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Simvastatin reduces venous stenosis formation in a murine hemodialysis vascular access model

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Venous neointimal hyperplasia (VNH) is responsible for hemodialysis vascular access malfunction. Here we tested whether VNH formation occurs, in part, due to vascular endothelial growth factor-A (VEGF-A) and matrix metalloproteinase (MMP)-9 gene expression causing adventitial fibroblast transdifferentiation to myofibroblasts (α-SMA-positive cells). These cells have increased proliferative and migratory capacity leading to VNH formation. Simvastatin was used to decrease VEGF-A and MMP-9 gene expression in our murine arteriovenous fistula model created by connecting the right carotid artery to the ipsilateral jugular vein. Compared to fistulae of vehicle-treated mice, the fistulae of simvastatin-treated mice had the expected decrease in VEGF-A and MMP-9 but also showed a significant reduction in MMP-2 expression with a significant decrease in VNH and a significant increase in the mean lumen vessel area. There was an increase in terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and decreases in α-SMA density, cell proliferation, and HIF-1α and hypoxyprobe staining. This latter result prompted us to determine the effect of simvastatin on fibroblasts subjected to hypoxia *in vitro*. Simvastatin-treated fibroblasts had a significant decrease in myofibroblast production along with decreased cellular proliferation, migration, and MMP-9 activity but increased caspase 3 activity suggesting increased apoptosis. Thus, simvastatin results in a significant reduction in VNH, with increase in mean lumen vessel area by decreasing VEGF-A/MMP-9 pathway activity.

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In the United States, approximately 600,000 patients have end-stage renal disease, with the vast majority of patients requiring chronic hemodialysis for long-term survival, and this population of patients will double in the coming decades.[1] Arteriovenous fistula (AVF) is the preferred vascular access; however, venous stenosis formation and lack of maturation are major problems, and thus 1-year patency rates are estimated to be 62%.[2] Venous stenosis occurs in AVFs because of neointimal hyperplasia.[3–5] Histologic analysis of AVF specimens reveals that there is angiogenesis located within the neointima and adventitia of the vessel, accompanied by increased proliferation of cells staining positive for α-smooth muscle actin (α-SMA) in the neointima.[3–5] Recent experimental studies have demonstrated a pivotal role for adventitial and medial fibroblasts that convert to myofibroblasts (α-SMA-positive cells) and can subsequently contribute to the formation of venous neointimal hyperplasia.[6–8] As a consequence, over a billion dollars are spent annually to maintain the functioning of hemodialysis AVFs and grafts.[1] Effective, non-invasive treatments, which would prevent and/or reduce AVF stenosis, could greatly benefit patients with end-stage renal disease.

Vascular endothelial growth factor-A (VEGF-A) has been shown to be involved in the pathogenesis of arterial stenosis, vein bypass grafts, and venous neointimal hyperplasia associated with hemodialysis vascular access.[5,9–18] Previous work from our laboratory and other laboratories has demonstrated increased expression of VEGF-A and other profibrotic genes, including matrix metalloproteinase-2 (*MMP-2*) and metalloproteinase-9 (*MMP-9*), at the site of venous stenosis in murine and porcine models of hemodialysis AVF and AV graft failure. However, the mechanism(s) through which VEGF-A and MMPs have a role in venous neointimal hyperplasia formation has not been carefully investigated.[17,19]

Our investigations were performed in a murine model of chronic kidney disease in animals with an AVF. Simvastatin has been shown to decrease VEGF-A and MMP expression.[20–22] We tested the hypothesis that the reduction of

VEGF-A and MMP-9 gene expression via systemic delivery of simvastatin before the placement of an AVF leads to a reduction in venous neointimal hyperplasia with positive vascular remodeling. Gene and protein expression studies, as well as histomorphometric analyses, were performed at the outflow vein removed from animals treated with either simvastatin or controls. We ascertained whether simvastatin reduces fibroblast to myofibroblast (α-smooth muscle actin–positive cells) differentiation induced by hypoxia, and we determined its effect on several important cellular functions including proliferation and migration, with increased caspase 3 activity and decreased MMP-9 activity.

RESULTS

Surgical outcomes

Sixty-nine male C57BL/6 mice weighing 25–30 g were used for the study. Five mice died after nephrectomy, and two mice (control) had thickened arteries at the time of AVF placement and were excluded. Therefore, 62 mice comprise this study[18,19] (Figure 1a and b). Either 40 mg/g of simvastatin (SV, $n = 35$) or phosphate-buffered saline only (control, C, $n = 27$) was given intraperitoneally every other day starting 1 week before fistula placement until the time of killing (Figure 1c).

Serum BUN and creatinine after nephrectomy

The serum blood urea nitrogen (BUN) and creatinine was used to assess the kidney function. After nephrectomy, the average BUN was significantly higher for the simvastatin and the control group at all time points ($P < 0.001$) when compared with baseline (Figure 1d). At 5 to 8 weeks after nephrectomy, the average BUN was significantly higher in the control group when compared with the simvastatin group (Figure 1e). At 8 weeks after nephrectomy only, the average serum BUN was significantly higher in the control group when compared with the simvastatin group ($P < 0.001$). At 8 weeks after nephrectomy, the average serum creatinine was significantly increased in the control group when compared with the simvastatin group ($P < 0.001$).

Simvastatin-treated vessels have a significant reduction in average gene expression of VEGF-A at the outflow vein at days 7 and 14

VEGF-A expression is increased in failed hemodialysis vascular accesses (AV fistulas or AV grafts) and in experimental animal models.[5,6,17,18] By day 7, the mean gene expression of VEGF-A at the simvastatin-treated vessels was significantly lower than the control vessels (average reduction: 44%, $P < 0.01$ (Figure 2a)), and also at day 14 (average reduction: 49%, $P < 0.01$). Taken collectively, these results indicate that the average gene expression of VEGF-A is reduced at the outflow vein in simvastatin-treated vessels when compared with control vessels.

Simvastatin-treated vessels have a significant reduction in gene expression of MMP-9 at the outflow vein at days 7 and 14

Studies have shown increased expression of MMP-9 in failed hemodialysis vascular accesses (AV fistulas or AV grafts) and in experimental animal models.[6,17,23] By day 7, the average gene expression of MMP-9 was significantly lower in the simvastatin-treated vessels when compared with controls (average reduction: 69%, $P < 0.0001$ (Figure 2b)), and also at day 14 (average reduction: 41%, $P < 0.0001$). Overall, these results indicate that simvastatin-treated vessels have a significant reduction in MMP-9 when compared with control vessels.

Because the expression of the protein can lag behind the gene expression, we performed zymography at day 14 to assess MMP-2 and MMP-9 activity in simvastatin-treated vessels as compared with controls (Figure 2c). There was significant reduction in both the MMP-9 and MMP-2 activity in the simvastatin-treated vessels when compared with controls (MMP-9—average reduction: 18%, $P < 0.01$ (Figure 2d); pro-MMP-2—average reduction: 33%, $P < 0.0001$; and active MMP-2—average reduction: 18%, $P < 0.0001$ (Figure 2e)).

Kidneys in simvastatin-treated animals have decreased gene expression of VEGF-A, MMP-2, and MMP-9 at 4 weeks

Because we observed a decrease in average serum BUN and creatinine in the simvastatin-treated animals when compared with controls at 8 weeks, we determined whether the improvement in kidney function was due to a decrease in genes implicated in causing chronic kidney disease, such as VEGF-A (Figure 2f), MMP-2 (Figure 2g), and MMP-9 (Figure 2h). The average gene expression of VEGF-A, MMP-2, and MMP-9 was significantly reduced at day 28 (VEGF-A—average reduction: 24%, $P < 0.01$; MMP-2—average reduction: 42%, $P < 0.001$; and MMP-9—average reduction: 57%, $P < 0.01$) in the simvastatin-treated kidneys when compared with controls.

Simvastatin-treated vessels have positive vascular remodeling at days 14 and 28

On hematoxylin- and eosin-stained sections, we were able to differentiate between the neointima and media/adventitia (Figure 3a). Semiquantitative histomorphometric analysis was performed on sections removed from the outflow veins of simvastatin-treated vessels and control vessels for the following: the area of the neointima (Figure 3b), media/adventitia (Figure 3c), and lumen vessel (Figure 3d). There was a significant reduction in the average area of the neointima of the simvastatin-treated vessels when compared with the controls by days 14 to 28 (average reduction: 56%, $P < 0.0001$; day 28—average reduction: 45%, $P < 0.001$). By day 14, the average area of the media/adventitia was significantly lower in the simvastatin-treated vessels when compared with the control group (average reduction: 43%, $P = 0.0028$).