Abstract

Saphenous vein (SV) grafts are commonly used to bypass coronary arteries that are diseased due to atherosclerosis. However, the development of intimal hyperplasia in such grafts can lead to patency-threatening stenosis and re-occlusion of the vessel. The proliferation and migration of smooth muscle cells (SMC) play key roles in the development of intimal hyperplasia, and an agent that inhibits both of these processes therefore has therapeutic potential. A prerequisite for SMC proliferation and migration in vivo is degradation of the basement membrane, achieved by secretion of the matrix-degrading gelatinases matrix metalloproteinase-2 (MMP-2) and MMP-9. Statins are cholesterol-lowering drugs that also have direct effects on SMC function. Here we report that neointima formation in organ-cultured human SV segments is inhibited by simvastatin, an effect that is associated with reduced MMP-9 activity. Additionally, our work shows that simvastatin not only inhibits proliferation, but importantly also inhibits invasion (migration through a matrix barrier), of cultured human SV SMC. Thus simvastatin treatment appears to inhibit neointima formation as a result of combined inhibition of SMC proliferation and invasion. The potential intracellular mechanisms by which statins affect SMC proliferation and migration, and thus attenuate intimal hyperplasia, are discussed, with particular emphasis on the role of MMP-9.

Vein graft stenosis and intimal hyperplasia (IH)

Coronary artery bypass grafting using the autologous saphenous vein (SV) is used routinely to revascularize patients with atherosclerotic coronary artery disease. However, occlusions in such grafts are common, resulting in long-term patency rates of approx. 50% after 10 years [1]. The prevention of graft stenosis, rather than treatment of an established lesion, would make a significant impact on long-term patency, and in view of the large numbers of patients receiving venous bypass grafts, the development of preventative therapeutic approaches is an important aim. A large number of pharmacological agents have been shown to reduce stenosis in animal models, although no systemic agent has yet proven effective in humans.

The underlying pathological lesion of stenosis is IH, the characteristic histological features of which are a thickened intima (neointima) containing smooth muscle cells (SMC) in a stroma of mucopolysaccharide, collagen and elastin [2]. IH is a complex process that is initiated in the vessel wall following endothelial injury/denudation as a consequence of bypass grafting. Venous bypass graft failure is related principally to the biological properties of SMC and endothelial cells, and, in areas of endothelial loss, platelet aggregation favours thrombus formation and vascular occlusion [3]. Furthermore, SV SMC proliferate in response to a host of released growth factors and cytokines, including platelet-derived growth factor (PDGF), thrombin and endothelin-1 [3–5],

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Key words: HMG-CoA reductase inhibitor, MMP-9, smooth muscle cell migration, smooth muscle cell proliferation.

Abbreviations used: FCS, foetal calf serum; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IH, intimal hyperplasia; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; SMC, smooth muscle cell(s); SV, saphenous vein.

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which may contribute to the poor long-term patency rates of SV grafts. It has been shown that both migration and proliferation of SMC play key roles in the development of IH in vein grafts [4]. These processes are associated with activation of the matrix-degrading gelatinases matrix metalloproteinase-2 (MMP-2) and MMP-9 [6-8]. In further support of a detrimental role for MMP-9 in vascular disorders characterized by neointima formation, a recent study identified a polymorphism in the promoter region of the MMP-9 gene that is associated with an increase in the severity of atherosclerosis [9].

Therapeutic strategies for ameliorating IH that target either SMC proliferation or migration alone have had limited success [10,11]; a combined anti-proliferative and anti-migratory strategy may be more effective, but remains untested.

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins)

**In vivo studies**

The HMG-CoA reductase inhibitors (statins) are a class of cholesterol-lowering drugs that are used extensively for the prevention and treatment of atherosclerosis. A number of large clinical trials, which include the Scandinavian Simvastatin Survival Study [12] and the West of Scotland Coronary Prevention Study [13], have demonstrated a significant improvement in cardiovascular morbidity and mortality. However, the clinical benefits of statin therapy appear greater than would be expected from a simple lowering of cholesterol levels, and these additional effects have been claimed to be due to direct modulation of SMC function [14].

Some recent animal studies have reported a beneficial effect of statins in the prevention of IH following arterial injury. Lovastatin reduced neointima formation in rabbit carotid artery following collar placement [15]. Similarly, simvastatin [16] and cerivastatin [17] were found to ameliorate neointimal thickening following arterial angioplasty in the rat and rabbit respectively.

The few reports to date on the use of statins in humans for the control of clinical events characterized by neointima formation are, as yet, inconclusive. Simvastatin has been shown to prevent the progression of carotid artery stenosis [18] and has a beneficial effect on lumen size after angioplasty of the femoro-popliteal artery [19]. Furthermore, simvastatin was shown recently to reduce the development of vein graft occlusions in a relatively small cohort of patients [20]. However, fluvastatin, lovastatin and pravastatin have all been reported to confer no clinical benefits following coronary angioplasty in humans [21-23].

**In vitro organ culture studies**

We have previously established and validated the in vitro organ-cultured human SV as being a useful model of vein graft IH [24]. Using this model, we have shown that the neointima that develops in culture closely resembles the pathological lesion associated with stenosis in vein bypass grafts in vivo. We have also observed that increased gelatinase (both MMP-2 and MMP-9) activity occurs concomitantly with neointima formation in SV organ culture [25,26], in agreement with a previous report [27]. The organ-cultured human SV is therefore an appropriate model for studying potential therapeutic agents for the amelioration of vein graft stenosis [25,26].

In order to investigate the ability of simvastatin to reduce IH, human SVs were obtained, with permission, from 10 patients undergoing coronary artery bypass grafting and prepared for organ culture as follows. Paired SV segments were cultured in RPMI-1640 medium supplemented with 30% (v/v) foetal calf serum (FCS) for 14 days in the absence or presence of simvastatin (0.5–5 μM) or of simvastatin (1 μM) plus mevalonate (100 μM). Following paraffin-embedding and immunostaining of 4 μm sections, we measured neointimal thickness using a computerized image analysis system, as described previously [24]. Simvastatin dose-dependently reduced neointimal thickness by a median of 59%, 68% and 75% at concentrations of 0.5, 1 and 5 μM respectively (Figure 1a). Statins act by inhibiting the conversion of HMG-CoA into mevalonate (see below). Supplementation with mevalonate reversed the inhibitory effect of simvastatin and restored neointimal thickness to 93% of control values (Figure 1a), confirming that the effects of simvastatin were due to a decrease in intracellular mevalonate.

**In vitro cell culture studies**

Neointima formation occurs as a result of a combination of both SMC proliferation and migration, which requires the degradation of surrounding matrix proteins, a process accomplished by secretion of specific MMPs from the SMC themselves [6,28]. In order to determine whether the inhibitory action of simvastatin on SV neointima formation was due to inhibition of SMC...
Simvastatin reduces SV neointima formation and SV SMC proliferation and invasion

(a) Neointima formation. SV segments were cultured for 14 days in serum-supplemented growth medium (30% FCS) in the presence or absence of simvastatin or mevalonate at the indicated concentrations. Horizontal bars represent median values. **P < 0.01 compared with control (no simvastatin); N.S., not significantly different (Wilcoxon paired rank test).

(b) Cell proliferation was determined in 10% FCS/RPMI medium (control) or medium supplemented with 1 μM simvastatin. Data are expressed as means ± S.E.M. Statistical analysis was performed on the areas under the curves. ***P < 0.001 compared with control (n = 5; Student's t test).

(c) Cell invasion (migration through Matrigel) in response to migration medium alone (control) or migration medium containing PDGF-BB (10 ng/ml), with or without 1 μM simvastatin, was determined using modified Boyden chambers. Data are expressed as the means ± S.E.M. of the number of invasive cells per high-power field. **P < 0.01 for the effect of simvastatin (n = 5; Student's t test).

Statins and MMP-9

The ability of statins to reduce vascular SMC invasion may be due, at least in part, to inhibition of gelatinase activity. Statin treatment has been shown to lower MMP-9 activity in monocytes [32,33], macrophages [34,35], NIH 3T3 fibroblasts [36] and breast cancer cell lines [37]. Importantly, simvastatin has been shown to lower proliferation or migration, we investigated the effects of simvastatin in isolated cultures of SV SMC. Cells were first rendered quiescent in 0.4% (v/v) FCS for 72 h before being plated into 24-well plates at a density of 1 x 10⁴ cells per well in 10% FCS/RPMI medium (control) or medium supplemented with 1 μM simvastatin. Medium and drugs were replaced every 2 days. Cell number was determined every 2 days over a 10-day period in duplicate wells using a haemocytometer. This enabled the construction of growth curves, from which the area under each curve was calculated and statistical analysis was performed. Simvastatin (1 μM) significantly reduced the serum-stimulated proliferation of SV SMC to 54% of the control (no simvastatin) value (Figure 1b), in agreement with previous studies in human vascular SMC [17,29–31].

To investigate the effects of simvastatin on SMC migration through a matrix barrier (i.e. cell invasion), we used a modified Boyden chamber technique in which the polycarbonate transwell membranes were coated with Matrigel (65 μg/cm²), an artificial basement membrane matrix comprising laminin, collagen IV, entactin and heparan sulphate proteoglycan. SV SMC were first rendered quiescent in 0.4% (v/v) FCS for 72 h. Using PDGF (10 ng/ml) as a chemotactic stimulus in the lower wells of the Boyden chambers, SMC were loaded into the upper chambers (1 x 10⁵ cells in 0.25% BSA/Dulbecco's modified Eagle's medium) and incubated for 24 h at 37°C in the absence or presence of simvastatin (1 μM). After fixation, invasive cells on the underside of the membranes were visualized by staining with haematoxylin and eosin (30 s each). Membranes were rinsed in water and mounted on microscope slides for examination. Cell nuclei were counted in ten random high-power fields (×400) for each membrane. As can be seen in Figure 1(c), simvastatin significantly reduced the number of SMC invading the Matrigel and migrating through the pores in the membrane to 31% of that observed with PDGF alone.
Figure 2

Simvastatin decreases MMP-9 levels in organ-cultured human SVs

Segments of human SVs were cultured in the absence or presence of simvastatin (0.5–5 μM) and/or mevalonate (100 μM) for 14 days before metalloproteinases were extracted and gelatin zymography was performed. MMP-9, pro-MMP-2, and MMP-2 levels were visualized as bands of lysis with molecular masses of 92 kDa, 72 kDa, and 66 kDa respectively. Conditioned medium from HT-1080 cells was included as a positive control.

We have reported previously that increased tissue gelatinase activity (both MMP-2 and MMP-9) is observed during neointima formation in organ-cultured human SVs [26]. To investigate whether simvastatin treatment decreases gelatinase activity in this model, we cultured segments of human SVs in the absence or presence of simvastatin (0.5–5 μM) for 14 days before extracting the metalloproteinases and performing gelatin zymography, as described previously [26]. Simvastatin significantly reduced MMP-9 activity, but had no effect on MMP-2 activity (Figure 2). In keeping with the effects on neointima formation, the inhibitory effect of simvastatin on MMP-9 activity was fully reversed by co-supplementation with mevalonate (Figure 2). We have reported previously that the broad-spectrum MMP inhibitor marimastat significantly reduces neointima formation in organ-cultured human SVs [25], consistent with an essential role for MMP activation in the development of neointima formation in this model. Thus the mechanism by which simvastatin inhibits neointima formation appears to be the result of a combined inhibition of both SMC proliferation and migration, the latter being associated with inhibition of MMP-9.

Intracellular mechanisms by which statins inhibit SMC function

The cellular effects of statins arise from the inhibition of HMG-CoA reductase, a key enzyme in the mevalonate metabolic pathway (Figure 3). In addition to its central role in cholesterol synthesis, mevalonate is also an important precursor of many isoprenoid compounds, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Both FPP and GGPP are required for post-translational prenylation of a variety of proteins, including small G-proteins (reviewed in [40]). Prenylation of these small G-proteins, which include the Ras and Rho families, is essential for their membrane localization and function. Ras and Rho regulate gene expression, and Rho is also important for cytoskeletal reorganization. Both of these small G-protein families have been shown to be important in the mechanisms of vascular SMC proliferation, migration, and neointima formation [41–43]. A number of previous studies have suggested that the inhibitory effects of statins on vascular SMC proliferation are attributable to inhibition of the prenylation of either Ras or Rho [29,31,41]. Moreover, statins have also been shown to induce apoptosis of vascular SMCs via a mechanism involving the inhibition of Ras and/or Rho prenylation [44,45].

The mechanisms by which statins inhibit vascular SMC invasion have yet to be fully elucidated. The process of cell invasion requires two distinct phases: degradation of the basement membrane and migration towards a chemotactic stimulus. Statins have been shown to reduce the latter of these two processes in other cell types via a mechanism involving the inhibition of Rho prenylation and subsequent disruption of the cytoskeleton [37,46]. However, it is likely that statins also decrease cell invasion by inhibiting MMP-9 activity, and thereby the capacity of SMC...
Intracellular mechanisms underlying the effects of statins on vascular SMC function

Statins inhibit HMG-CoA reductase, resulting in deprivation of intracellular mevalonate. In addition to its central role in cholesterol synthesis, mevalonate is also an important precursor of many isoprenoid compounds, including FPP and GGPP. FPP and GGPP are required for the prenylation of Ras- and Rho-family G-proteins, post-translational modifications that are necessary for their function. The effects of statins on vascular SMC proliferation, migration and invasion may be mediated via inhibition of Ras and Rho prenylation.

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\begin{align*}
\text{HMG-CoA} & \xrightarrow{\text{STATINS}} \text{Mevalonate} \\
\downarrow & \\
\text{IPP} & \downarrow \\
\downarrow & \\
\text{FPP} & \xrightarrow{\text{Ras prenylation}} \\
\downarrow & \\
\text{Squalene} & \xrightarrow{\text{GGPP}} \\
\downarrow & \\
\text{Cholesterol} & \xrightarrow{\text{Rho prenylation}} \\
\end{align*}
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Proliferation
Migration
Invasion

Statins have been shown to reduce MMP-9 activity in several different cell types [32–37].

Intracellular mechanisms by which statins affect MMP-9 activity

MMP-9 activity can be regulated at the transcriptional level in response to a variety of stimuli, including certain growth factors and cytokines [7]. The promoter region of the MMP-9 gene contains both AP-1 (activator protein-1) and nuclear factor-κB (NF-κB) transcription factor binding sites [47]. Thus MMP-9 gene expression can be increased following stimulation of several signal transduction pathways, including the mitogen-activated protein kinase (MAPK) and NF-κB pathways [48,49]. Statins have the potential to attenuate MMP-9 gene expression by inhibiting the prenylation of Ras and Rho, upstream activators of both the MAPK and NF-κB pathways [50,51]. However, although statins are reported to inhibit MAPK in some cell types [52,53], they do not appear to inhibit MAPK activity in human vascular SMC (N. A. Turner and K. E. Porter, unpublished work; [30,31]). However, statins have been shown to inhibit the NF-κB pathway in cultured vascular SMC [54,55]. In addition, the ability of statins to inhibit the Rho/NF-κB pathway and to reduce MMP-9 gene expression and cell invasion has been demonstrated very recently in a human breast carcinoma cell line [37].

Concluding remarks

The ability of statins to inhibit both the proliferation and the migration/invasion of vascular SMC suggests that they have potential therapeutic value in the prevention of vein graft stenosis. The intracellular mechanisms underlying the effects of statins on SMC function appear to be mediated predominantly via inhibition of the prenylation of small G-proteins. A prerequisite for both SMC proliferation and migration in vitro is the activation of gelatinases (MMP-2 and MMP-9) which facilitate basement membrane degradation. In addition to their direct effects on SMC proliferation and motility, statins may also have the capacity to inhibit cell invasion by reducing MMP-9 activity via mechanisms involving inhibition of small G-protein prenylation.

References


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Proteases and Anti-proteases: Molecular Mechanisms and Novel Therapeutic Targets

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Role of plasminogen activators in peritoneal adhesion formation

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Abstract

Intra-abdominal adhesion formation is a major complication of serosal repair following surgery, ischaemia or infection, leading to conditions such as intestinal obstruction and infertility. It has been proposed that the persistence of fibrin, due to impaired plasminogen activator activity, results in the formation of adhesions between damaged serosal surfaces. This study aimed to assess the role of fibrinolysis in adhesion formation using mice deficient in either of the plasminogen activator proteases, tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). We hypothesize that, following serosal injury, mice with decreased peritoneal fibrinolytic activity will be more susceptible to adhesion formation. Adhesion formation was induced in tPA- and uPA-deficient and wild-type mice following either surgical trauma to the serosa with haemorrhage and acute or chronic intra-peritoneal inflammation. Adhesion formation was assessed from 1 to 4 weeks post-injury. Mice deficient in tPA were more susceptible to adhesion formation following both a surgical insult and a chronic inflammatory episode compared with uPA-deficient and wild-type mice. In addition, the time of maximal adhesion formation varied depending on the nature of the initial insult. It is proposed that the persistence of fibrin due to decreased tPA activity following surgery or chronic inflammation plays a major role in peritoneal adhesion formation.

Key words: fibrin, inflammation, plasmin, post-operative, tissue repair.

Abbreviations used: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; WT, wild-type; PAI, plasminogen activator inhibitor.

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Introduction

Peritoneal adhesions are defined as fibrous bands of tissue that join together organs that are normally separated and/or the internal body wall. They are a common consequence of serosal repair, occurring in 93–100% of patients following laparotomy, and may lead to serious complications such as intestinal obstruction, pelvic pain and infertility [1–3]. One-third of intestinal obstructions and nearly one-quarter of infertility cases in women are a consequence of adhesions, with removal often resulting in recurrence. The magnitude of the problem was highlighted by a survey performed over a 10-year period suggesting that 5.5% of all hospital re-admissions were directly attributable to adhesions [4]. However, despite their clinical importance, information regarding their formation is sparse, and current prevention is based on careful surgery and the occasional use of physical barriers that are effective in only a proportion of patients [5].

Fibrin persistence and peritoneal adhesion formation

Peritoneal adhesions form when closely apposed visceral and/or parietal peritoneal surfaces are damaged due to surgery, thermal or ischaemic injury, inflammation or a foreign body reaction. The protective surface mesothelial layer is disrupted and a fibrinous exudate ('fibrinous' adhesion) is deposited between the damaged, closely apposed serosal surfaces. These filmy adhesions are often transient and are degraded by proteases of the fibrinolytic system within a few days of injury, leading to restoration of the normal peritoneal surface in association with re-epithelialization by mesothelial cells [6,7]. Alternatively, if there is insufficient peritoneal fibrinolytic activity,

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