading to a series of coordinated remodelling events that trigger cell migration, proliferation or both.

VSMC phenotypic remodelling – the transition from the contractile to the synthetic phenotype – affects both proliferation and migration. Signalling pathways that are activated when exposed to mitogens, growth factors or peptides often trigger pathways that stimulate both proliferation and migration. To date, published data addressing the relation between cell migration and proliferation in VSMCs or other cell types in response to mitogens suggest that the two processes are not interdependent. However, this relation between migration and proliferation was not studied for all mitogens known to induce both processes in VSMCs. In this section, we will discuss the recent data that suggest independence between proliferation and migration in VSMCs and other cell types.

Recent work looking at AngII-induced porcine SMC remodelling in organ culture showed that blocking DNA synthesis with aphidicolin did not inhibit cellular migration or reduce intimal formation compared with cells that were not treated (14). However, blocking metalloproteinases inhibited

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neointimal formation. This analysis suggested that VSMC migration is the earliest step to take place in VSMC remodelling in that culture system. Although it was implied that cellular proliferation occurs subsequent to migration, proliferation of the cells was not examined (14).

Another study (15) investigating the relation between proliferation and migration in response to PDGF activation showed that the induction of cell proliferation takes place only under the influence of high PDGF concentrations (greater than 5 ng/mL), whereas cell migration is activated at lower concentrations of PDGF (1 ng/mL). Interestingly, it was reported that the migration index is negligible at higher PDGF concentrations. According to this study, dose-dependent differential activation of intracellular signalling was observed in response to PDGF. At low PDGF concentrations, maximal activation of signalling pathways was linked to cytoskeleton rearrangement, a process needed for cell motility, whereas high PDGF concentrations activated pathways linked to proliferation and cell division. This study suggested cells switch from a migrating to a proliferating phenotype by sensing the local PDGF concentration. This study was conducted in rodent fibroblasts and has not yet been repeated in VSMCs; however, PDGF as a growth factor is known to induce both VSMC proliferation and migration (15). Further analyses investigating a differential dose effect of other growth factors and peptides are needed to establish what dose may function as a regulator between migration and proliferation on cell phenotypic remodelling.

One possible mechanism that may influence the decision to migrate or proliferate is cell cycle arrest in a specific phase. VSMCs can migrate during the G_1 phase of the cell cycle but not in other phases (16,17). It was shown that the cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ are upregulated on vascular injury (18). Those CDK inhibitors prevent progression through the cell cycle by blocking CDK from binding to cyclins, a step that is essential for G_1/S transition. In the same context, collagen degradation products that result from activation of surface proteases during cell migration were shown to upregulate $p27^{Kip1}$ levels in VSMC (18,19). However, the relationship between $p27^{Kip1}$/$p21^{Cip1}$ levels and cell migration needs further investigation. It is known, however, that exogenous overexpression of $p27^{Kip1}$ decreases neointimal thickening and hyperplasia in rodent models (16,18).

THE ROLE OF VSMC MIGRATION IN ATHEROGENESIS

Atherosclerotic lesions (atheroma) are asymmetric thickenings of the innermost layer of the artery, the intima. Atherosclerosis is a chronic inflammatory response of the arterial wall initiated by injury resulting from chemical insults (hyperglycemia), modified low-density lipoprotein (LDL) or physical forces (hypertension) (9). The earliest changes that contribute to the formation of atherosclerotic lesions take place in the endothelium and result in endothelial cell dysfunction (20). The changes in endothelial cells in response to injury may include decreased production of nitric oxide or increased permeability to lipoprotein, leukocyte adhesion and thrombotic potential (21). In the early atherogenic process, arterial endothelial cells begin to express intracellular adhesion molecule 1 that binds various classes of leukocytes. Vascular cell adhesion molecule 1 binds monocytes and T lymphocytes. After monocytes adhere to endothelial cells, they migrate between endothelial cells to localize in the intima, transform into macrophages after stimulation with chemokines and avidly engulf oxidized LDL. Macrophages produce interleukin-1 and tumour necrosis factor, which increase adhesion of leukocytes (21). Macrophages also generate several chemokines, including monocyte chemotactic protein 1, that recruit more leukocytes into the plaque. Toxic oxygen species produced by macrophages cause oxidation of LDL. When LDL particles become trapped in the vessel wall, they undergo progressive oxidation and can be internalized by macrophages through scavenger receptors, which leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol ester, resulting in the formation of foam cells (22). The next stage is fatty streak formation. The fatty streak initially consists of lipid-laden monocytes and macrophages that are engorged with oxidized LDL (foam cells) together with T lymphocytes. Subsequently, VSMC migration increases the number of cells in the fatty streak (21). In the centre of an atheroma, foam cells and extracellular lipid droplets form a core region that is surrounded by a cap of SMCs and a collagen-rich matrix (23).

VSMCs constitute a major component of the vascular media. During blood vessel formation, the phenotype of VSMCs in the medial layer of the wall changes such that secretion of ECM protein is reduced and the formation of intracellular filament is increased. This is the transition from the synthetic to the contractile phenotype that is required for the VSMC to perform its functions of controlling vascular contractility and blood flow in the healthy vessel. However, the inflammatory response in the microenvironment of the atherosclerotic lesion stimulates migration of VSMCs. If these responses continue unabated and are accompanied by accumulation of new ECM, the artery wall will thicken (24). Recent studies have provided insight into the mechanisms that trigger migration and proliferation of VSMCs, and the matrix metalloproteinases (MMPs) that facilitate removal of the basement membrane around VSMCs and allow their migration (25). The presence of both inflammatory cytokines and growth factors in atherosclerotic blood vessels could, therefore, increase the production of MMPs with the ability to remodel basement membranes and the components of interstitial matrix.

Phenotypic regulation of VSMCs is a very complex process, and cellular motility may be induced via several distinct mechanisms including a random increase in motility, as in wound healing, that is independent of a concentration gradient (chemokinesis), a flow of the cell up or down a chemical concentration gradient (chemotaxis), or a response to different interactions with ECM (26).

VSMCs synthesize the majority of the ECM proteins found in the atherosclerotic lesion (27). The arrival of VSMCs and their elaboration of ECM yield fibrous fatty lesions instead of just the accumulation of macrophage-derived foam cells. The synthesized ECM molecules stabilize the atherosclerotic plaque; however, activated inflammatory and immune cells in the plaque can lead to the death of intimal VSMCs by apoptosis. This is usually followed by plaque rupture in the late stages of atherosclerosis (27).

The mechanics of VSMC migration in atherosclerotic lesions involves formation of plasma membrane-leading

lamellae (leading edge) that are in contact with the ECM. In parallel, the interaction with ECM proteins enables binding of integrin transmembrane receptors to form focal complexes that then secure the focal adhesions (28). A cascade of intracellular signal transduction events involving G proteins and tyrosine kinases results in actin filament alignment and myosin contraction within the leading edge. This is followed by disengagement of the focal adhesions, allowing contractile forces to propel the cell forward in the direction of the anchored leading edge (29,30).

THE ROLE OF VSMC MIGRATION IN RESTENOSIS

Percutaneous coronary intervention is considered a common treatment for coronary artery disease, but its major limitation is restenosis (31). Stent placement is accompanied by stretching of the entire artery, de-endothelialization and compression of plaque, which often results in dissection of the tunica media and, occasionally, dissection of the adventitia (32). Restenosis after stent deployment is considered a result of an exaggerated wound healing response after vascular injury, and is characterized by the sequence of inflammation, granulation, ECM remodelling, and VSMC proliferation and migration (33). The series of events that follow this surgical intervention leads to neointimal thickening and restenosis (34,35). A layer of platelets and fibrin are first deposited on the injured and de-endothelialized vessel surface. Activated platelets on the injured vessel surface express adhesion molecules, such as P-selectin, that are secreted from alpha-granules (36). Leukocytes then adhere firmly to the vessel surface through the leukocyte integrin macrophage-1 antigen. These inflammatory processes activate migration and proliferation of VSMCs, which remodel the ECM, leading to neointimal thickening (36). Macrophage-1 antigen is thought to be an important signalling protein in the mechanism of restenosis due to the fact that its blockage reduced neointimal thickening after experimental angioplasty and stenting (37).

PROGENITOR CELLS IN ATHEROSCLEROSIS

The original theory of intimal thickening in restenosis considers VSMC migration and proliferation to have equivalent roles in atherogenesis. In support of this theory, studies have shown that VSMCs accumulating in the fibrous cap express fewer contractile markers and, based on telomere length assessment, have undergone more population doublings than cells in the normal media, which suggests that existing arterial VSMCs contribute to arterial healing in response to injury (38,39). However, recent studies looking at markers expressed by VSMCs accumulating in the media suggest that intimal thickening after vascular injury may include progenitor SMCs that either locally reside in the arterial wall at the site of injury or mobilize from bone marrow (40,41). These studies suggest that progenitor cells differentiate at the site of injury and contribute to intimal thickening (42). Evidence in support of these hypotheses has been demonstrated by inducing vascular injury in sex-mismatched heart and bone marrow transplantation mouse models, and monitoring the cells that accumulate at the site of vascular injury using in situ hybridization (43). However, the presence and extent of VSMC progenitors in lesions may depend on the model, time and species studied.

MECHANISMS OF VSMC MIGRATION AND INVASION

Cell migration is essential for tissue formation at early developmental stages or for tissue maintenance and regeneration in developed tissue and organs. For most cells, including epithelial, stromal and neuronal cells, migration is typically confined to morphogenesis and ceases with terminal differentiation. They become reactivated only for tissue regeneration. For other cell types, such as leukocytes, migration is integral to their function and this capability is retained for their entire life span. Some cell types can migrate only through a specific microenvironment. Epithelial cells, for instance, can only move along the basement membrane and not through interstitial tissues, whereas other cell types, such as leukocytes, which are able to interact with all connective tissue in the body, are motile regardless of the substrate to which they are exposed.

The complexity of cell migration lies in the fact that it is a process controlled by both cellular (molecular) and environmental (physical) parameters. These parameters are integrated together for each cell type and appear to be tunable, thereby influencing the mode of migration and affecting the switch between physiological and pathological migration. In this section, we discuss the mechanisms that control cellular migration and cellular invasion as well as the regulation of those mechanisms.

Cell migration from normal to pathological

The common process underlying all forms of migration for nucleated mammalian cells is a polarized actomyosin-driven shape change of the cell body (10,44). This basic program is regulated and 'shaped' by several distinct yet interdependent physical and molecular parameters of the tissue and the cell itself that together determine how a cell migrates. In response to environmental determinants, the actomyosin cytoskeleton adapts in a dynamic manner and generates different geometries in space and time, ranging from flat and spread out, to roundish, elongated or multipolar shapes (45). To transmit actomyosin-driven forces to surrounding tissue structures, the cell either develops actin polymerization-driven protrusions that bind to adhesion sites of the tissue through adhesion receptors (46), or it uses poorly adhesive intercalation and propulsion (47). In both cases, subsequent to leading edge protrusion, actomyosin contraction leads to retraction of the cell rear and translocation of the cell body (47,48). The cyclic repetition of those basic steps results in cellular movement that will vary from one cell type to another based on the molecular pathways that are activated and the surrounding extracellular medium. The activation of surface proteases that remodel the surrounding tissue will increase the efficiency of cell migration and modulate the migration mode, enabling the switch to pathological cell migration as in atherogenesis or cancer metastasis (49).

Cells with activated surface proteases can proteolytically remodel the surrounding ECM and generate gaps, a process that is a hallmark of pathological cell migration. Without protease activation, cell motility is limited to filling available spaces within the cell group body (50).

Cell adhesions to ECM ligands are predominantly generated by integrins via coupling to cytoskeletal proteins in response to specific signalling pathways. The strength and turnover rates of

cell attachments to the extracellular environment determine which cell shapes and forces are being generated during migration (10). High integrin expression levels are mandatory for high attachment forces, but are also associated with relatively slow turnover of adhesion sites and, consequently, associated with slow migration (51,52). Strong cell-substrate adhesions thus promote the cellular contractile phenotype and the formation of elongated spread-out or spindle-shaped morphologies (53,54). If cell adhesion is reduced to a moderate or low level, such as by interfering with the integrin-talin axis, focal adhesions and stress fibres do not form or do not reach full maturation (55). As a consequence, the cells convert to a less elongated or spread-out morphology that is more vulnerable to a switch in the synthetic migratory phenotype.

A key determinant of how cells move is whether cell-cell junctions are retained (56). Cell-cell adhesion is mainly mediated by cadherins. Strong junctions inhibit single-cell migration and only allow collective migration. In this situation, cell movement in groups interferes with ECM penetration. If cell-cell contacts are absent, cells move independently in terms of both speed and direction (57). Thus, the presence or absence of stable cell-cell junctions determines whether collective translocation or single-cell migration takes place.

In a given differentiation state, each cell type employs a particular 'default' phenotype; however, naturally occurring (or experimentally induced) modifications of either the environment or cell properties may result in de novo adaptation, a process that can alter the preferred phenotype and switch the cell into its migratory mode. As discussed, most of the parameters controlling migration modes are switchable. For example, modulation of adhesion receptor expression or the availability of cytoskeletal adaptor proteins could be altered as a result of activating or silencing the expression of one gene that is downsteam of a growth factor signalling pathway.

One major pathway controlling the switch between different migratory modes is the balance between Rac and Rho signalling (58). In several experimental systems, the transition from contractile, migratory and invasive modes depended on pathways that downregulate Rac and/or upregulate Rho signalling (59). Similarly, inhibition of chemokine-meditated Rac activation favours the switch to a motile phenotype in quiescent cells (60). On the other hand, pathways that activate Rho promote entry into the quiescent state (61).

Another reversible parameter that controls cell migration mode is the upregulation/activation or downregulation/inactivation of beta (β) 1 , β2 and β3 integrins (62). Furthermore, in loose interstitial tissues with gaps that accommodate the cell body, the inhibition of surface protease activity leads to protease-independent gap-filling migration that also involves a shape change but in the absence of tissue remodelling (63).

Migration versus invasion

Cell migration is essential for several cell types during development and for other cell types such as immune response cells, in which motility is essential for cell function. In the case of VSMCs, mobility is required for the physiological process of wound healing. However, there are several types of cell migration mechanisms that can take place, depending on whether migration is random or directional, and whether migration involves degradation of the ECM (10).

Directional migration takes place as a response to extracellular signals including chemotactic, hepatotactic, durotactic or electrotactic signals, and the migration direction takes place toward the source of the signal (10). In VSMCs, random and directional movement involves the formation of lamellipodia, subcellular structures that have both a leading edge and a tailing edge; however, cell migration involving lamellipodia is not associated with the secretion of metalloproteinases or modification of the ECM. In fact, directional cell migration is often influenced by the two-dimensional and three-dimensional organization of the ECM, as well as by the composition of the matrix (64).

If ECM degradation takes place, cell motility in this case is called invasion rather than migration and involves subcellular structures – different from lamellipodia – named invadopodia and podosomes. For both, migration and invasion cell motility involves actin severing to create barbed ends, followed by actin polymerization to form a plasma membrane protrusion. Furthermore, lamellipodia, invadopodia and podosomes all share a number of components including integrins and other focal adhesion proteins such as focal adhesion kinase (FAK), cofilin, paxillin, talin and Src, among others (65).

There are a number of structural and nonstructural differences between lamellipodia, and invadopodia and podosomes. Structurally, lamellipodia have one leading edge and one tailing edge that extend diagonally along the cell. Invadopodia and podosomes are subcellular protrusions lying along the ventral side of the cell. Nonstructurally, invadopodia and podosomes are characterized by the ability to break down or digest the ECM via activation of metalloproteinases, a process that does not take place in lamellipodia-based cell migration. Another difference is the mechanism of activation of proteins involved in cell motility. For example, cofilin activation, which is essential in both lamellipodia-based and invadopodia/podosome-based migration, is coupled to distinct regulatory pathways for each of those subcellular structures. This will be further discussed in another section of the present review.

Invadopodia are usually observed in metastatic cells rather than untransformed (noncarcinogenic) cells. This could be due to limitations in the imaging techniques being used for characterization of metastatic cells in vivo and the inability to detect the initial steps in metastasis. It is proposed that podosome structures form during the initial steps of cellular invasion whether metastatic or not, and then podosomes switch to invadopodia. To date, however, this mechanism is still hypothetical and has not been shown in any cell type. It is worth mentioning that invadopodia differ from podosomes in size, number and turnover rate, with invadopodia being larger in size but fewer in number and with longer turnover rates than podosomes. Thus, invadopodia are able to facilitate cell migration in a faster and more aggressive manner.

Podosomes were first observed and reported in a mouse SMC line in 2002 (66,67). The formation of podosomes in this cell line appears to be protein kinase C- and Src-dependent, and involves classical podosome components such as cortactin, Arp2/3 complex and vinculin (67,68). It was also shown in the same cell line that podosome structures mediate ECM degradation. Recently, it was shown that microRNA-143 and -145, which regulate the switch from the contractile to the synthetic phenotype, also regulate podosome formation in SMCs in vitro

and in mice in vivo. The same study reported that the formation of podosomes in mouse aorta SMCs is sensitive to PDGF treatment (69).

In support of the fact that SMC migration following vascular injury resembles 'cell invasion' mediated via podosomes, we recently reported (70) that cortactin is phosphorylated and translocated to the plasma membrane on AngII treatment. These data also suggest that AngII induces podosome formation in porcine VSMC; however, further analysis is still needed to confirm this hypothesis.

KEY PLAYERS IN VSMC MIGRATION

In this section, we will discuss the role of various signalling pathways that control VSMC migration and invasion. We will also discuss, wherever applicable, the differential regulation of those players between migration and invasion. We will focus on integrins, integrin-linked kinase (ILK), FAK, cortactin-cofilin and metalloproteinases.

Integrins

Integrins are a family of transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions (71). All known members of this superfamily are noncovalently associated heterodimers composed of one alpha (α) and one β subunit. At present, at least eight β and 18 α subunits have been characterized, and these subunits associate to generate at least 24 different integrins. For example, subunit β3 associates with subunits αIIb and αv to generate integrins αIIbβ3 and αvβ3, respectively. Integrins are type I membrane proteins composed of a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (72).

The interaction between integrins and their ligands, besides mediating cell adhesion, plays a role in a number of cellular processes. The heterodimer αvβ3 integrin is one of the most highly expressed integrins in cells originating from mesenchymal origin, including endothelial cells and SMCs (73). The αvβ3 integrin is known to mediate many biological events, especially migration of vascular SMCs. The αvβ3 heterodimer is highly active due to its ability to bind various ligands including fibronectin, vitronectin and fibrinogen through the Arg-Gly-Asp motif (74).

Integrin-mediated adhesion of cells to the ECM leads to bidirectional intracellular signalling events that regulate cell migration, as well as survival and proliferation (75). In outside-in signalling, ligand binding activates intracellular signalling pathways. In inside-out signalling, signals received by other receptors activate intracellular signalling pathways that impinge on integrin cytoplasmic domains and change the extracellular domain conformation for binding to ligands (76). Recent studies have shown that αvβ3 expression in SMCs is subject to regulation and is increased by treatment with thrombin and several other growth factors and mitogens (76).

In addition to ligand binding, which activates signalling pathways inside the cell, transmembrane integrins have been shown to serve as mechanical sensors. In SMCs, integrins are located in membrane-associated dense plaques that are structurally analogous to focal adhesions (77). β-integrins interact with ECM on the outside of the membrane and link to actin filaments on the inside of the membrane. The structural linkage of matrix to the actin cytoskeleton by integrins facilitates mechanical force transmission between the contractile apparatus and ECM (77). Mechanical signals sensed by integrins may trigger intracellular signals that regulate remodelling of the actin cytoskeleton. FAK, c-Src, protein tyrosine kinase 2 (PYK2), v-CRK-associated tyrosine kinase substrate p130 protein and paxillin are associated with integrins on the membrane. Activation of integrins, either mechanically or via ligand binding, leads to FAK autophosphorylation at Tyr^{397} (73). Integrin-mediated activation of tyrosine kinases in turn modulates the functional status of downstream molecules such as paxillin and Hic-5 that mediate remodelling of the actin architecture (78).

In VSMCs, the expression and distribution of integrins is sensitive to growth factors and various hormones. Studies in cultured rodent VSMCs showed that, following vascular injury, the bioavailability of PDGF influenced the relocalization of αvβ3 to the leading edge of migrating cells. In contrast, αvβ3 was evenly distributed on the surface of VSMCs grown in the absence of PDGF. In humans, the αvβ3 heterodimer is present in both the normal artery and the site of SMC accumulation in atherosclerotic plaques. In the normal artery, αvβ3 is generally detectable only on the luminal surface, with minimal expression in the media (79).

Several studies in animal models have shown that arterial injury is a stimulus for the expression of αvβ3 by endothelial cells and medial SMCs (73). In the porcine coronary stent model, there is early upregulation of αvβ3 at sites of cell accumulation within the neointima and adventitia seven days after arterial injury. This is followed by persistent high levels of αvβ3 expression within the media and neointima for up to 21 days, followed by a decrease toward baseline by 28 days (80).

ILK

ILK, a serine-threonine protein kinase containing a catalytic domain at its C terminus, has a central pleckstrin homology-like domain and four ankyrin-like repeats at the N-terminus, and is an important component of the focal adhesion complex, anchoring actin filaments to integrin receptors and the cell membrane. Even with this information available, its function in the arterial response to injury is not completely understood. It has been shown that ILK activates Akt and inhibits glycogen synthase kinase-3β, and it has been implicated in cancer cell growth and vascular development through modulation of these downstream targets (81,82).

In VSMCs, ILK appears to have two functions that may be distinct. First, ILK may function as a scaffold protein at focal adhesion sites. In cultured rat VSMCs, ILK co-localizes and interacts with several focal adhesion proteins, particularly paxillin, PINCH1 and parvins. Such interactions coordinate actin polymerization and reorganization, and control cell spreading. Second, ILK can activate signal transduction via its kinase domain (83).

Interestingly, different groups examining the role of ILK in VSMC migration using rat models in vitro and in vivo have obtained contradicting data. Silencing endogenous ILK using small hairpin RNA in VSMCs increased cellular migration and accelerated wound healing. This observation led to the conclusion that ILK expression was required for focal adhesion stability/formation, contractility and quiescence in VSMCs.

Silencing ILK, however, did not affect Akt/glycogen synthase kinase-3 phosphorylation, which suggests that the kinase function of ILK is dispensable for VSMC migration and it is the scaffolding function of ILK that maintains the stability of focal adhesions (84). Conversely, studies published by other groups using conditional overexpression of ILK in rat VSMCs showed increased cell migration and accelerated wound healing. In those experiments, the expression of an ILK mutant deficient in kinase function failed to accelerate VSMC migration (85). Further studies are needed to assess how ILK affects VSMCs and whether its functions are cell-type or species dependent. An important question that also needs to be addressed is, "How are the scaffolding and kinase functions of ILK coordinated?"

FAK

FAK is a 125 kDa protein tyrosine kinase (PTK) composed of an N-terminal protein 4.1, ezrin, radixin and moesin homology (FERM) domain, followed by a 40-residue linker region, a central kinase domain, a proline-rich low-complexity region and a C-terminal focal adhesion-targeting domain. The crystal structure of the FERM/catalytic domain complex suggests that FAK is maintained in an inactive conformation by molecular interactions between the FERM domain and the catalytic domain (86). This interaction blocks the active site of the catalytic domain by inhibiting access to the ATP and substrate binding sites. When FAK is activated, Tyr^{397} within the linker region between the FERM domain and the catalytic domain gets exposed by conformational changes. This allows FAK phosphorylation at Tyr^{397}, with the consequent creation of a high-affinity binding site for the Src homology 2 (SH2) domains of the Src family kinases (87).

Once phosphorylated, the Tyr^{397} site recruits and activates Src. The interaction between Tyr^{397}-phosphorylated FAK and Src leads to a cascade of tyrosine phosphorylations at multiple sites in FAK (residues Tyr^{576}, Tyr^{577} and Tyr^{925}) and other signalling molecules such as the 130 kDa adaptor protein Crk-associated substrate (p130Cas) and paxillin (88). Active FAK can also affect the organization of the actin cytoskeleton via Rho family GTPases and other downstream signalling pathways, including Ras and the mitogen-activated protein kinases (extracellular signal-regulated kinases 1/2 [ERK1/2]) (88). Furthermore, phosphorylation of Tyr^{397} appears to be important for the recruitment of other SH2-containing proteins, including the 85 kDa subunit of phosphatidylinositol 3-kinase (PI3K), which can promote activation of Akt (88).

Once Src phosphorylates the other tyrosine residues, FAK starts to serve as a docking site for SH2 domain proteins (89). However, to date, the autophosphorylation site (Y397) appears to be the main binding site for the molecules that associate with FAK through an SH2 domain, including Src, Shc, PI3K, phospholipase C and Grb7. Although the binding partners of pY407, pY861 and pY925 are not entirely clear, the phosphorylation of these residues may be important for other cell events.

FAK has been implicated in the regulation of cell motility. The first evidence describing a role for FAK in cell adhesion and migration was published in the early 1990s (90). At present, FAK localization in focal adhesion is considered a general mechanism underlying migration of all cell types. Enhanced phosphorylation of FAK in VSMCs has been observed on vascular injury or stimulation with growth factors or Ang II.

Although FAK function appears to be mediated largely through its action as a scaffolding molecule, FAK also directly phosphorylates several proteins such as talin, paxillin, v-CRK-associated tyrosine kinase substrate family proteins and neuronal Wiskott-Aldrich syndrome protein (N-WASP), which is a member of the WASP family of proteins that modulate actin cytoskeletal remodelling (91,92).

FAK was initially described as the prototype of a new large family of PTKs (93). However, to date, the focal adhesion PTK family contains only two members: FAK and PYK2. In VSMCs, activation of PYK2 and FAK has been linked not only to cell migration but also to the transmission of survival signals. Inhibition of PYK2 expression resulted in the complete inhibition of AngII-induced protein synthesis and reduced the activation of ERK and PI3K (94). It was also shown that ERK and PI3K directly interact with a complex containing PYK2, p130Cas, Shc and growth factor receptor-bound protein 2 (95).

On the other hand, the overexpression of FAK-related nonkinase reduces the phosphorylation of FAK at Tyr^{397} and at tyrosines phosphorylated by Src, and consequently attenuates AngII-stimulated VSMC migration (96). Also, it has been hypothesized for VSMCs that differential signalling from FAK and PYK2 may be modulated according to their localization. While FAK is mainly localized to the cell membrane, the perinuclear enrichment of ERK1/2 observed on AngII treatment is associated with translocation of PYK2 from the plasma membrane to the cytosol (97).

Cofilin and cortactin

The actin-severing protein cofilin is essential for directed cell migration and chemotaxis. Specifically, cofilin increases the number of free barbed ends capable of initiating actin polymerization. This process is required for actin-based protrusion in distinct subcellular structures including lamellipodia, invadopodia and podosomes. The severing activity of cofilin is tightly regulated because inhibition of actin severing limits cell motility. The regulation of cofilin is characteristic to the subcellular structures that mediate cell motility (lamellipodia, invadopodia or podosomes). In lamellipodia, cofilin activity is regulated by phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2), which controls the initial activation of cofilin at the leading edge (98). In invadopodia/podosomes, which mediate focal degradation of ECM in metastatic carcinoma cells and in muscle cells via MMP activity, cortactin controls the initial activation of cofilin (99).

Loss of cofilin binding to PI(4,5)P2 enables activation of cofilin at the leading edge of mammary carcinoma cells and in muscle cells. In resting cells, cofilin is directly bound to PI(4,5)P2 at the plasma membrane. On stimulation, cofilin is phosphorylated and released from the membrane to trigger actin severing for the purpose of creating barbed ends (100).

Cofilin also localizes to invadopodia and plays the same role in creating barbed ends to facilitate cellular motility; however, in this case, cofilin is regulated by cortactin (99). Cortactin is a multidomain scaffolding protein that is known to activate the Arp2/3 complex and bind to the branch points of actin filaments and stabilize them (101).

Cofilin and cortactin interact directly and cortactin inhibits cofilin's severing activity. Tyrosine phosphorylation of cortactin promotes dissociation of cortactin and cofilin, thereby releasing cofilin's actin binding and severing activities to create free barbed ends for actin polymerization. Within minutes, cortactin is dephosphorylated and the cofilin-cortactin interaction is restored, which inhibits further cofilin activity (102).

Cortactin phosphorylation not only directly regulates cofilin activity at invadopodia, but regulates the activity of the Arp2/3 complex through a phosphorylated cortactin/Nck1/N-WASP pathway. This pathway was first described in vitro and was later validated in vivo (99,102).

We have recently shown (70) that cortactin is phosphorylated via an Src-dependent pathway in porcine VSMCs on AngII stimulation. Phosphorylated cortactin transiently translocates to the focal adhesions and then relocates to its original cellular compartment. These data suggest that the cofilin-cortactin pathway regulating cellular migration in invadopodia/podosomes may be active in VSMCs migrating after vascular injury. However, it is not known whether cortactin is directly phosphorylated by Src or whether this phosphorylation occurs downstream of Src.

MMPs

MMPs are a family of enzymes that degrade the ECM and other connective tissue proteins. In normal physiological vascular remodelling, MMP activity is tightly controlled at different levels. However, factors that promote vessel remodelling upregulate MMP activity. Also, uncontrolled MMP activity can result in degradation of ECM, enabling VSMCs to migrate and proliferate. Partial degradation of the ECM surrounding VSMCs is a necessary step for allowing repositioning of cells during remodelling (103). The human MMP family includes 26 members, including both secreted and membrane-bound enzymes, characterized by their ability to degrade ECM and by their dependence on Zn^{2+} binding for proteolytic activity (104). MMPs are divided into five major subclasses: collagenases (eg, MMP-1, -8 and -13), gelatinases (eg, MMP-2 and -9), stromelysins, matrilysins and membrane-type (MT) MMPs (eg, MT1-MMP to MT6-MMP) (105).

The activity of MMPs is regulated by three mechanisms: transcription, secretion and activation of the latent pro-enzymes. As well, many interact with the tissue inhibitors of MMPs (TIMPs), which are able to inhibit MMP activity by forming noncovalent complexes with active MMPs (106). Most MMPs are secreted as inactive zymogens or proenzymes, and activation requires disruption of the Cys-Zn^{2+} interaction (proteolytic removal of a prodomain) (107). Most pro-MMPs are thought to be activated by tissue or plasma proteinases. Several proteases such as plasmin, trypsin, kallikrein, tryptase and chymase were reported to activate pro-MMPs extracellularly (108). Pro-MMP-2 activation is typically thought to take place on the cell surface via MT1-MMP. Recently, it has been suggested that this activation process requires both active MT1-MMP and the TIMP-2-bound MT1-MMP (109).

MT1-MMP, MMP-2 and MMP-9 are expressed in VSMCs and were shown to be upregulated in response to vascular injury as a result of PI3K activation (14). Pharmacological inhibitors of MMPs or overexpression of endogenous TIMP proteins postinjury block intimal thickening. MMP expression and activation in VSMCs were shown to be sensitive to AngII and growth factor stimulation. In porcine coronary artery organ culture, it was shown that MMP-2 is more important than MMP-9 in triggering intimal thickening. In the same experimental system, it was also shown that the activation of MMP-2 is under the control of MT1-MMP (14). However, the post-translational mechanism by which MT1-MMP is activated in VSMCs is still elusive. Recently, it was proposed that MT1-MMP is activated by the hormone convertase furin; however, our group has shown (14,110) that, in porcine VSMCs, the activation of MT1-MMP is furin independent.

STIMULATORY FACTORS RELEASED FOLLOWING INJURY

After arterial injury, the release of mitogenic and chemotactic factors from intracellular and extracellular elements within the wounded lesion and from aggregated platelets on the damaged intimal surface appears to initiate the neointimal response. Significant contributors to the neointimal response are VSMC migration and proliferation, two processes that are mediated by the released factors. Those factors may include members of several families such as transforming growth factors, growth factors, cytokines and hormonal peptides. Some factors have been well studied due to their importance in the formation of neointimal lesions that lead to vascular disease. We will elaborate specifically on the role of AngII, VEGF and PDGF in the induction of VSMC migration on vascular injury.

AngII

AngII, an octapeptide hormone, is the active component of the renin-angiotensin system. AngII is produced both systemically and locally via tissue-specific renin-angiotensin systems. AngII contributes to the regulation of blood pressure and plasma volume via aldosterone-regulated sodium excretion and sympathetic nervous activity (111). AngII is also involved in the regulation of several cellular processes such as proliferation, differentiation, regeneration and apoptosis. The multiple actions of AngII are mediated via specific, highly complex intracellular signalling pathways that are stimulated following an initial binding of the peptide to its cell surface receptors (112). The mechanisms controlling the formation and degradation of AngII are important in determining its final physiological effect. AngII is formed from enzymatic cleavage of angiotensinogen to angiotensin I (AngI) by the aspartyl protease renin, with subsequent conversion of AngI to AngII by angiotensin-converting enzyme (ACE) (113). Recently, the enzyme carboxypeptidase angiotensin-converting enzyme 2 (ACE2) was identified and was shown to cleave one amino acid from either AngI or AngII, decreasing AngII levels and increasing the metabolite Ang-(1-7). This cleavage event regulates the turnover of AngII. Thus, the balance between ACE and ACE2 is an important factor in controlling AngII levels (114,115). In VSMCs, the duration of exposure to AngII appears to be crucial; while acute exposure is necessary for normal cell physiology including tissue repair after injury, chronic exposure leads VSMCs to proliferate, migrate and secrete ECM via AngII receptors. This process is implicated in the development and maintenance of neointima formation and restenosis.

The biological actions of AngII are mediated by two distinct G protein-coupled angiotensin receptors, AngII type 1 receptor (AT1R) and AngII type 2 receptor (AT2R), which are seven transmembrane glycoproteins with only 32% to 34% sequence homology (116). Studies using pharmacological inhibitors for both receptors revealed high specificity, selectivity and affinity for each receptor to its antagonist molecules, suggesting specific functions downstream of each signalling receptor. For a long time, it was believed that AT1R facilitated AngII-induced proliferative and mitogenic actions while AT2R balanced AT1R signalling by facilitating apoptotic nonproliferative signalling (117). However, recent studies suggested that the functions of each receptor may be species dependent because the level of expression of each receptor is different in rodents than in pigs and humans (118). For example, in rodent VSMCs, AT2R is expressed during the early stages of fetal development and is decreased in adulthood. In contrast, adult porcine and human VSMCs express AT2R. Our recent studies (118) showed that both receptors contribute to VSMC proliferation. Further analyses are needed to characterize the signalling pathways downstream of each receptor and determine possible avenues of crosstalk between them.

VEGF

In VSMCs, VEGF is expressed under physiological conditions. Vascular injury, however, leads to VEGF overexpression. It was shown that overexpression of VEGF in VSMCs may take place under the influence of different injuring factors, including hypoxia, ultraviolet light, reactive oxygen species or mechanical injury (119). VEGF receptors, which were initially thought to be expressed only in endothelial cells, have recently been shown to be expressed by other cell types including VSMCs (120).

Recently, some studies (121,122) have shown that hypoxia, reactive oxygen species and fibroblast growth factor 2 can stimulate endothelial cells to also produce VEGF, especially after vascular injury, thus having an effect on VSMCs. VEGF appears to assist with the repair of vascular injury by stimulating MMP production by VSMCs, which consequently facilitates VSMC migration and intimal thickening (123). It is worth noting that, to date, there are no reports that VEGF induces the proliferation of VSMCs.

PDGF

PDGFs comprise a family of homo- or heterodimeric growth factors. PDGFs are produced by platelets, macrophages, vascular endothelium, fibroblasts and keratinocytes (124). These ligands bind to two different transmembrane tyrosine kinase receptors (α and β). Ligand binding causes receptor dimerization, leading to autophosphorylation of the receptors. This creates a docking site for SH2 domain-containing signalling molecules, whereby several signalling pathways are activated (125,126).

Stimulation of VSMCs to migration in SMCs by PDGF is mediated by a specific membrane receptor – the PDGF β-receptor. Migrating VSMCs show high expression of the PDGF β-receptor gene, which contributes to the migratory capacity of intimal VSMCs. Accordingly, the failure to downregulate expression of the PDFG β-receptor after wound repair may contribute to neointimal thickening and vascular disease (127,128).

PDGF plays a role in each stage of wound healing. Initially, PDGF is expressed at low or undetectable levels in normal vessels (PDGF-A, PDGF-B). On injury, the expression of PDGF and its receptor gradually increases. PDGF is released from degranulating platelets and is present in the wound fluid (129). PDGF was shown to stimulate VSMC migration to the wound site (130). In rodent models, PDGF induces migration of VSMCs from the media into the intima approximately two to three days after injury and continues until the appearance of the neointima approximately one week after injury (131). In a different model, it was shown that the use of an antibody to neutralize the activity of PDGF-AA, PDGF-BB and PDGF-AB reduced VSMC migration by 79% (132). The effect of PDGF-BB stimulation on injured arteries was also examined in a porcine vascular injury model using the exogenously expressed PDGF-BB gene. Transduction with PDGF-B induced an eightfold increase in the intimal to medial ratio compared with control transduced arteries 21 days after transduction (133). However, in this study, the individual contributions of PDGF to cell migration or proliferation were not distinguished.

CONCLUSIONS

In the present review we examined recent developments regarding VSMC migration and its contribution to vascular disease. We summarized what is known about VSMC modes of migration, and the role and regulation of key players involved in activating such a process as a response to vascular injury. The response to vascular injury is a multicellular event that involves the activation of a cascade of signalling pathways. One major process that takes place is the activation of VSMC migration, which leads to cell movement from the media to the intima. This process is sensitive to several activators that appear to cooperate to accelerate vascular repair and wound healing. Unfortunately, the failure to downregulate those activators can leave VSMCs in the synthetic phenotype, which leads to vascular disease. The ultimate goal of this area of research is to understand these regulatory mechanisms to develop novel interventions that will permit tissue repair but prevent neointimal thickening. However, to achieve this goal, several aspects still need to be clarified, including the characterization of the components of each signalling pathway; the identity of pathways that are sensitive to different activators or, in some cases, the same activator; the mode of VSMC migration involved in vascular injury; the mechanisms responsible for the switch from migration mode to invasion mode on chronic exposure to certain activators; and characterization of the degree of homology between rodent experimental models that are widely used to study vascular diseases and humans with respect to VSMC migration. Finally, it will be important for future work to choose appropriate experimental systems in vivo or in vitro that mimic human vascular biology to extrapolate data acquired from experimental systems to humans.

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