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Regulation of VSMC behavior by the cadherin-catenin complex

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1. ABSTRACT

Vascular smooth muscle cells (VSMCs) are the predominant cell type within blood vessels. In normal vessels VSMC have low rates of proliferation, migration and apoptosis. However, increased VSMC proliferation, migration, and apoptosis rates radically alter the composition and structure of the blood vessel wall and contribute to vascular diseases such as atherosclerosis, in-stent restenosis and vein graft failure. Consequently, therapies that modulate VSMC proliferation, migration and apoptosis may be useful for treating vascular diseases. In this review article we discuss recently emerging research that has revealed that homophilic cell-cell contacts mediated by the cadherin:catenin complex and Wnt/beta-catenin signalling are important regulators of VSMC behaviour.

2.INTRODUCTION

Cardiovascular disease is the most frequent cause of premature death in modern industrialized countries, accounting for 4.35 million deaths each year in Europe, and 35% of deaths in UK (1-2). It is caused by thickening of the artery wall, due to a pathology known as atherosclerosis. Atherosclerosis, which occurs over several decades, leads to the formation of a plaque containing extracellular lipid and inflammatory cells within the artery wall. In some cases, the atherosclerotic plaque restricts blood flow and causes symptoms such as angina, while in others, the plaque ruptures, leading to an occlusive thrombosis, and causes myocardial infarction or stroke.

Normal arteries are composed of three layers: intima, media and adventitia. The medial (middle) layer

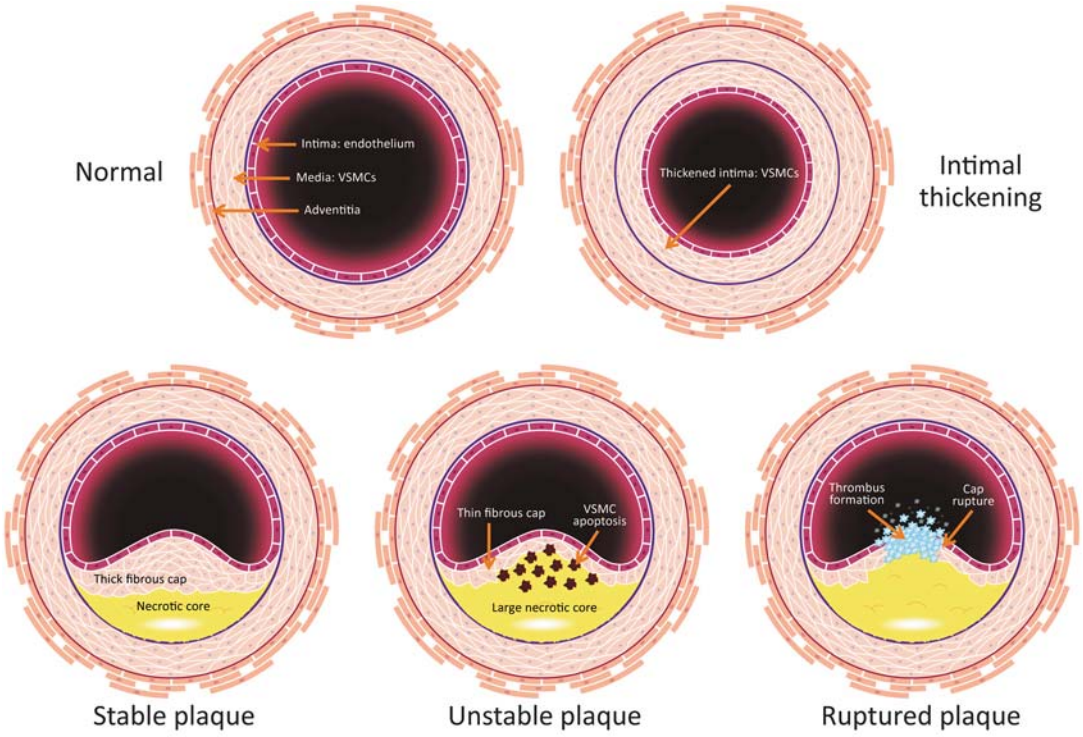

Figure 1. Schematic representation of the role of VSMCs in intimal thickening and atherosclerosis. In normal blood vessels, VSMCs reside in the media and exhibit a low rate of proliferation and migration. Injury to the blood vessel (vein graft, balloon-injured artery or stented artery) results in migration of VSMCs into the intima where they proliferate and deposit extracellular matrix. As a result the intima becomes thickened. Stable atherosclerotic plaques possess a thick VSMC-rich fibrous cap overlying the necrotic core. Apoptosis of VSMCs results in thinning of the fibrous cap. Consequently, the cap is weakened making the plaque unstable and is liable to rupture. Rupture of the fibrous cap exposes the contents of the necrotic core to the blood constituents, which induces thrombus formation.

contains extracellular matrix and VSMCs, which exhibit a contractile phenotype with a low rate of proliferation and migration, despite the presence of growth factors. In fact, exposure of intact blood vessels to exogenous growth factors, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), both *in vivo* and *in vitro*, does not induce rapid proliferation (as reviewed in (3)). During atherosclerosis, VSMCs are activated and de-differentiate into a synthetic phenotype, which exhibits an increased proliferative rate and migrate from the media into the intima (the innermost layer), that is normally composed only of endothelial cells (4). VSMCs de-differentiate in response to various factors including growth factors and chemokines, which are released by damaged endothelial cells, degranulating platelets that have adhered to the damaged endothelium, infiltrating inflammatory cells, and lipoproteins (5). The VSMCs in the intima have a heightened proliferative rate, deposit newly synthesized extracellular matrix proteins, and thereby lead to intimal thickening (6-7) (Figure 1). The thickening of the intima can act as a 'soil' for the subsequent processes involved in the formation and progression of the atherosclerotic plaque (4). Intimal thickening, resulting from VSMC migration and proliferation, is also an undesirable response to injury that occurs after coronary artery bypass vein grafting, balloon angioplasty and stent implantation. Although these treatments are designed to relieve the symptoms of atherosclerosis, unfortunately intimal thickening occurs in approximately 30-50% of patients receiving these treatments (8-9). Injury of the blood vessel induces rapid VSMC proliferation and migration, suggesting that inhibitors of proliferation and migration may exist in the normal vessel wall preventing VSMCs from responding to growth factor stimulation. Inhibitory signals that prevent VSMC proliferation are thought to be cell-matrix interaction and soluble mediators, including growth factors such as transforming growth factor-β and nitric oxide (3, 10). Recently, it has been revealed that homophilic cell-cell contacts mediated by cadherins and catenins also regulate VSMC proliferation and migration. The evidence to support this will be outlined in the subsequent sections of this review.

Later in advanced atherosclerotic plaque development, the plaque contains a large quantity of lipid covered by a stabilising layer of VSMCs and extracellular matrix, called the fibrous cap (Figure 1). The fibrous cap confers strength to the plaque and assists in resisting plaque rupture which can lead to clinical symptoms (11). Recent studies in animal models have clearly shown that apoptosis of VSMCs in the fibrous cap leads to cap thinning which increases the likelihood of rupture, thrombus formation and

possible occlusion of the blood vessel (12-13). Reducing VSMC apoptosis in the fibrous cap could therefore increase plaque stability and reduce clinical events. Apoptosis of VSMCs is also an important modulator of aneurysm formation and has been observed in human aneurysm samples and in animal models (14-18). Aneurysms are a small but significant cause of premature death in the UK, and are characterised by localised structural deterioration of the vessel wall, in particular thinning of the medial layer and degeneration of the internal elastic lamina (19). This results in progressive vessel dilatation and can lead to complications such as vessel rupture, which leads to massive internal bleeding, or blood leaking into the brain, for aortic and intracranial aneurysms, respectively. Apoptosis of medial VSMCs is an important component in aneurysm formation as it removes the only cells capable of repairing and producing connective tissue. Therefore, reducing VSMC apoptosis is also a potential therapeutic strategy to reduce aneurysm formation and progression. VSMC survival is promoted by several factors including soluble growth factors such as insulin-like growth factor (IGF-1) (20-21) and survivin (22-23), and cell-matrix contacts (24). We have recently revealed that cell-cell contacts play an important role in VSMC survival (25-26), and the evidence for this involvement will be discussed later in this review.

Healing of atherosclerotic plaque ruptures also occurs in over 50% of hearts which previously suffered myocardial infarctions (27). These were identified as buried VSMC-rich fibrous layers in the atherosclerotic plaque and have been subsequently identified in a mouse model of atherosclerosis (28). Healed ruptures favour the accumulation of immature smooth muscle cells at repair sites, with a cellular proliferation index of 0.40±0.09%, significantly higher than the index at the sites of rupture (27), indicative that VSMC proliferation (as well as migration) is important for plaque healing after rupture. Paradoxically therefore promotion of VSMC migration and proliferation, and as a result plaque healing, may be beneficial for plaque stabilisation after a rupture.

In summary, altered rates of VSMC migration, proliferation and apoptosis influence atherosclerotic plaque formation, progression and stability, in-stent restenosis, vein graft failure and aneurysms. A greater understanding of the factors that regulate VSMC behaviour, may therefore be useful in the design of novel therapeutic approaches for the treatment of atherosclerosis, intimal thickening and aneurysm. Consequently, in this review article we will discuss the role of the cadherin:catenin complex in the regulation of VSMC proliferation, migration and apoptosis.

3. THE CADHERIN:CATENIN COMPLEX

3.1. The cadherin structure and cadherin:catenin complex

Cadherins are a superfamily of calcium-dependent cell–cell adhesion proteins that form the basis of adherens junctions. They mediate homophilic adhesion, and therefore cadherin expression mediates cell sorting and segregation (29). As a result, cadherins are vital for embryonic development, and different cadherin types are distributed in different spatio-temporal patterns in the embryo, and are tissue specific (29). Many cadherins are named according to the cell-types in which they were first identified, for example, N-cadherin was originally found in neural tissue and E-cadherin was originally found in epithelial cells. The cadherin superfamily is divided into subfamilies based on molecular characteristics. The type I classic subfamily of cadherins, which includes E-, N-, P and VE-cadherin, will be the focus of this review as it is this subfamily of single pass proteins that forms a complex with catenins.

Classic type I cadherins are transmembrane glycoproteins with three domains: the extracellular domain, the transmembrane domain, and the intracellular domain (Figure 2). The extracellular domain of classic cadherins is composed of extracellular cadherin domains 1–5 (EC1–5) containing calcium binding sites (30). Calcium binding by the EC domains leads to a conformational change, from a loose disorganised structure to a rigid, elongated rod-like structure (30-31). This rigid structure facilitates cis homo-dimerisation on the cell surface, which is required for cadherin function and adherens junction formation. Trans homo-dimers can then form on adjacent cells and enable the formation of adherens junctions in a zipper-like fashion. Cadherin adhesion is mediated, at least in part, by a highly conserved tripeptide, His-Ala-Val (HAV), located in EC1 domain (32). However, it has also been suggested that it is not just the EC1 regions that interact, but the entire extracellular domain (EC1–EC5) (33).

The intracellular domain of the classical cadherins interacts with a family of adaptor proteins known as catenins (Figure 2). The catenins mediate the assembly of the cytoplasmic cell adhesion complex, which is critical for the formation of extracellular cell–cell adhesion (34). Beta-Catenin or gamma-catenin bind directly to the cytoplasmic domain, permitting the binding of alpha-catenin to the beta- or gamma-catenin. This cadherin:catenin complex then interacts with the actin cytoskeleton via binding of other proteins including α-actinin and vinculin. In addition, p120-catenin binds directly to the cytoplasmic domain of the cadherin molecule close to the plasma membrane, and is thought to regulate the stability of the cadherin:catenin complex and consequently cell–cell adhesion (35).

In addition to mediating cell–cell adhesion, classic cadherins can modulate various cellular signalling pathways via the intracellular domain (Figure 3). The most-well studied is the beta-catenin/Wnt pathway (36).

3.2. Wnt pathways

3.2.1. The beta-catenin/Wnt pathway

Wnt genes were first identified in mutant wingless *Drosophila melanogaster,* subsequently nineteen Wnt isoforms have been identified in humans (37). Wnts are a family of extracellular matrix-associated growth factors, which are involved in the regulation of numerous biological processes including embryonic development, tumour regulation, cell fate, and cell proliferation (38). Wnt

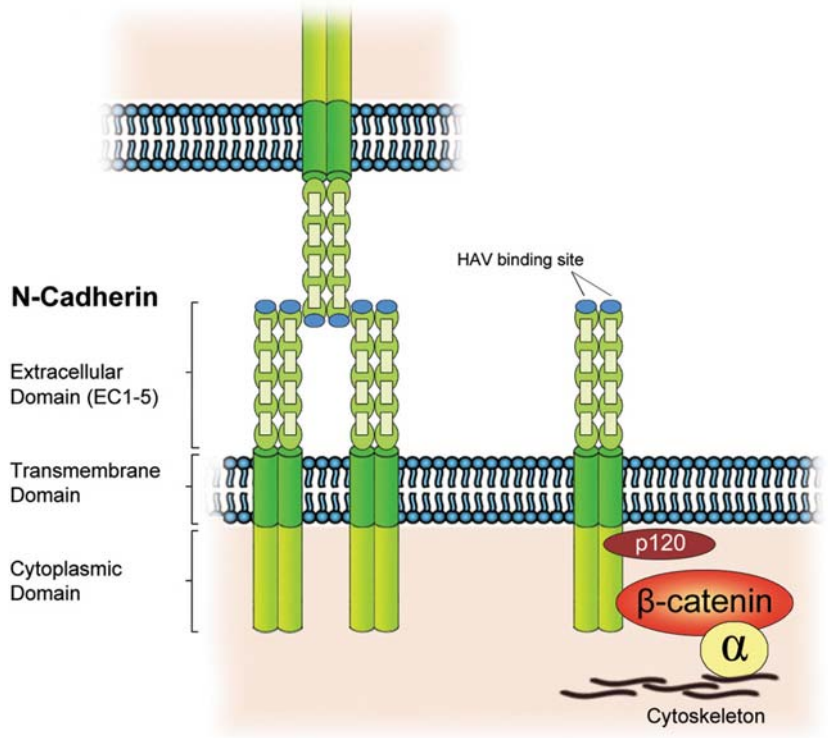

Figure 2. The cadherin:catenin complex and homophilic cell–cell adhesion. Classical type I cadherins are composed of three domains; extracellular, transmembrane and cytoplasmic. Binding of calcium to the extracellular domain permits dimerization and exposure of the HAV binding site. The HAV binding site within the extracellular region of the cadherin forms homophilic cell–cell adhesion in a zipper-like fashion between adjacent cells. The cytoplasmic domain contains binding sites for p120-, alpha - and beta-catenin. The binding of the catenins permits association with the actin cytoskeleton.

proteins can signal through canonical and non-canonical pathways depending on the cell type and receptor expression (39-40) (Figure 3). During canonical signalling, which predominantly controls cell fate, Wnts interact with the 7-membrane spanning Frizzled receptors (Fzds) and the co-receptors (low density lipoprotein receptor related protein 5/6, LRP5/6), to initiate a beta-catenin dependent pathway leading to altered transcription of at least 54 mammalian genes (see http://www.stanford.edu/~rnusse/wntwindow.html for complete list). In the absence of Wnt signalling, cytoplasmic beta-catenin levels are kept low by a beta-catenin-destruction complex containing adenomatous polyposis coli (APC), Axin and glycogen synthase kinase 3-beta (GSK3-beta) (41). This complex targets free beta-catenin for ubiquitination and proteosomal degradation through phosphorylation of specific sites. However, in the presence of Wnt, this complex is inhibited and consequently beta-catenin escapes degradation through de-phosphorylation of destruction important sites. This permits accumulation of de-phosphorylated beta-catenin, which is not bound to cadherins, in the cytoplasm, and ultimately activation and translocation to the nucleus where beta-catenin binds to the transcription factors, lymphoid enhancer factor (LEF) or T-cell factor (TCF). Binding to LEF/TCF stimulates up- or down-regulation of target genes (42), which include cell cycle activators, such as cyclin D1 (43) and c-myc (44), as well as matrix-degrading metalloproteinases (MMPs (such as MMP-7)) (45). This altered gene expression can result in modulation of cell proliferation, migration, differentiation and survival. Thus, beta-catenin has one of two roles: it can enable the formation of the adhesion complex by binding to the cytoplasmic domain of the cadherin molecule, and/or it can regulate gene transcription by translocating to the nucleus and interacting with the transcription factors, LEF and TCF. Consequently, beta-catenin signalling can be up-regulated as a result of adherens junction dismantling and Wnt pathway activation.

Non-canonical Wnt signalling is beta-catenin independent and activates the Wnt/planar cell polarity pathway (PCP) and the Wnt/Ca^{2+} pathway (Figure 3). Despite the absence of direct involvement of β-catenin, the non-canonical signalling pathways can modulate Wnt/beta-catenin signalling. The Wnt/planar cell polarity pathway activates Rac/Rho and Jun N-terminal kinase (JNK), resulting in phosphorylation of c-Jun. The Wnt/Ca^{2+} pathway leads to activation of calcium-dependent effector molecules such as calcium/calmodulin dependent kinase II (CamKII), protein kinase C (PKC), and nuclear factor associated with T cells (NFAT). Since the non-canonical pathway is beta-catenin-independent it's role in the regulation of VSMC behaviour will not be discussed in this review.

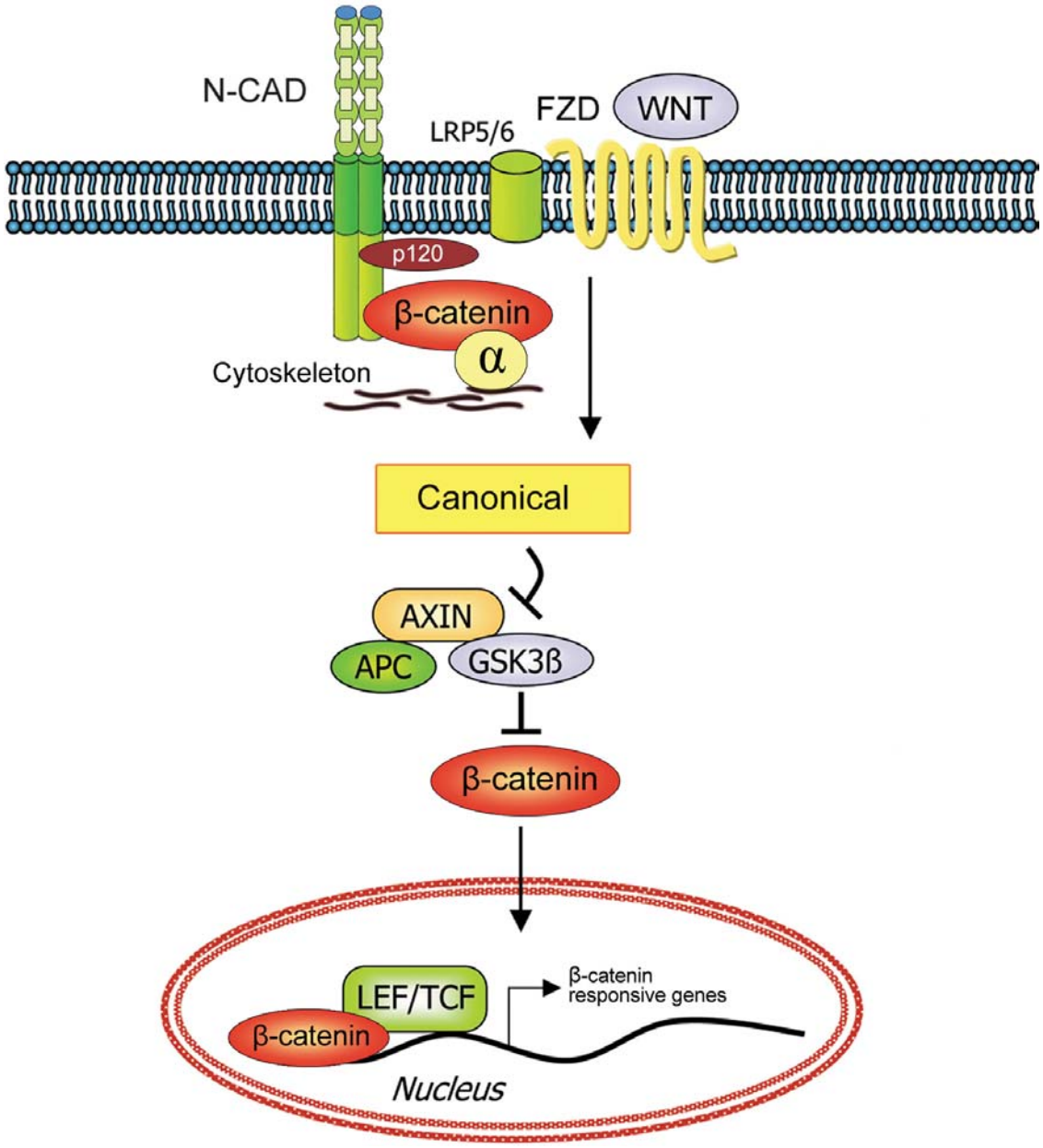

Figure 3. Wnt/beta-catenin signalling pathways. Binding of Wnt proteins to the receptors Frizzled and LRP5/6 inhibits the APC/Axin/GSK-3b complex that normally targets beta-catenin for proteosomal degradation. As a result beta-catenin is stabilized, which permits translocation to the nucleus, where it interacts with the transcription factors lymphoid enhancer factor (LEF) and T cell factor (TCF). Together LEF/TCF and beta-catenin modulate the expression of numerous target genes, including cyclin D1 (CD1) and c-jun.

3.2.3. Wnt pathway inhibitors

Wnt signalling is antagonized by several soluble inhibitors, including secreted Frizzled related proteins (sFRP, such as FrzA and Fzbeta−1) and Dickkopf-related protein 1 (DKK-1). sFRPs bind the Wnt proteins thereby inhibiting their interaction with Frizzled and blocking Wnt canonical and non-canonical signalling. DKK-1 on the other hand, inhibits the interaction of Wnts with LRP5/6 and thereby selectively retards canonical Wnt signalling.

4. ROLE OF CADHERIN:CATENIN COMPLEX IN REGULATION OF VSMC BEHAVIOUR

4.1. Role of the cadherin:catenin complex in the regulation of VSMC proliferation

Although the cadherin:catenin complex has a recognized role in controlling the proliferation of human cancers (see review (46)), its role in VSMCs has only recently been considered. However, in the early 1990s, N-cadherin was detected in uninjured blood vessels and was

thought to mediate cell–cell contacts between VSMCs and to mediate adhesion between endothelial cells and VSMCs (47). Furthermore, adherens junctions have been identified in the media and intima of large arteries, including coronary and carotid arteries (48-49). More recently, our group showed that N-cadherin, the predominant classical cadherin in human saphenous vein VSMCs, retards VSMC proliferation, most likely by modulating beta-catenin-mediated intracellular signalling (50). We observed that N-cadherin is shed from the surface of human saphenous vein VSMCs during proliferation, and is accompanied by translocation of beta-catenin to the nucleus and induction of beta-catenin signalling (50-51). Furthermore, adenoviral over-expression of dominant negative N-cadherin, composed of the transmembrane and cytoplasmic domains and capable of inhibiting endogenous full length classical N-cadherin, increased VSMC proliferation (50). Conversely elevating N-cadherin levels, by over-expression of membrane bound beta-catenin suppressed VSMC proliferation (52). Dismantling of cadherin:catenin complexes occurs not only in isolated cells but also during medial VSMC proliferation in balloon injured rat carotid arteries, and is associated with increased levels of total and nuclear beta-catenin and elevated expression of the beta-catenin target gene, cyclin D1 (52-53). In subsequent studies we have shown that PDGF-induced proliferation is regulated by beta-catenin-induced down-regulation of p21 as well as beta-catenin-dependent up-regulation of cyclin D1 (51). Bedel and colleagues also showed that oxidized low-density lipoprotein (ox-LDL) induced VSMC proliferation and caused dismantling of adherens junctions (54). In this case they studied E-cadherin and observed a reduction in E-cadherin, an increase in nuclear translocation and activity of beta-catenin, and elevated cyclin D1 expression, in conjunction with proliferation in human aortic VSMCs after treatment with ox-LDL (54). Analysis of human carotid artery atherosclerotic plaques also revealed elevated levels of proliferation and active beta-catenin in disrupted plaques compared to stable plaques, suggestive of a role for β-catenin signalling in proliferation within atherosclerotic plaques (54). However, in this study it was not examined in which cell type this occurred (i.e. in VSMCs, macrophages or other cell type). Moreover previous studies have expressed concerns regarding the specificity and reliability of the anti-active beta-catenin antibody used for immunohistochemistry in this study (55).

This newly identified functional role for cadherins implies that dismantling of the cadherin:catenin complex is required for VSMC proliferation. One potential mechanism is cleavage of the cadherin by proteases including presenilin (56), calpain (57), plasmin (58) or MMPs (50, 59-62). Our group has demonstrated that MMPs control growth factor-induced VSMC proliferation by regulating cell–cell attachments (63). Disruption of N-cadherin cell–cell contacts, mediated in part by MMPs, released beta-catenin into the cytoplasm and triggered intracellular signalling, leading to cell proliferation (63). Inhibition of MMPs with a synthetic inhibitor, and over-expression of tissue inhibitors of metalloproteinases (TIMPs), inhibited shedding of the extracellular domain of N-cadherin into the conditioned media, elevated N-cadherin levels on the cell membrane, and reduced the translocation of beta-catenin to the nuclei (50). We have recently observed that MMP-9 and MMP-12 are at least in part responsible for cleavage of N-cadherin and release of beta-catenin and up-regulation of cyclin D1 during VSMC proliferation (60).

It appears that beta-catenin signalling may induce VSMC proliferation due to Wnt signalling, as well as adherens junction disassembly, since retardation of Wnt signalling using a dominant negative LRP significantly reduced VSMC proliferation (64) and a sFRP (FrzA), delayed VSMC entry into S-phase (65). Although Wnt1 is capable of inducing beta-catenin signalling and cyclin D1 expression in VSMCs *in vitro (*64), characterization of the subtypes of Wnt and frizzled receptors involved in VSMC proliferation is incomplete. Puzzlingly, down-regulation of Fzd-1 and Fzd-2 was observed at 1 and 4hours after arterial injury and in proliferative VSMCs *in vitro*, suggesting they have a negative relationship to proliferation (66). On the other hand, increased expression of Fzb-1, an endogenous antagonist of the Wnt cascade, was observed in the rat arterial wall at 4 days and 3 weeks after *in vivo* injury and in quiescent isolated VSMCs (66). Since Fzb-1 is a sFRP that inhibits Wnt signalling by binding and thereby preventing their interaction with Fzds, it was suggested that this increased Fzb-1 expression when arrest of VSMC proliferation is observed in the media and neointima, indicates that Fzb-1 may suppress proliferation. Consequently this also suggests that Wnt signalling has a positive role in proliferation prior to the up-regulation of Fzb-1.

In summary, the data presented above highlights that research in the last few years has revealed that both the cadherin:catenin complex and Wnt/beta-catenin signaling may modulate VSMC proliferation. Although it has emerged that the cell–cell adhesion and intracellular signalling mediated by β-catenin may both participate in regulating VSMC proliferation through regulating genes such as cyclin D1 and p21, the complete set of target genes affected by beta-catenin signalling is still unknown. At the same time, much remains to be learnt concerning the cross-talk between the Wnt and cadherin pathways. Future studies will elucidate the precise role of these proteins in the pathogenesis of atherosclerosis and intimal thickening.

4.2. Role of the cadherin:catenin complex in the regulation of VSMC migration

Chemoattractants, including growth factors and remodelled extracellular matrix components, lead to the activation of integrins and signalling pathways that initiate the migration process (67). Studies have suggested that cadherin mediated cell-cell adhesion may modulate VSMC migration. However, these studies have yielded what appears to be opposing findings for the role of N-cadherin on VSMC migration. One study demonstrated that N-cadherin was up-regulated after wounding in VSMCs *in vitro,* and promoted VSMC migration and neointima formation (68). In support of this, we have observed that inhibition of N-cadherin using various approaches,

including over-expression of a dominant negative N-cadherin, neutralising antibodies and inhibitory peptides, significantly reduced VSMC migration (69). We observed that this reduction in VSMC migration correlated with a significant increase in VSMC apoptosis by between 1.5- and 3.3-fold at the wound edge (69) suggesting this appears to be, at least in part, due to reduced activation of Akt and increased VSMC apoptosis caused by inhibition of N-cadherin function. As discussed later in this review, we previously showed that N-cadherin is essential for VSMC survival via activation of Akt (25) and therefore this study corroborates the pro-survival effect of N-cadherin on VSMCs. Importantly we also assessed the effect of N-cadherin function in an *ex vivo* model of intimal thickening, inhibition of N-cadherin function by infection of human saphenous vein segments with RAd dn-N-cadherin significantly reduced VSMC migration and increased VSMC apoptosis (69). As a result, intimal thickening was significantly suppressed by approximately 50% Importantly, there was no detrimental effect of dn-N-cadherin on endothelial coverage; in fact, it was significantly increased, as was survival of cultured human saphenous vein endothelial cells (69). Therefore we suggest that cell-cell adhesion mediated by N-cadherin regulates VSMC migration via modulation of viability. Disruption of N-cadherin-mediated cell-cell contacts may therefore be a potential strategy for reducing VSMC migration and intimal thickening. In contrast to both our study and that of Jones and colleagues (68), Blindt and colleagues previously showed that VSMC migration was increased by the neutralising anti-N-cadherin antibody GC-4 (70). Although the reason for this discrepancy is not fully elucidated, we suggest two possibilities. Firstly, to assess migration Blindt *et al* used chemotaxis assays rather than the wound injury model. In the chemotaxis assay, VSMCs are placed in the chamber as single cells, whereas in the wound injury model cell-cell contacts are established prior to the treatment which is more akin to the *in vivo* situation for VSMC migration. Secondly, the concentration of the neutralising anti-N-cadherin antibody was higher in the study by Blindt *et al* compared to our study, which may have induced cross-linking and activation of N-cadherin rather than neutralisation of function. Furthermore in this study they showed that over-expression of N-cadherin inhibited migration of VSMCs via deactivation of RhoA (70). One possible reason for these apparently discrepant findings is that the precise level of expression may be critical to determine whether N-cadherin inhibits or promotes VSMC migration. Additionally as proposed by Sabatini *et al* proposed that the distribution of N-cadherin on the cell surface may be the key controller of migration (71). They demonstrated that asymmetrical distribution of N-cadherin to the posterior-lateral cell edge is essential for establishment of cell polarity and efficient directional migration (71). In summary these studies provide a convincing case that N-cadherin plays an essential role in VSMC migration A recent study has also suggested that the atypical cadherin, Fat-1 interacts with atrophin, a protein essential for cell polarity, and thereby also participates in the regulation of VSMC migration (72). In cancer cells a switch in cadherin expression occurs during migration/invasion (73-74), however, to date, it has not been established whether a switch in cadherin expression occurs during VSMC migration.

The precise mechanism of how N-cadherin modulates cell migration has not been resolved in VSMCs. Studies from other cell types suggest that since N-cadherin regulates several cell signalling pathways, including organisation of the actin cytoskeleton via GTPases, fibroblast growth factor receptor (FGF-R) signalling, Akt signalling (75-76), or modulation of beta-catenin (and consequently genes involved in migration such as MMP-7 (77), versican (78) and MT1-MMP (79)), therefore suppression of these signalling pathways by cadherins may reduce migration. In neuronal and cancer cells, N-cadherin was found to promote migration via interaction with the FGF-R through a common HAV binding domain (80-82). N-cadherin associates with FGF-R-4 in tumour cells via EC4 of the cadherin molecule (83), in a complex that includes many effector molecules, including neural cell adhesion molecule (N-CAM) (82), and leads to sustained activation of MAPK and migration. Furthermore, the observation that PI-3K-mediated activation of Akt regulates migration in squamous cell lines via down-regulation of E-cadherin (84), suggests that activation of PI-3K and Akt on N-cadherin-mediated cell-cell contact formation may also regulate VSMC migration. Little is known of the role of Wnt/beta-catenin signalling in VSMC migration. Although it appears likely that it is involved since animal models of intimal thickening in which both VSMC proliferation and migration occur have shown altered expression of cadherins and increased beta-catenin levels (52-53), VSMC migration has not been assessed directly in these studies. We currently have studies underway to directly examine the role of Wnt/beta-catenin signalling in VSMC migration and hope to report our findings soon.

4.3. Role of cadherin:catenin complex in the regulation of VSMC apoptosis

4.3.1. N-cadherin and VSMC apoptosis

Cadherin mediated cell-cell contacts increase survival in many cell types (85-87). Because of the therapeutic interest in reducing VSMC apoptosis in atherosclerosis and aneurysm formation, we have investigated the effect of cadherin contacts on VSMC apoptosis. We previously found a direct relationship between the presence of N-cadherin cell-cell contacts and prevention of apoptosis in human VSMCs (25). Over-expression of full length N-cadherin prevented apoptosis, while inhibition of N-cadherin function via various approaches including antagonists, neutralising antibodies or dominant negative N-cadherin induced apoptosis (25). This study also revealed that N-cadherin promoted VSMC survival via activation of phosphatidylinositol-3-kinase (PI-3K), that caused phosphorylation of Akt and as a result phosphorylation (and inactivation) of the pro-apoptotic Bcl family protein, Bad. To study the link between N-cadherin and apoptosis further, we induced VSMC apoptosis using a variety of approaches, including treatment of VSMCs with Fas-ligand and hydrogen peroxide, and examined N-cadherin protein expression by Western blotting (26). We observed that induction of VSMC apoptosis resulted in the cleavage of the full length N-cadherin and the production

of a fragment of approximately 35kDa (26). Using MMP inhibitors, recombinant proteins and VSMCs from mice deficient in various MMPs, we discovered that MMP-7 was responsible for the cleavage of N-cadherin during both human and mouse VSMC apoptosis (26). Interestingly, VSMCs from MMP-7 null mice exhibited lower rates of apoptosis, and atherosclerotic plaques from the arteries of MMP-7/Apoplipoprotein E null mice exhibited significantly less apoptosis compared to those from control mice (26). Importantly, we also confirmed that active MMP-7 was present in apoptotic VSMCs and that active MMP-7, apoptosis and N-cadherin cleavage were more prevalent in human atherosclerotic plaques than in non-diseased control arteries (26). Together this data indicates that N-cadherin is important for VSMC survival and that cleavage of N-cadherin by MMP-7 leads to VSMC apoptosis and contributes to atherosclerotic plaque formation and progression.

4.3.2. Soluble N-cadherin and VSMC apoptosis

As mentioned above we have shown that whilst inhibition of N-cadherin contacts increased VSMC apoptosis, over-expression of N-cadherin reduces VSMC apoptosis, and is equally effective in maintaining cell survival as cell-matrix contact (25). N-cadherin is a large, insoluble molecule, so with the aim of designing a more therapeutically useful agent we investigated a smaller soluble form of N-cadherin (SNC), consisting of the extracellular domain alone. We found that SNC acts as a mimetic of the full length cadherin and provides a pro-survival effect in VSMCs *in vitro* (88). We found that this effect was mediated by an interaction between SNC and the FGF-R (that have a common HAV binding motif), which results in activation of PI-3K and Akt signalling leading to increased cell survival by inactivating Bad (88). This confirmed previous findings from other cell types that demonstrated cadherins can activate PI-3K and Akt signalling (89-92) and the FGF-R via the HAV motif (93-94), and that the cadherin-FGF-R interaction can result in increased cell survival (85-86, 95). Importantly, SNC also reduced apoptosis *in vivo* within atherosclerotic plaques in the Apolipoprotein E null mouse model of atherosclerosis. Additionally, SNC increased features of plaque stability such as a greater fibrous cap coverage, reduced macrophage content, and reduced occurrence of buried layers (88). Together this data highlights the important role of N-cadherin in maintenance of VSMC survival and thereby stability of the atherosclerotic plaque.

4.3.3. Beta-catenin and VSMC apoptosis

Although we observed that SNC had no effect on beta-catenin signalling (88) this does not rule out the involvement of Wnt/beta-catenin signalling in the regulation of VSMC survival. Others have shown that full length cadherins can regulate apoptosis via beta-catenin signalling. The role of beta-catenin in apoptosis appears to be cell-type specific, (96-100). Whilst beta-catenin reduces apoptosis in an embryonic liver culture system (99), in fibroblasts (97) and in epithelial cells in suspension culture (102), it increases apoptosis in other cell types including COS7 and 293 cells (98) and several tumour cell lines (96). It has been suggested that Wnt/beta-catenin signalling inhibits VSMCs apoptosis, since perturbation of Wnt/beta-catenin signalling, using a dominant negative form of TCF-4 or LRP, increased VSMC apoptosis (64, 101), although the precise mechanism of action was not investigated. Recent findings suggest that a reduction in beta-catenin protein levels through inhibition of a cell cycle protein peptidyl-prolyl cis/trans isomerase (Pin1) increases the rate of VSMC apoptosis, which further intimates a role for β-catenin protein in VSMC survival (102). Over 54 beta-catenin target genes have been identified, but only two of these genes, survivin and IGF-1, have a well defined role in cell survival (22-23). However, numerous transcription factors, including c-jun, fra-1, Sox9, and ID2 are β-catenin responsive genes, which could activate the expression of other anti-apoptotic genes. Consequently, further studies are essential to dissect the precise role of beta-catenin signalling in VSMC apoptosis.

5. EVIDENCE FOR THE INVOLVEMENT OF CADHERIN-CATENIN COMPLEX AND WNTS IN HUMAN ATHEROSCLEROSIS, INTIMAL THICKENING AND ANEURYSMS

The evidence presented above outlines the current data that clearly demonstrates a role for N-cadherin, beta-catenin and Wnts in VSMC proliferation, migration and survival. Consequently, one can intimate that these factors are likely to play an important role in atherosclerosis, aneurysm formation and intimal thickening. Although a direct assessment of the involvement of the cadherin:catenin complex in atherosclerosis has not been presented, a few descriptive studies have assessed the expression of several cadherins and beta-catenin in atherosclerotic lesions and supports this notion. As mentioned above we have shown that the expression of N-cadherin is lower in atherosclerotic lesions than in control arteries and is associated with VSMC apoptosis which may contribute to plaque instability (26). Morevover, elevated levels of active beta-catenin were detected in disrupted plaques compared to stable plaques, suggesting beta–catenin signalling contributes to plaque instability (54). Furthermore, E-cadherin was detected in 96% of atherosclerotic aortic lesions, suggesting that E-cadherin is also involved in the formation and progression of atherosclerotic lesions (103). E-cadherin was expressed by atherosclerotic intimal cells during foam cell transformation and may be important in the alteration of metabolism and accumulation of intracellular lipids as well as re-organisation of cell-cell interaction in atherogenesis (103). VE-cadherin was also expressed by endothelial cells in human carotid atherosclerotic plaques and its expression was associated with neovascularisation, plaque instability, a high degree of stenosis and clinical events (104). Moreover, soluble VE-cadherin appears to be a marker of plaque progression, indicating that VE-cadherin shedding occurs in atherosclerotic plaques (104). Together this suggests a role for the cadherin:catenin complex in atherosclerosis but it is clear that more studies are essential to eludicate the precise involvement.

There is also some evidence in the literature for the involvement of the Wnt pathway in atherosclerosis.

Recently Christman and colleagues demonstrated expression of Wnt5a in human and murine atherosclerotic lesions, indicating a potential role for Wnt5a signalling in atherosclerosis (105). The potential involvement of the Wnt pathway in atherosclerosis is supported by studies in which the expression and significance of the canonical Wnt pathway co-receptor LRP5/6 in cardiovascular disease has been examined (106-107). Firstly, there is evidence for reduced LRP6 receptor expression levels in human carotid atherosclerotic lesions indicating that retarded canonical Wnt signalling may contribute to atherosclerosis, perhaps by reducing the VSMC survival, proliferation and migration that is required in healing plaque ruptures (107). Secondly mutations in the co-receptor LRP6, essential for Wnt/beta-catenin signalling, was associated with hypertension, type 2 diabetes, high circulating LDL cholesterol levels and early coronary artery disease (106-107). Thirdly, Magoori and colleagues suggested a potentiation of atherosclerotic lesions in LRP5 null mice (18). Additionally, elevated levels of Wnt inhibitor DKK-1 are present in advanced carotid plaques and since DKK-1 promotes platelet mediated endothelial cell activation, it was proposed that this could lead to enhanced inflammation in the atherosclerotic lesion (109), however it may also lead to reduced VSMC survival, proliferation and migration.

6. SUMMARY

In this review we present strong evidence for the involvement of the cadherin:catenin complex and Wnt/beta-catenin signalling in the regulation of VSMC proliferation, migration and survival. Most of these findings however have been generated using *in vitro* approaches and *in vivo* models using healthy young animals. It is fair to say therefore that currently there is little direct evidence for the involvement of these factors in atherosclerosis, intimal thickening and aneurysms. Future studies are therefore required to directly assess the role of N-cadherin, beta-catenin and Wnt pathway components in these vascular diseases and determine the therapeutic potential of modulating these factors for vascular diseases.

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Abbreviations: APC: Adenomatous polyposis coli, bFGF: Basic fibroblast growth factor, CamKII: Calcium/calmodulin dependent kinase, DKK-1: Dickkopf-related protein-1, EC: Extracellular, ECM: Extracellular matrix, ERK : Extracellular signal-regulated kinase , FGF-R: Fibroblast growth factor receptor, GSK-3b: Glycogen synthase kinase-3b, HAV: His-Ala-Val, IGF-1: Insulin-like growth factor, JNK: Jun N-terminal kinase, LEF: Lymphoid enhancer factor, LRP: Low density lipoprotein receptor related protein, MAPK: Mitogen-activated protein kinase, NCAM: Neural cell adhesion molecule, NFAT: nuclear factor associated with T cells, MMP: Matrix-degrading metalloproteinase, Ox-LDL: Oxidized low-density lipoprotein, PCP: Planar cell polarity, PDGF: Platelet-derived growth factor, PI-3K: Phosphatidylinositol-3-kinase, PKC: Protein kinase C, sFRP: Soluble Frizzled related protein, SNC: Soluble N-cadherin, TCF: T-cell factor, TIMP: Tissue inhibitor of metalloproteinase, VSMC: Vascular Smooth Muscle Cells

Key Words: Vascular Smooth Muscle Cell, Proliferation, Migration, Apoptosis, Cadherin, beta-catenin, Wnt, Review

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