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Regulation of cell–matrix contacts and β -catenin signaling in VSMC by integrin-linked kinase: implications for intimal thickening

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Abstract

Vascular smooth muscle cell (VSMC) proliferation and migration is responsible for intimal thickening that occurs in restenosis and atherosclerosis. Integrin-linked kinase (ILK) is a serine/threonine protein kinase implicated in signaling pathways involved in cell proliferation and migration. We studied the involvement of ILK in intimal thickening. ILK expression was significantly increased in two models of intimal thickening: balloon-injured rat carotid arteries and human saphenous vein organ cultures. Over-expression of a dominant negative ILK (DN-ILK) significantly reduced intimal thickening by approximately 50% in human saphenous vein organ cultures, demonstrating an important role in intimal thickening. ILK protein and activity was reduced on laminin and up-regulated on fibronectin, indicating ILK protein expression is modulated by extracellular matrix composition. Inhibition of ILK by siRNA knockdown and DN-ILK significantly decreased VSMC proliferation and migration while wild type ILK significantly increased proliferation and migration on laminin, confirming an essential role of ILK in both processes. Localization of paxillin and vinculin and protein levels of FAK and phospho-FAK indicated that inhibition of ILK reduced focal adhesion formation. Additionally, inhibition of ILK significantly attenuated the presence of the cell–cell complex proteins *N*-cadherin and β -catenin, and β -catenin signaling. We therefore suggest ILK modulates VSMC proliferation and migration at least in part by acting as a molecular scaffold in focal adhesions as well as modulating the stability of cell–cell contact proteins and β -catenin signaling. In summary, ILK plays an important role in intimal thickening by modulating VSMC proliferation and migration via regulation of cell–matrix and cell–cell contacts and β -catenin signaling.

Keywords

smooth muscle; intimal thickening; cell signaling; focal adhesions; cadherins

Introduction

The interaction of vascular smooth muscle cells (VSMCs) with extracellular matrix (cell–matrix adhesion) and with adjacent cells (cell–cell adhesion) plays an important role in proliferation and migration of VSMCs during vascular development and during pathological conditions which involve intimal thickening such as restenosis and atherosclerosis [2, 6]. Extracellular matrix regulates VSMC proliferation [21] and migration [16] largely via

integrins that activate intracellular signaling molecules via various effector proteins. Integrin-linked kinase (ILK) is one such effector.

Integrin-linked kinase is a serine/threonine protein kinase that interacts with the cytoplasmic domain of β_1 and β_3 integrins [28]. ILK has the capacity to phosphorylate numerous substrates including glycogen synthase kinase-3 β (GSK-3 β), protein kinase B(PKB)/Akt, β_1 and β_3 integrin tails, myosin light chain, myosin light chain phosphatase and several myosin light chain phosphatase regulators (see for review [19]). Consequently, ILK regulates multiple signaling pathways and has been implicated in the regulation of numerous cellular processes including proliferation, migration, cell spreading, invasion, differentiation, transformation, and survival in various cell types (see for review [19]).

In addition, ILK has an essential role in regulating cell–matrix adhesions and actin cytoskeletal organization. ILK is a molecular scaffold in focal adhesions as it acts as an adaptor protein binding numerous other proteins, including paxillin and actopaxin [24]. It is therefore an important contributor to the modulation of cell–matrix contacts mediated by focal adhesion complexes, which are composed of numerous proteins including paxillin, actopaxin, vinculin, and focal adhesion kinase (FAK). ILK activity thereby plays an essential role, via activation of Rac-1 and Cdc42, in actin cytoskeleton reorganization upon extracellular matrix engagement [7]. ILK also regulates cell–cell adhesion since cross-talk between cell–matrix and cadherin-mediated cell–cell contacts occurs as a result of a physical response to integrin-mediated adhesion, complex cell differentiation processes or direct signaling pathways that link the two adhesion systems (see for review [3]). ILK has also been implicated in both GSK-3 β dependent and independent regulation of β -catenin stability [26] and therefore may also affect proliferation and migration via the modulation of β -catenin responsive genes.

Integrin-linked kinase is expressed in VSMC and is known to mediate calcium-independent myosin diphosphorylation and contraction of VSMCs [38]. Additionally, Friedrich et al. [8] recently demonstrated that ILK modulates VSMC proliferation and migration. However, a direct involvement in intimal thickening and the mechanism of action has not been shown. Consequently, in this study we tested the hypothesis that ILK plays a pivotal role in intimal thickening. In addition, we sought to provide an insight into the mechanism by which ILK regulates VSMC proliferation and migration by determining the effect of ILK on cell–matrix adhesions and the cadherin/catenin system in VSMCs.

Methods

Adenoviral construct and expression

pSP72 cloning vectors containing V5-tagged cDNA encoding the wild-type and mutant (E359K) dominant negative ILK [4] were obtained from Professor Dedhar (University of British Columbia, Vancouver, BC, Canada). Coding sequences were cloned and recombined with the adenovirus genomic plasmid by cotransfection into 293 cells.

Human saphenous vein organ culture

Surplus segments of human saphenous vein ($n = 10$) were obtained from consenting patients undergoing coronary artery bypass surgery and placed in organ culture as described previously [11]. This study was reviewed by the relevant ethics committee (Research Ethics Committee number 04/Q2007/6), and therefore was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Five segments were infected with adenovirus to express dominant negative ILK (RAdDN-ILK) and RAdLacZ (control, to express β -galactosidase) prior to organ culture as described previously [9]. Three micrometre paraffin-wax sections were stained with Haematoxylin and Eosin, and Miller's

elastic van Gieson stain. Intimal thickness was quantified using image analysis (Image Pro-plus, MediaCybernetics, Wokingham, UK) by dividing the intimal area by the length of the vein segment, as described previously [11].

Rat carotid injury

Rat carotid arteries were subjected to balloon injury as described previously [31]. The 'Principle of laboratory animal care' (NIH publication No. 86-23, revised 1985) was followed as well as the UK Home Office guidelines.

Culture and adenoviral infection of VSMCs

Vascular smooth muscle cells were grown from human saphenous vein as described previously [35]. VSMCs were quiesced in serum-free DMEM media for 72 h and were used at passages 2–10. VSMCs were infected with 300 plaque-forming units (pfu) per cell of RAdDN-ILK, RAdWT-ILK or RAdLacZ in serum-containing (10% foetal calf serum; FCS) DMEM culture media for 18 h (infection period). After washing, cells were cultured in fresh serum-containing DMEM for 24 h; in some cases cells were grown for a further 24 h in serum-free DMEM. In some experiments infected cells were grown on 20 ng laminin- or fibronectin-coated 24 well plates. For β -catenin signaling assays, VSMCs were grown from aortas of TOPGAL mice as previously described [29].

ILK knockdown using siRNA

Two silencing RNA oligonucleotides (siRNA) for ILK and GFP were purchased from Qiagen (catalogue numbers SI00064743, SI00288183). Transfections of human and TOPGAL VSMCs were performed with a Nucleofector device and VSMC kit (Amaxa, Inc., Cologne, Germany) following the manufacturer's instructions. Briefly 8×10^5 cells were subjected to nucleofection with 250 pmol of ILK or GFP siRNAs using U-25 and A-33 program for human and mouse VSMCs, respectively. Treated cells were analysed 24 h after nucleofection.

Bromodeoxyuridine incorporation

Proliferation was determined by bromodeoxyuridine (BrdU) incorporation as previously described [29].

Migration

The migration of VSMCs was determined by plating 4×10^4 cells in uncoated and laminin-coated (upper side) Transwells (Corning, UK) with 20 ng/ml PDGF-BB used as a chemotactic agent and by a scratch wound assay as described previously [30] at 24 h after the transfection or infection period. VSMCs migration after 24 h was quantified by counting the total number of cells on the underside of the Transwell in 10 high power fields or measuring the size of the wound (distance between the edges of the injured monolayer) at 20 points using computer-aided image analysis (Image Pro-plus, MediaCybernetics, Wokingham, UK).

Immunocytochemistry

Paxillin, vinculin, smooth muscle α -actin and ILK were detected by immunocytochemistry using 2.5 μ g/ml mouse anti-paxillin antibody (clone 349, BD Biosciences), anti-vinculin mouse ascites fluid diluted 1:1,000 (Sigma, Poole, UK), 1.2 μ g/ml mouse anti-smooth muscle α -actin antibody (clone 1A4, Sigma), 3.5 μ g/ml ILK antibody (clone 65.1.9, Upstate Biotechnology, Cambridge, UK), as previously described [18, 35]. Bound antibodies were detected with biotinylated goat anti-mouse IgG (Dako, Ely, UK) and ExtravidinTM-FITC. Coverslips were mounted on glass slides with Vectashield mounting medium (Vector

Laboratories, Peterborough, UK) with or without 4', 6-diamidino-2-phenylindole (DAPI). Controls, where primary antibody was substituted with non-immune mouse IgG, were always included.

Immunohistochemistry

Serial 3 μ m paraffin sections were dewaxed and rehydrated. ILK and smooth muscle α -actin protein were detected using 0.7 μ g/ml mouse anti-ILK (clone 65.1.9, Upstate Biotechnology) and anti- β -actin mouse ascites fluid diluted 1:500 (Sigma) and a previously described protocol [11]. Co-location of ILK and BrdU was determined using this protocol and dual immunohistochemistry for BrdU incorporation as described previously [31]. Controls, where primary antibody was substituted with non-immune mouse IgG, were always included.

Western blot analysis

Vascular smooth muscle cells were lysed in RIPA protein extraction buffer and equal protein concentrations were subjected to Western blotting using mouse anti-V5 antibody diluted 1:5,000 (Invitrogen), 0.7 μ g/ml mouse anti-ILK antibody (clone 65.1.9, Upstate Biotechnology), mouse anti-PCNA antibody diluted 1:500 (Dako), 0.1 μ g/ml mouse anti-N-cadherin antibody (clone 32, BD Biosciences), 1.25 μ g/ml mouse anti- β -catenin antibody (clone 14, BD Biosciences), rabbit anti-Akt antibody diluted 1:1,000 (#9272 Cell Signaling Hertfordshire, UK), rabbit anti-pAkt (Ser473) antibody diluted 1:1,000 (#9271 Cell Signaling), 0.5 μ g/ml mouse anti-GSK-3 β antibody (Calbiochem), rabbit anti-phospho-GSK-3 β (Ser9) antibody diluted 1:1,000 (#9336 Cell Signaling), 0.25 μ g/ml mouse anti-FAK (BD Biosciences), 0.25 μ g/ml mouse anti-phospho-FAK (BD Biosciences), 2 μ g/ml mouse anti-p21 (BD Biosciences), 2 μ g/ml mouse anti-cyclin D1 (BD Biosciences), or anti- β -actin mouse ascites fluid diluted 1:1,000 (Sigma) and previously described protocol [31]. Optical densitometry (O.D.) data (O.D. $\times$ mm²) was normalised by loading control protein.

Apoptosis

In situ end labelling (ISEL) of DNA was used to quantify VSMC apoptosis as previously described [9]. Apoptotic cells identified as brown fragmented nuclei were counted and expressed as a percentage of the total number of cells. Additionally, immunocytochemistry for cleaved caspase-3 immunocytochemistry was performed as follows. VSMCs were fixed with 3% paraformaldehyde for 10 min and then blocked for 1 h with 5% goat serum (DAKO) in PBS/Triton. Anti-cleaved caspase-3 antibody (R&D systems) was added at 1 μ g/ml and incubated at 4 °C overnight. Following three washes with PBS, the secondary antibody (Alexa fluor 488, goat anti-rabbit, Molecular Probes) was added at a 1:200 dilution and incubated for 1 h at room temperature, followed by three washes with PBS and mounting with Vectashield (Vector Laboratories) with DAPI. The number of green (apoptotic) and blue cells was counted under the fluorescent microscope and recorded.

β -Catenin signaling

Vascular smooth muscle cells grown from aorta of TOPGAL mice were utilised to assess β -catenin signaling as previously described [29]. VSMCs were cultured in serum-free media or 10% FCS on uncoated or 20 ng laminin- or fibronectin-coated 24 well plates for 24 h. Additionally, VSMCs were subjected to nucleofection with siRNA and then cultured in serum-free media or 10% FCS media for 24 h. VSMCs were lysed and β -galactosidase activity was determined by using the Galacto-Light Plus kit (Tropix, Bedford, UK) following the manufacturer's instructions. Luminescence was measured by using a Glomax luminometer (Promega) and normalised by total protein concentrations.

Statistics

Experiments were performed at least in triplicate with VSMCs cultured from different vein segments. Mean $\pm$ standard error of the mean (SEM) was analysed using ANOVA for multiple comparisons and paired Student's *t* test for paired analysis between two groups. Significance was accepted when $P < 0.05$.

Results

ILK expression is increased during intimal thickening and DN-ILK inhibits intimal thickening

Integrin-linked kinase protein expression was low in uninjured rat carotid arteries and was greatly increased in the media at 2 days and the neointima at 10 days after injury (Fig. 1A–C). Immunohistochemical analysis for BrdU demonstrated that proliferation was undetectable at 24 h and the rate of medial proliferation per day was relatively constant at approximately 10% between 48 and 118 h (Fig. 1D). A higher rate of proliferation (approximately 35%) was detected in the intima at 10 days. To investigate whether this increase in ILK expression is exclusive to proliferative cells, we performed dual staining for ILK and BrdU incorporation. As shown in Fig. 1E, F, ILK protein is not exclusively expressed in proliferating cells (red nuclei) at both 2 and 10 days after injury, suggesting it is involved in other cellular processes in addition to proliferation. Immunohistochemistry for α -smooth muscle actin revealed that ILK is expressed predominantly in VSMCs (Fig. 1G, H). Similarly, ILK protein was low in uncultured vein segments but increased both in the intima and media during intimal thickening in the human saphenous vein organ culture model (Fig. 2A, B). No staining was observed when the anti-ILK antibody was substituted with non-immune mouse IgG (data not shown).

Adenoviral infection was assessed by X-gal staining as previously described [10] and revealed efficient transfection along the luminal surface (insets in Fig. 2C, D). Luminal infection of saphenous vein with DN-ILK significantly inhibited intimal thickening after 7 and 14 days (Fig. 2C–G).

Modulation of ILK expression

Integrin-linked kinase protein was abundant in quiescent cultured human VSMCs and was not affected by addition of FCS (Fig. 3A). Significantly less ILK protein was detected when VSMCs were cultured on laminin compared to when grown on tissue culture plastic (2.3 ± 0.3 vs. 3.6 ± 0.1 O.D. $\times$ mm², $n = 3$, $P < 0.05$, Fig. 3B). In addition, significantly more ILK protein was detected when VSMCs were cultured on fibronectin compared to when grown on tissue culture plastic (5.2 ± 0.4 vs. 3.7 ± 0.1 O.D. $\times$ mm², $n = 3$, $P < 0.05$, Fig. 3B). The amount of phosphorylated GSK-3 β , indicative of ILK activity, was also significantly reduced by approximately 50% (0.8 ± 0.2 vs. 1.6 ± 0.2 O.D. $\times$ mm², $n = 3$, $P < 0.05$) when cultured on laminin, and increased by 1.8-fold when cultured on fibronectin (2.8 ± 0.2 vs. 1.5 ± 0.24 O.D. $\times$ mm², $n = 3$, $P < 0.05$), (Fig. 3B).

Infection of VSMCs grown on laminin with an adenovirus encoding WT-ILK increased ILK protein expression and phosphorylation of GSK-3 β as expected (Fig. 3C). Nucleofection of VSMCs with ILK siRNA significantly reduced expression of ILK protein by $60 \pm 13\%$ compared to GFP siRNA controls (Fig. 3D). The amount of phosphorylated GSK-3 β and Akt were also significantly reduced to 64 ± 13 and $24 \pm 11\%$ of controls (0.93 ± 0.28 vs. 1.46 ± 0.28 and 0.18 ± 0.08 vs. 0.78 ± 0.08 O.D. $\times$ mm², respectively, $n = 5$, $P < 0.05$), without an effect on total levels of these proteins (Fig. 3D).

ILK modulates VSMC growth

Proliferation was significantly increased by over-expression of WT-ILK in VSMCs grown on laminin in serum-free media compared to VSMCs infected with control virus (Fig. 4A, $n = 3$, $P < 0.05$). VSMC proliferation during 24 h treatment with FCS was significantly reduced by after ILK silencing compared to VSMCs treated with control siRNA (Fig. 4B, $n = 4$, $P < 0.05$). In addition, proliferation during 24 h treatment with FCS was also reduced in cells expressing DN-ILK compared to control cells and control infected (β -gal) cells (Fig. 4B, $n = 3$, $P < 0.05$). Further support for the involvement of ILK in proliferation was gained by Western blotting for proliferating cell nuclear antigen (PCNA) protein in which ILK silencing and expression of DN-ILK, significantly reduced PCNA expression compared to controls (Fig. 4C, $n = 3$, $P < 0.05$).

ILK modulates VSMC migration

Over-expression of WT-ILK significantly increased migration in laminin-coated chemotaxis chambers compared to control cells (Fig. 5A). However, over-expression of WT-ILK had no significant effect on migration through uncoated membranes or fibronectin coated membranes (Fig. 5A). In contrast, infection of VSMCs with DN-ILK significantly inhibited migration through uncoated membranes and membranes coated with either laminin or fibronectin (Fig. 5A). Additionally, nucleofection with ILK siRNA significantly reduced migration through uncoated chemotaxis membranes compared to controls (Fig. 5B). Further support for the involvement of ILK in VSMC migration was obtained using wounding assays. The wound size was significantly greater in VSMCs subjected to ILK silencing ($193 \pm 43 \mu\text{m}$) compared to VSMC treated with control siRNA ($138 \pm 12 \mu\text{m}$), indicating ILK promotes migration (Fig. 5C, $n = 5$, $P < 0.05$). Similar results were observed with expression of DN-ILK. The wound size was significantly greater in VSMCs expressing DN-ILK ($509 \pm 56 \mu\text{m}$) compared to uninfected control cells ($92 \pm 11 \mu\text{m}$) and control infected (β -gal) cells ($65 \pm 17 \mu\text{m}$) (Fig. 5C, $n = 4$, $P < 0.001$).

ILK modulates cell–matrix and cell–cell contacts

We examined the effect of ILK inhibition on some markers of cell–matrix contacts (focal adhesions). Fluorescent immunocytochemistry showed decreased localization of paxillin in focal adhesions in cells treated with ILK siRNA and cells expressing DN-ILK compared to control cells (Fig. 6), despite no changes in total amount of paxillin proteins detected by Western blotting (data not shown). Similar effects were observed with another focal adhesion protein, vinculin (Fig. 6). In addition, disorganization of actin cytoskeleton was observed in cells expressing DN-ILK (Fig. 6). Western blotting revealed the total amount of FAK and phospho-FAK were reduced by ILK siRNA and DN-ILK (Fig. 7). Cleavage of FAK identified by the presence of smaller molecular weight fragments was not observed at 24 h after the infection period. However, a 60 kDa fragment was apparent with extended culture to 72 h (data not shown).

To examine cadherin-mediated cell–cell contacts we carried out Western blotting for *N*-cadherin (the predominant cadherin in VSMCs [34]) and β -catenin. Expression of DN-ILK significantly reduced *N*-cadherin and β -catenin protein compared to control infected (β -gal) and uninfected cells (1.4 ± 0.3 vs. 3.1 ± 0.6 and 3.7 ± 0.7 and 3.8 ± 0.8 vs. 4.7 ± 0.6 and 5.4 ± 0.4 O.D $\times$ mm², respectively, Fig. 7, $n = 8$, $P < 0.05$). Similarly cells treated with ILK siRNA showed significantly decreased levels of *N*-cadherin and β -catenin protein compared to control cells (0.5 ± 0.2 vs. 2.5 ± 0.6 and 0.8 ± 0.3 vs. 2.2 ± 0.2 O.D $\times$ mm², respectively, Fig. 7, $n = 5$, $P < 0.05$).

Long-term inhibition of ILK reduces VSMC survival

At 24 h after treatment inhibition of ILK using DN-ILK did not significantly affect cell viability assessed by ISEL compared to uninfected and control infected (β -gal) VSMCs (1.5 ± 1 vs. $1.0 \pm 0.3\%$ and $0.3 \pm 0.2\%$, $n = 3$). Interestingly, however, at 72 h after the infection period cell death was significantly elevated in VSMCs expressing DN-ILK compared to uninfected cells and control infected (β -gal) cells (31.0 ± 2.8 vs. $0.7 \pm 0.3\%$ and $0.6 \pm 0.3\%$, respectively, $n = 3$, $P < 0.05$). Immunocytochemistry for cleaved caspase-3 confirmed these findings (Fig. 7).

ILK modulates β -catenin signaling

Using TOPGAL VSMCs we observed that culture on different extracellular matrix proteins alters β -catenin signaling. Culture on laminin significantly reduced β -catenin signaling while fibronectin increased signaling (Fig. 8A). Growth factors (FCS) significantly increased β -catenin signaling in TOPGAL VSMCs grown on all matrices. Induction of β -catenin signalling after FCS treatment was significantly less in TOPGAL VSMCs subjected to ILK silencing compared to GFP controls (Fig. 8B). Additionally, we observed that the expression of two β -catenin responsive genes were regulated by ILK. First, ILK silencing significantly reduced cyclin D1 protein (0.4 ± 0.2 vs. 0.6 ± 0.3 O.D. $\times$ mm², $n = 3$, $P < 0.05$, Fig. 8C). Second, ILK silencing significantly increased p21 protein (0.7 ± 0.1 vs. 0.3 ± 0.1 O.D. $\times$ mm², $n = 3$, $P < 0.05$, Fig. 8C).

Discussion

In this study, we observed that ILK is an important regulator of intimal thickening. We have shown for the first time increased ILK expression in the intima and media in two models of intimal thickening. Furthermore, the significant inhibition of intimal thickening by 50% with a dominant negative ILK directly indicates its involvement in intimal thickening. Following balloon injury of the rat carotid artery, VSMCs in the media undergo an early, reversible de-differentiation, followed by proliferation and migration [1]. We observed that ILK is up-regulated as early as 2 days after injury and therefore participates in the induction of VSMC proliferation and migration. Although a previous study has demonstrated the ability of ILK to regulate VSMC proliferation and migration [8] this is the first demonstration that ILK is elevated during intimal thickening and inhibition of ILK effectively retards intimal thickening. We confirmed the ability of ILK to modulate VSMC proliferation and migration in cultured cells and suggest that this is mediated at least in part by modulation of cell–matrix contacts and cross-talk between cell–matrix and cell–cell contact proteins and β -catenin signaling.

To investigate the mechanism by which ILK is upregulated during intimal thickening we utilised cultured VSMCs. We observed that ILK protein expression was high both in the presence and absence of FCS when VSMCs were grown on plastic tissue culture wells, indicating that expression was not regulated by growth factors. Since low levels of ILK were observed in vivo in the presence of extracellular matrix we attempted to modulate ILK levels by growing VSMCs on laminin a basement membrane component that normally surrounds VSMCs and maintains the contractile phenotype [13]. ILK was significantly, albeit partially, reduced by culture on laminin, which illustrates the ability of the extracellular matrix to modulate ILK expression as observed previously with collagen type I in fibroblasts [22]. In support of this we also observed that ILK expression was elevated by culture on fibronectin, an extracellular matrix protein associated with the synthetic phenotype and intimal thickening and atherosclerosis [5, 33].

We examined the role of ILK by increasing ILK levels using over-expression of WT-ILK and by inhibiting ILK using siRNA and over-expression of DN-ILK. Over-expression of WT-ILK significantly increased both proliferation and migration of VSMC grown on laminin in vitro, corroborating previous findings [8]. Interestingly, enhanced migration was not observed by over-expression of WT-ILK when VSMC were grown on uncoated or fibronectin-coated membranes. We suggest that in these conditions where ILK activity is elevated over-expression of ILK is unable to further enhance migration, since the ILK-dependent pathway is maximally stimulated. The inhibitory experiments confirmed the ability of ILK to stimulate proliferation and migration on all matrices. Importantly no significant induction of VSMC death was observed with over-expression of ILK, after ILK silencing or in DN-ILK expressing cells at the time of the migration and proliferation assays (24 h) so enhanced cell death cannot be responsible for the reduced proliferation and migration. Interestingly, however, sustained inhibition of ILK with DN-ILK for 72 h significantly increased VSMC apoptosis. We suggest that extended culture of VSMCs that have reduced levels of FAK, *N*-cadherin, and pAkt which provide vital survival signals for VSMCs [14, 18] render the VSMC more susceptible to apoptosis.

A key function of ILK is to provide a molecular scaffold in focal adhesions and therefore a potential mechanism for these effects on VSMC behaviour is the modulation of focal adhesion complexes. Indeed, studies in ILK-null systems in *Drosophila melanogaster* and *Caenorhabditis elegans* questioned the role of ILK kinase activity in cellular regulation and suggested a more dominant role for ILK as a molecular scaffold [20, 42]. This is supported by the observation that ILK mutants which retain kinase activity act as a dominant negative by blocking paxillin binding and ILK localization to focal adhesions [23, 40]. In this study we observed decreased localization of paxillin in focal adhesions despite no effect on total levels of this protein in VSMCs subjected to ILK silencing or expressing DN-ILK. This is consistent with previous findings with this DN-ILK and with siRNA inhibition of ILK in other cell types [12, 17, 24, 36]. In addition, we observed reduced vinculin localization in focal adhesions and attenuated levels of FAK, indicative of decreased numbers of focal adhesions. Although we were unable to detect cleavage of FAK at 24 h after the infection period after extended culture for 72 h a 60 kDa fragment was detected, indicating that FAK cleavage may be responsible for the reduction in FAK expression (data not shown). It is likely that reduced FAK is the result of attenuated levels of paxillin in focal adhesions since Yano et al. [41] demonstrated that paxillin is essential for the recruitment of FAK to focal adhesions. FAK is a cytoplasmic tyrosine kinase, which functions as an integrin-activated scaffold in focal adhesions for assembly of signaling and structural proteins involved in cell migration (see for review [39]). Activation of FAK is also necessary for optimal growth factor-mediated VSMC proliferation [32]. Furthermore, we observed that ILK inhibition led to disorganization of the actin cytoskeleton, this may be the result of reduced Rac- and Cdc-42 activity as observed previously in epithelial cells [7]. Therefore our findings suggest that via acting as a molecular scaffold for paxillin binding and organization of focal adhesions and the actin cytoskeleton, ILK regulates VSMC proliferation and migration and thereby intimal thickening.

Although it is fair to say that controversy exists as to whether ILK is a *bona fide* kinase, many studies have shown that activation of ILK phosphorylates downstream effectors, including Akt and GSK-3 β [4]. Consequently, ILK is an important modulator of several signaling pathways including the canonical Wnt/ β -catenin pathway [25-27], which regulates expression of cell cycle genes including cyclin D1, p21 and c-myc and migratory genes including MMP-7. Indeed in this study, silencing of ILK caused a dramatic reduction in the phosphorylation of GSK-3 β and Akt and therefore this may contribute to the observed effects on intimal thickening by modulation of signaling pathways including the Wnt/ β -catenin pathway. In support of this we observed that silencing of ILK significantly reduced

β -catenin signalling. Additionally, culture on laminin which decreased ILK expression reduced β -catenin signaling, while the contrary was observed with fibronectin. We and others have shown that β -catenin is increased during intimal thickening and is a key regulator of VSMC proliferation [29, 31, 35, 37]. However, the mechanism responsible for up-regulation of β -catenin during intimal thickening has not been fully elucidated. We suggest therefore that increased ILK activity contributes to increased β -catenin signaling during intimal thickening and thereby alters the expression of β -catenin responsive genes including two cell cycle regulators, cyclin D1 and p21. Oloumi et al. have also highlighted that ILK may regulate β -catenin stability in a GSK-3 β independent manner [26] and therefore we cannot rule out that this also contributes to β -catenin stability and signaling.

Previous studies have demonstrated cross-talk between cell-matrix and cell-cell contacts, which may result from a physical response to integrin-mediated adhesion, complex cell differentiation processes or direct signaling pathways that link the two adhesion systems (see for review [3]). We and others have previously demonstrated that *N*-cadherin-mediated cell-cell contacts modulate VSMC proliferation and migration [15, 29, 35]. Consequently, it is pertinent to determine the mechanisms by which cell-cell adhesion proteins are regulated. To determine therefore whether ILK activity affects cell-cell adhesion proteins, we investigated the levels of *N*-cadherin, which is important for cell-cell contacts in VSMCs [35]. We observed decreased levels of *N*-cadherin in VSMCs subjected to ILK siRNA knockdown and expressing DN-ILK compared to controls. We observed no effect on mRNA levels (data not shown) and therefore we suggest that this is a post-translational effect, for example shedding by MMPs as we previously observed [35]. Therefore we propose that reduced focal adhesions lead to a concomitant loss of *N*-cadherin and thereby cell-cell contacts. In agreement with our results Yano et al. [41] have previously demonstrated that siRNA-mediated knockdown of FAK and paxillin resulted in impaired assembly of *N*-cadherin-mediated cell-cell contacts due to impaired FAK and Rac1 activity. It is logical that reduced levels of *N*-cadherin may inhibit VSMC migration since *N*-cadherin is required for VSMC migration [15]. From our previous studies, one would predict that reduced *N*-cadherin levels would promote VSMC proliferation [31, 35, 37]. However, we have shown that this requires β -catenin signaling [29] and therefore since β -catenin stability and signaling is reduced by ILK inhibition [26], loss of *N*-cadherin may be unable to promote proliferation due to attenuated levels of β -catenin. Consequently, we suggest that inhibition of ILK regulates intimal thickening via attenuation of VSMC migration and proliferation is mediated at least in part by loss of *N*-cadherin and down-regulation of β -catenin signaling.

In summary, ILK plays an important role in intimal thickening via regulation of VSMC proliferation and migration. We observed that ILK is a key intermediate regulator of several pathways which control VSMC proliferation and migration and therefore small molecule inhibitors of ILK such as QLT-0267 may be useful for attenuating intimal thickening.

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Fig. 1. Integrin-linked kinase (*ILK*) is elevated during intimal thickening. Representative immunohistochemical staining for ILK (brown) in rat carotid arteries from sham-operated controls (A), 2 days (B), and 10 days (C) after balloon injury. Quantification of proliferation in media (black bars) and intima (white bar) at various time points after injury assessed by BrdU incorporation 24 h prior to carotid artery removal (D). Dual immunohistochemistry for ILK (brown) and BrdU (red) at 2 days (E) and 10 days (F). Immunohistochemistry for smooth muscle α -actin (brown) at 2 days (G), and 10 days (H). Dotted lines indicate the intima:media boundary. Nuclei are stained blue. Scale bar in A represents 50 μ m and applies to all panels. $n = 4$ per time-point

Fig. 2.

Integrin-linked kinase modulates intimal thickening. Representative ($n = 4$) immunohistochemical staining for ILK (brown) of uncultured saphenous vein (A) and saphenous vein after 14 days of culture (B). Scale bar in A represents 100 μm and applies to A and B. Dotted lines indicate the intima:media boundary. Representative sections from human saphenous vein infected with RADLacZ (β -gal: C, E) and RADDN-ILK (D, F), stained with haematoxylin and eosin, 7 (C, D) and 14 (E, F) days after infection. Dotted lines indicate intima:media boundary. Scale bar in C represents 50 μm and applies to C–F. Inset in C and E show X-gal staining of saphenous vein; blue colour identifies β -galactosidase expression. G Quantification of intimal thickness in RADDN-ILK infected segments, asterisk indicates significant difference from β -gal control ($P < 0.05$, $n = 5$)

Fig. 3.

Modulation of ILK in cultured VSMCs. Immunocytochemistry and Western blotting for ILK in VSMCs cultured in presence (+FCS) or absence (-)FCS of serum (A). Scale bar represents 10 μ m; green colour indicates ILK and nuclei are stained blue with DAPI. Representative Western blots for ILK and phosphorylated GSK-3 β in lysates of VSMCs cultured in serum-free media for 24 h on tissue culture plastic or laminin, $n = 3$ (B). Western blotting for V5-tag, ILK, phosphorylated GSK-3 β and total GSK-3 β in lysates of uninfected VSMCs and VSMCs infected with RAdLacZ (β -gal) and RAdWT-ILK (C). Representative Western blots for ILK, phosphorylated GSK-3 β , total GSK-3 β , phosphorylated Akt, and total Akt in lysates of VSMCs treated with GFP siRNA and ILK siRNA ($n = 5$) (D). β -actin is shown as a loading control.

Fig. 4.

Integrin-linked kinase modulates proliferation. Proliferation assessed by BrdU incorporation in VSMCs infected with RAdLacZ (β-gal) or RAdWT-ILK and then cultured for 24 h on laminin in serum-free media (**A**). *Asterisk* indicates significant difference from β-gal control, $P < 0.05$, $n = 3$. Proliferation assessed by BrdU incorporation (**B**) and representative Western blotting for PCNA and β-actin (**C**) in VSMCs treated with GFP siRNA and ILK siRNA and uninfected VSMCs, and VSMCs infected with RAd-LacZ (β-gal) and RAd DN-ILK and then cultured for 24 h in FCS. The number of BrdU positive cells is expressed as a percentage of the total. *Asterisk* indicates significant difference from GFP siRNA and *dollar* indicates significant difference from uninfected and β-gal controls, $P < 0.05$, $n = 3$ and $n = 4$, respectively

Fig. 5.

Integrin-linked kinase modulates migration. Migration of VSMCs infected with RADLacZ (β -gal) RAD DN-ILK or RADWT-ILK and then placed in uncoated, or fibronectin- or laminin-coated chemotaxis chambers for 24 h (A). *Asterisk* indicates significant difference from β -gal control and uninfected control, $P < 0.05$, $n = 3$. Migration assessed by 24 h in uncoated chemotaxis chambers in VSMCs treated with GFP siRNA and ILK siRNA (B). Migration assessed 18 h after wounding in VSMCs treated with GFP siRNA and ILK siRNA and uninfected VSMCs, and VSMCs infected with RADLacZ (β -gal) and RAD DN-ILK (C). *Asterisk* indicates significant difference from GFP siRNA control, $P < 0.05$, $n = 3$. Scale bar represents 250 μ m

Fig. 6.

Effect of DN-ILK on focal adhesion proteins and cell viability. Immunocytochemical staining for paxillin, vinculin, and α -actin in VSMCs treated with GFP siRNA and ILK siRNA, and VSMCs infected with RAdLacZ (β -gal) and RAdDN-ILK (DN-ILK) 24 h after the infection period. Immunocyto-chemical staining for cleaved caspase-3 in VSMCs infected with RAdLacZ and RAdDN-ILK 24 and 72 h after the infection period. *Arrows* indicate positive cells. *Green colour* indicates the presence of protein; nuclei are stained *blue* with DAPI in all panels except α -actin. *Scale bars* represent 20 μ m

Fig. 7.

Dominant negative-ILK reduces focal adhesions and cell–cell contacts. Western blots for total FAK, phosphorylated FAK, *N*-cadherin, β-catenin, and β-actin at 24 h after treatment with GFP siRNA and ILK siRNA (**A**) or 24 h after infection period in uninfected VSMCs and VSMCs infected with RAd LacZ (β-gal) and RAd DN-ILK (**B**). *Numbers* represent molecular weights of proteins, *n* = 8 DN-ILK and *n* = 5 siRNA

Fig. 8.

Integrin-linked kinase regulates β -catenin signaling. β -Catenin signaling in VSMCs grown on plastic, laminin or fibronectin in the presence (+FCS) or absence of FCS (–FCS) for 24 h. *Asterisk* and *dollar* indicate significant difference ($P < 0.05$) from plastic and –FCS, respectively, $n = 3$. Induction of β -catenin signaling 24 h after the addition of FCS was assessed in TOPGAL VSMCs subjected to GFP or ILK siRNA. Results are expressed as a percentage of the quiescent VSMCs (control), $n = 3$, *asterisk* indicates significant difference from GFP siRNA $P < 0.05$ (B). Western blots for cyclin D1, p21 and β -actin at 24 h after treatment with GFP siRNA and ILK siRNA. *Numbers* represent molecular weights of proteins, $n = 3$ (C)