Prevention of Venous Neointimal Hyperplasia by a Multitarget Receptor Tyrosine Kinase Inhibitor

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Key Words

Arteriovenous graft · Neointimal hyperplasia · Perfused organ culture · Sunitinib · Growth factors

Abstract

Background/Aims: Venous neointimal hyperplasia (NH) is the predominant cause of stenosis in hemodialysis arteriovenous grafts (AVG), but there is currently no clinically used therapy to prevent NH. **Methods:** A porcine AVG model was used to identify potential pharmacological targets to prevent NH. Sunitinib, a broad-spectrum tyrosine kinase inhibitor, was examined as a potential anti-NH drug utilizing in vitro and ex vivo models. **Results:** In an in vivo porcine model, PDGF, VEGF and their receptors PDGFR- α and VEGFR-2 were upregulated at the venous anastomosis within 2 weeks after AVG placement, with NH development by 4 weeks. Sunitinib inhibited PDGF-stimulated proliferation, migration, phosphorylation of MAPK and PI3K/Akt proteins and changes in the expression of cell-cycle regulatory proteins in vascular smooth-muscle cells as well as VEGF-stimulated endothelial cell proliferation in vitro. In an ex vivo model, sig-

nificant NH was observed in porcine vein segments perfused for 12 days under pathological shear stress. Sunitinib (100 nM) inhibited NH formation, with the intima-to-lumen area ratio decreasing from 0.45 ± 0.25 to 0.04 ± 0.02 ($p < 0.05$) with treatment. **Conclusion:** These findings demonstrate sunitinib to be a potential NH-preventive drug as well as the utility of an ex vivo model to investigate pharmacotherapies under pathophysiological flow conditions.

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Introduction

While a well-functioning native arteriovenous fistula is the preferred form of vascular access for chronic hemodialysis, up to 60% of arteriovenous fistulas fail to mature [1–4]. A synthetic arteriovenous graft (AVG), consisting of expanded polytetrafluoroethylene tubing anastomosed between an artery and vein, is a common alternative.

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AVGs do not require maturation and can typically be used within 2–3 weeks of placement. However, AVGs are associated with high stenosis rates, followed by thrombosis and occlusion, with 50 and 75% failure rates at 1 and 2 years, respectively, after implantation [5, 6]. Currently, AVG stenosis is treated by angioplasty, but the vascular wall injury associated with this procedure predisposes to restenosis [3].

Neointimal hyperplasia (NH) accounts for practically all stenotic lesions associated with AVGs [3, 7]. Histologically, NH consists of proliferating smooth-muscle cells (SMCs) and myofibroblasts, mostly derived from the medial and the adventitial layers of the vessel wall [6–12]. Progressive NH occurs much more frequently at the graft-venous anastomosis (hereafter referred to as venous anastomosis) than at the graft-arterial anastomosis (arterial anastomosis) of an AVG [3, 7, 13]. The AVG shunts arterial blood flow directly into the vein, exposing the vein to high blood pressures and pulsatile and disturbed flow patterns, resulting in highly aberrant wall shear stress (WSS). In addition to the multiple pathogenic factors, such as inflammation enhanced by the foreign graft material and surgical damage to the protective adventitia and endothelium, drastically altered hemodynamics at the venous anastomosis likely predisposes this location to NH development.

The early stage of NH development requires the growth and migration of cells in the vascular wall. Platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) have been implicated in the pathogenesis of NH in arterial injury models [14–18], and the receptor for VEGF (VEGFR) and the receptor for PDGF (PDGFR) have been reported to mediate mechanotransduction signaling in vascular endothelial cells (ECs) and SMCs, respectively [19, 20]. We examined the expression of PDGF and VEGF and their receptors in a porcine AVG model. Sunitinib, a multitarget receptor tyrosine kinase (RTK) inhibitor with well-established antiproliferative properties [21, 22], was also examined for its potential to prevent NH. The efficacy of sunitinib in inhibiting growth factor-stimulated proliferation was first examined in cultured SMCs and ECs, and then tested in an ex vivo perfused organ culture model using explanted whole porcine jugular vein segments. This ex vivo system enables us to recapitulate the pathological hemodynamic parameters occurring at the venous anastomosis in vivo, such as very low or very high WSS, with the additional advantage that it can be utilized to efficiently test pathogenic mechanisms and drugs under these and other specific isolated conditions. Whole-vessel segments retain the vessel wall

architecture as well as cell-cell and cell-extracellular matrix interactions that are more relevant than primary monolayer cell cultures to the in vivo environment.

Materials and Methods

Materials

All culture reagents were purchased from Cascade Biologics (Portland, Oreg., USA) except for fetal calf serum (FCS), which was purchased from Atlanta Biologicals (Lawrenceville, Ga., USA). Sunitinib maleate was provided by Pfizer Inc. (New York, N.Y., USA). Recombinant human PDGF-BB and human VEGF₁₆₅ were purchased from R&D Systems (Minneapolis, Minn., USA). Antibodies against PDGF-B chain, VEGF, PDGFR- α and VEGFR-2 were purchased from Thermo Scientific (Fremont, Calif., USA). Antibodies against von Willebrand factor (vWF) and Ki-67 were purchased from DAKO Pharmaceuticals (Carpinteria, Calif., USA). Anti-smooth-muscle α -actin antibody was purchased from Sigma (St. Louis, Mo., USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam (Cambridge, Mass., USA). Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). All other antibodies were obtained from Cell Signaling Technologies (Danvers, Mass., USA).

Porcine Model of AVG Stenosis

All animal procedures were performed in accordance with a protocol that was approved by the Institutional Animal Care and Use Committees at the Veterans Affairs, Salt Lake City Health Care System, and the University of Utah. Female Yorkshire-cross domestic pigs (Sigma Livestock, Salt Lake City, Utah, USA) weighing approximately 30 kg were implanted with a spiral-reinforced polytetrafluoroethylene graft (C.R. Bard, Murray Hill, N.J., USA) unilaterally between the common carotid artery and the ipsilateral external jugular vein (EJV) as we have described previously [23, 24]. Animals were euthanized at the indicated time points, and the AVG and adjoining blood vessels were retrieved en bloc.

Histology and Morphometry at Venous Anastomosis in the Porcine AVG Model

Explanted EJV segments were perfusion-fixed in formalin. Paraffin-embedded tissue cross sections from the center of the venous anastomosis were stained with elastic Van Gieson's stain (Accustain Elastic Stain, Sigma). The NH index was calculated as the ratio of the NH area to the combined area of the graft and media, as previously described [25]. Tissue sections from the same anastomotic region were also immunostained for the nuclear proliferation marker, Ki-67, and for the EC marker, vWF. The proliferation index (PI) was calculated as the ratio of the number of Ki-67-positive cells relative to the number of all cells in the same area, as described previously [6]. Direct manual counting of the vWF-positive neovessels in the NH and the adventitia in a given image field (i.e. neovessel density) was performed separately by 3 independent observers under light microscopy at a $\times 20$ magnification. Tissue sections from the same anastomotic region were also subjected to immunohistochemistry using antibodies against VEGF, VEGFR-2, PDGF-B and PDGFR- α .

Cell Culture

Primary human aortic SMCs (HASMCs), human saphenous vein SMCs (HVSVCs) and human umbilical vein ECs (HUVECs) were obtained from Cambrex (Chicago, Ill., USA). SMCs were maintained in Medium 231 supplemented with 10% FCS. ECs were grown in Medium 200 prepared with growth supplements. Cells at passages 3–7 were used.

In vitro Proliferation Assays

HASMCs, HVSVCs and HUVECs were seeded at a density of 5×10^3 cells/well and cultured to approximately 70% confluence. Before each experiment, cells were rendered quiescent by incubating for 24 h with their respective basal media containing 0.5% FCS but no additional growth factors. Cells were then pretreated with various concentrations of sunitinib for 1 h, followed by stimulation with either PDGF-BB (50 ng/ml) or VEGF₁₆₅ (50 ng/ml) for SMCs and ECs, respectively. After 48 h, DNA synthesis was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation (Roche Applied Science, Indianapolis, Ind., USA). At least 6 replicate experiments were analyzed for each sunitinib concentration for both SMCs and HUVECs.

In vitro Migration Assays

A suspension of 5×10^4 HASMC was loaded into the upper migration chamber of the InnoCyte™ Cell Migration Assay (Calbiochem, San Diego, Calif., USA) that contained media with 0.5% FCS but no additional growth factors. Cells were preincubated with various concentrations of sunitinib added to the media in both the upper and lower chambers for 1 h, and then PDGF-BB (50 ng/ml) was added to the lower chamber. Cells were allowed to migrate through the porous membrane towards the lower chamber for 4 h. Finally, the migrated cells that attached at the lower-chamber side of the membrane were dislodged and fluorescence-labeled with calcein AM, followed by quantification at 485 nm. At least 3 replicate experiments were analyzed for each sunitinib concentration.

Western Blotting

Quiescent HASMCs were pre-exposed to various concentrations of sunitinib for 1 h, then stimulated with PDGF-BB (50 ng/ml) for 10 min to determine early changes in signaling protein phosphorylation, for 24 h to assess changes in cell-cycle regulatory protein expression and for 6 h to determine the level of phosphorylated p53. As a positive control for apoptosis, cells were treated with 1 mM hydrogen peroxide for 6 h to induce the phosphorylation of p53. Cell lysates were prepared by washing cells with ice-cold PBS containing 150 mM NaCl, 10 mM HEPES, 0.6% NP-40 (pH 7.9) and protease inhibitors, followed by incubation on ice for 30 min, then centrifugation at 10,000 g for 10 min. Protein concentrations were determined using the BCA protein assay (Pierce Chemical, Rockford, Ill., USA). Equal amounts of protein were loaded in each well on precast 4–20% gradient gels and subjected to SDS-PAGE. After transfer of proteins to nitrocellulose membranes for immunoblotting, the membranes were incubated with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibody incubation for 1 h at room temperature. Chemiluminescent detection of antibodies was conducted according to the manufacturer's protocol. Anti-GAPDH antibody was purchased from Abcam. All other antibodies were obtained from Cell Signaling Technologies.

Ex vivo Perfused Vein Culture and Analysis

From the same pigs that had been used to create AVG, segments of the internal jugular vein (IJV) from the contralateral side were harvested at euthanasia for ex vivo organ culture using sterile techniques. An ex vivo perfusion system, similar to that described [26–28], with modifications of the vessel chamber and the long-term application of laminar flow with various WSS values, was used. Briefly, explanted IJV segments were suspended at their in vivo length (3–5 cm) between 2 stainless steel connectors using surgical sutures, and bathed in a vessel chamber filled with Dulbecco's modified eagle medium (DMEM) supplemented with 30% FCS and antibiotics but no additional growth factors. Vein segments were perfused with the oxygenated DMEM media (same as in the vessel chamber) that was recirculated at a laminar, constant volumetric flow rate to generate specific calculated WSS values at the lumen-vessel wall interface. Volumetric flow rate was measured manually through a flow valve positioned immediately downstream of the vessel chamber. Vein segments were cultured for 12 consecutive days with media flowing continuously ($n = 6$). Fresh media was supplied every 2–3 days. In the sunitinib-treated veins ($n = 3$), sunitinib was added to the circulating media as well as to the media that filled the vessel chamber on the second day of ex vivo culture. The delay in sunitinib administration was designed to allow the vessels to recover from the explantation procedure. Sunitinib was also added to the fresh replacement media to maintain a constant media drug concentration of 100 nM throughout the duration of the experiment.

WSS was calculated using the following equation: $WSS = (4\mu Q)/(\pi r^3)$, where μ is the viscosity of the culture media containing serum ($\mu = 0.01$ Poise), Q is the volumetric flow rate through the vessel adjusted to generate the desired WSS, and r is the inner radius of the vessel lumen measured at the time of harvest. The WSS inside the vessels was maintained at <1 dyn/cm² at the beginning of culture ($n = 5$), a value that is considered nonphysiologically low for the normal vein. One of the untreated control IJV segments had a small inner radius, such that we were unable to generate an initial low WSS value; therefore, this vein segment was subjected to a WSS of >50 dyn/cm² which is nonphysiologically high for the normal vein.

Explanted IJV segments were formalin-fixed at the end of each perfusion experiment by directly perfusing formalin into the lumen through the metal tubes and immersing the entire vessel in formalin for 24–48 h. Paraffin-embedded cross-sections were stained with either hematoxylin/eosin (Poly Scientific, Bay Shore, N.Y., USA) or Van Gieson's stain. The cross-sectional surface areas of various vessel wall regions were determined using planimetry under light microscopy, and were quantified using the NIH ImageJ software. The results were used to calculate the intima-to-media area (I/M) ratio and the intima-to-lumen area (I/L) ratio. In immunohistochemistry analysis, SMCs and/or myofibroblasts were identified by positive fluorescence immunostaining with anti-smooth-muscle α -actin, while apoptotic and proliferating cells were identified by positive staining with anti-cleaved caspase-3 and anti-Ki-67 antibodies, respectively. ECs were identified by positive immunostaining with anti-vWF antibody. Immunostained proteins were visualized using confocal microscopy.

For comparison, some IJV segments were kept in the static condition for 12 days. In these static experiments, vein segments of about 1–2 cm in length were cut longitudinally after harvest from the animal to expose the lumen, pinned onto a sterile mesh, and

Fig. 1. Time course of NH development, neovessel density and PI at the venous anastomosis in a porcine AVG model. **A** NH index at the venous anastomosis was calculated as the ratio of NH cross-sectional area to the combined area of the graft and media in tissues collected at various time points (n = 2, 3, 3, 7 and 5 animals at days 1, 7, 14, 21 and 28, respectively). * p < 0.05 versus day 1. **B, C** Anastomotic tissue sections were immunostained to detect neovessels and proliferating cells, using antibodies against vWF and Ki-67, respectively, within the NH (**B**) and the adventitia (**C**). PI was determined by dividing the number of Ki-67-positive nuclei by the total number of nuclei in the image field (n = 4 animals for each time point). * p < 0.05 versus day 1 for neovessel density; # p < 0.001 versus day 1 for PI. In all panels, each data point represents the mean and standard deviation.

cultured in DMEM containing 30% FCS in a tissue culture dish. Formalin-fixation, paraffin-embedding and Van Gieson's stain were performed as described above for the perfused veins.

Data Analysis

Results are reported as mean ± standard deviation. Two-tailed Student's t test and one-way analysis of variance (ANOVA) with Tukey's post hoc test were performed using GraphPad Prism (GraphPad Software, San Diego, Calif., USA). Statistically significant results are defined as p < 0.05. IC₅₀ was estimated using GraphPad Prism's built-in non-linear regression (least squares) curve fitting.

Results

NH in Porcine AVG Model

In the porcine model of AVG established in our laboratory, significant stenosis is usually present within

4 weeks after the graft placement [6, 23]. Here, we conducted a time course analysis to delineate the temporal relationships between NH, angiogenesis and cell proliferation in tissue sections obtained from the venous anastomosis. Progression of the NH index (fig. 1A), calculated as the ratio of the NH area to the combined area of the graft and media, exhibited the same trend as the time-dependent increase in angiogenesis within the NH lesions (fig. 1B). Specifically, both the NH index and angiogenesis in the NH lesions were increased at postoperative day 7 whereas the PI in the NH lesions was significantly increased after day 14. This time lag is consistent with the notion that angiogenesis is essential for further NH development by supplying oxygen and nutrients to proliferating cells. Interestingly, the neovessel density within the adventitia immediately increased after AVG placement, but was followed by a

Fig. 2. Enhanced expression of VEGF, PDGF and their cognate receptors at the venous anastomosis in a porcine AVG model. Expression of VEGF, VEGFR-2, PDGF-B and PDGFR- α in fresh EJV (left column) and in the venous anastomotic tissues at 14 days (middle column) and 28 days (right column) after AVG placement was assessed by immunohistochemistry. A rust-brown color indicates positive staining for the indicated proteins. Blue structures are hematoxylin-stained nuclei (colors refer to the online version only). When compared to fresh EJV, venous anastomotic tissues had early (14 days) robust upregulated expression of these growth factors and their cognate receptors as well as sustained (28 days) overexpression of both growth factor receptors. Stained sections were assessed. $\times 20$. The scale bar in the top left panel applies to all panels in this figure. L = lumen.

sharp decrease after day 7 (fig. 1C). The adventitia showed the highest PI at early time periods, and after day 7 the changes in adventitial PI largely paralleled the changes in neovessel density. This temporal profile of proliferating cells in the adventitia was different from that in the NH and is consistent with the notion that an early event in NH development is the transformation and proliferation of adventitial myofibroblasts which subsequently migrate into the NH and contribute to NH formation [8, 12]. Similar data on cell proliferation and angiogenesis occurring in the adventitia and in the NH lesions at selected time points after the graft placement, determined by Ki-67 and vWF immunostaining, respectively, were reported previously by our group [6].

Enhanced Expression of Pro-NH Factors in the Porcine AVG Model

Expression of VEGF and PDGF has been reported to be prominent in the venous NH lesions of specimens obtained from patients with failed AVG [11]. We therefore investigated whether these mediators of proliferation, migration and angiogenesis and their receptors were expressed at the venous anastomosis in our porcine AVG model. Figure 2 shows that in the NH lesions, VEGF, PDGF-B and their respective receptors, VEGFR-2 and PDGFR- α , stained intensely at 14 days after AVG placement, compared to the control EJV. Moreover, the expression of these growth factors and their cognate receptors remained high at 28 days postoperatively. Interestingly, we observed that VEGFR-2 expression was diffusely distributed across the NH area, suggesting that

Fig. 3. Sunitinib inhibits PDGF-BB-induced proliferation of SMCs and VEGF₁₆₅-induced proliferation of ECs in vitro. **A** HASMCs and HVSMCs were preincubated with the indicated concentrations of sunitinib for 1 h before exposure to PDGF-BB (50 ng/ml) for 48 h. **B** HUVECs were preincubated with the indicated concentrations of sunitinib for 1 h before exposure to VEGF₁₆₅ (50 ng/ml) for 48 h. Proliferation was assessed using the bromodeoxyuridine incorporation assay. At least 6 replicate experiments were analyzed for each sunitinib concentration. * $p < 0.001$ versus PDGF-stimulated no-sunitinib control HASMCs, PDGF-stimulated no-sunitinib control HVSMCs or VEGF-stimulated no-sunitinib control HUVECs; # $p < 0.01$ versus PDGF-stimulated no-sunitinib control HVSMCs.

under pathologic conditions, upregulation of this receptor type is also prominent in SMCs. In general, SMCs express low levels of VEGFR-2 [29], but certain stimuli related to NH pathogenesis (e.g. hypoxia) are thought to promote the expression of VEGFR in SMCs [30, 31]. These observations are consistent with the notion that VEGF, PDGF and their receptors play key roles in both the early development and sustained progression of NH.

Fig. 4. Sunitinib inhibits PDGF-BB-induced migration of SMCs in vitro. HASMCs in a migration assay chamber were preincubated with the indicated concentrations of sunitinib for 1 h, and then stimulated with 50 ng/ml of PDGF-BB. HASMCs were allowed to migrate for 4 h, and the migrated cells were fluorescence-labeled and counted. At least 3 replicate experiments were analyzed for each sunitinib concentration. * $p < 0.001$ versus PDGF-stimulated no-sunitinib control.

Accordingly, we examined the effects of sunitinib, a multitarget RTK inhibitor of VEGFR and PDGFR, on proliferation, migration and intracellular signaling in cell cultures and in an ex vivo perfused-vein model.

Sunitinib Inhibits Growth Factor-Induced Proliferation of Human Vascular Cells

Primary HASMCs and HVSMCs were stimulated with 50 ng/ml of PDGF-BB for 48 h, after pretreatment with various concentrations of sunitinib. Sunitinib inhibited the PDGF-BB-induced proliferation of SMCs in a dose-dependent manner, with complete inhibition occurring at approximately 100 nM (fig. 3A). The mean IC₅₀ was approximately 10 ± 3 nM for HASMCs and 40 ± 3 nM for HVSMCs. Sunitinib almost completely inhibited VEGF-induced proliferation of HUVECs at concentrations ≥ 10 nM (fig. 3B), with IC₅₀ of approximately 8 ± 4 nM. Gross cytotoxicity was not apparent under these conditions, as assessed by morphologic examination, and was manifested only at concentrations $> 10,000$ nM (data not shown).

Sunitinib Inhibits PDGF-BB-Induced Migration of Human Vascular SMCs

Because NH initiation and development are attributable, at least in part, to the migration of cells, including medial SMCs and presumably adventitial fibroblasts/

Fig. 5. Sunitinib inhibits PDGF-BB-induced changes in phosphorylation of MAPK and PI3K/AKT signaling proteins and expression of cell-cycle regulatory proteins of HASMCs in vitro. **A** Phosphorylation of the MAPK and the PI3K/Akt pathway after exposure of HASMCs to PDGF-BB for 10 min, in the absence or presence of sunitinib, is shown: ERK1/2 (**a**), Akt (**c**), p70S6k (**e**) and rpS6 (**g**). Levels of total ERK1/2 (**b**), Akt (**d**), p70S6k (**f**) and rpS6 (**h**) proteins are shown. GAPDH was used as a loading control (**i**). **B** Levels of various cell-cycle regulatory proteins after exposure

of HASMCs to PDGF-BB for 24 h, in the absence or presence of sunitinib, are shown: cyclin D1 (**a**), p21 (**b**) and p27 (**c**). GAPDH was used as a loading control (**d**). **C** The toxicity of sunitinib at concentrations that inhibited the PDGF-stimulated signaling pathways and cell-cycle regulatory proteins was evaluated by examining the phosphorylation of the p53 protein (**a**). Hydrogen peroxide-induced apoptosis served as the positive control. Levels of total p53 are shown (**b**). GAPDH was used as a loading control (**c**).

myofibroblasts, into the growing NH [6, 8, 12], we investigated whether sunitinib also attenuated the migration of HASMCs. Sunitinib inhibited the SMC migration stimulated by 50 ng/ml of PDGF-BB in a dose-dependent manner, with approximately 45% inhibition observed at 1 nM (fig. 4). Higher concentrations of sunitinib (100–1,000 nM) did not further inhibit SMC migration.

Effects of Sunitinib on Signaling Proteins and Cell-Cycle Regulatory Proteins in Human Vascular SMCs

As reported previously by others [32, 33], PDGF-BB at 50 ng/ml activated the MAPK and PI3K/Akt pathways, as indicated by the robust phosphorylation of ERK1/2, Akt, p70S6k and rpS6 (fig. 5A). The down-

stream proteins of these pathways, p70S6k and rpS6, regulate translation of mRNAs and the expression of proteins important for proliferation [34]. We found that sunitinib inhibited the phosphorylation of these proteins in a dose-dependent manner. PDGF-induced phosphorylation of ERK1/2 and Akt was almost completely abolished by 100 nM sunitinib, the same concentration that completely inhibited SMC proliferation, as shown in figure 3A. Sunitinib also inhibited, in a dose-dependent manner, the effects of PDGF-BB on the expression of the key cell-cycle regulatory proteins, cyclin D1, p21 and p27. At 100 nM sunitinib, the PDGF-induced expression of cyclin D1, a cyclin-dependent kinase activator and one of the earliest mitogenic regula-

Fig. 6. NH formation in porcine IJV segments maintained in an ex vivo perfused culture. Porcine IJV segments were exposed to non-physiological WSS in a perfused culture system for 12 days ($n = 3$). Cross-sections were stained with Van Gieson's stain (elastic fibers and nuclei appear black; collagen appears red). Representative cross-sectional views are shown (colors refer to the online version only). **A, B** IJV segment harvested after 2 days of perfused culture shows no NH lesions. Prominent NH lesions were observed after 12 days of perfused culture. **C** Tissue sections were coimmunostained for smooth-muscle α -actin (appearing green) and Ki-67 (appearing pink in the insert) and examined under confocal mi-

croscopy. $\times 20$. Blue staining denotes cell nuclei, and the dashed line demarcates the NH lesion from the media. Cells constituting the lesions contained positive for α -actin and Ki-67. **D** NH lesions formed at the venous anastomosis of an EJV in vivo and in ex vivo perfused vein segments were examined for ECs. Tissue sections were assessed. $\times 20$. Blue staining denotes cell nuclei. NH lesions formed in EJV 4 weeks after graft implantation had an intact endothelium that stained positive for vWF (appears green in the insert). NH lesions formed in IJV after 12 days of ex vivo perfused culture were negative for vWF staining.

Fig. 7. Sunitinib inhibits NH formation in porcine IJV segments maintained in an ex vivo perfused culture. **A** Representative histological images of the tissue cross-sections revealed healthy vein walls in both untreated control and sunitinib (100 nM)-treated vein segments perfused for 12 days. Van Gieson's stain. $\times 10$. **B** The I/M ratio decreased from 0.38 ± 0.24 in the untreated vessels ($n = 3$) to 0.13 ± 0.05 in the sunitinib-treated vessels ($n = 3$; $p < 0.15$). The I/L ratio decreased from 0.45 ± 0.25 to 0.04 ± 0.02 (* $p < 0.05$).

tors of cell-cycle progression, was nearly completely inhibited (fig. 5B). In contrast, the expression of p21 and p27, cyclin-dependent kinase inhibitors that function as antimitogenic regulators, were potently decreased by PDGF-BB stimulation, and this effect was reversed by concentrations of sunitinib as low as 10 nM (fig. 5B). Densitometric analyses of images are shown in online supplementary figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000442977). These observations are consistent with the notion that sunitinib inhibits the action of PDGF-BB by suppressing the expression of promotive regulatory proteins and enhancing the expression of cell-cycle progression inhibitors in vascular SMCs. Phosphorylation of p53, an event that promotes apoptotic cell death, was examined after sunitinib treatment, to assess the toxicity of this drug at pharmacologically relevant concentrations. Sunitinib, even at the maximal dose used to inhibit proliferation (100 nM) failed to induce apoptosis in cultured HASMCs (fig. 5C).

Sunitinib Inhibits Venous NH Formation in ex vivo Perfused Vein Culture

Explanted porcine IJV segments were perfused ex vivo under defined nonphysiological laminar-flow conditions for up to 12 days, or kept in a static-flow condition for comparison. All vein segments cultured ex vivo appeared intact and patent after 12 days. Histological examination showed significant NH formation in the perfused vein segments at 12 days in culture (fig. 6A, B). However, vein segments in static culture did not develop NH (data not shown), suggesting that perfusion per se is an important promoter of NH development. Cells in the NH lesions appeared disorganized, stained positive for smooth-muscle α -actin, and had elevated levels of proliferation as determined by Ki-67 immunostaining (fig. 6C). These histological features are similar to those observed in the NH lesions in our porcine model in vivo. However, in contrast to the in vivo lesions, we did not detect an intact endothelium or any neovessels in the NH lesions (fig. 6D).

Sunitinib (100 nM) markedly inhibited NH lesion formation in the vein segments perfused ex vivo (fig. 7A).

Fig. 8. Low level of apoptosis and cell proliferation in sunitinib-treated vein segments. Apoptotic cells appear bright yellow in representative tissue sections, while blue staining denotes cell nuclei, and the dashed line demarcates the NH lesion from the media (colors refer to the online version only). Both untreated control (**A**) and sunitinib-treated (**B**) vein segments perfused for 12 days showed low levels of apoptosis. Proliferating cells (appearing bright yellow) in the neointima in untreated control (**C**) and sunitinib-treated (**D**) vein segments perfused for 12 days were examined in representative tissue sections. Blue staining denotes cell nuclei. Sunitinib-treated vein samples had less proliferating cells than untreated vein samples.

The mean I/M ratio decreased in sunitinib-treated vein segments, although this difference did not reach statistical significance (0.38 ± 0.24 vs. 0.13 ± 0.05 ; $n = 3$ each; $p < 0.2$; fig. 7B). The mean I/L ratio, which takes into consideration variations in the lumen diameters, was, however, significantly decreased (0.45 ± 0.25 vs. 0.04 ± 0.02 ; $n = 3$ each; $p < 0.05$; fig. 7B). Anti-cleaved caspase-3 and anti-Ki-67 antibodies were used to identify apoptotic and proliferating cells, respectively, in the perfused vein samples. Similarly low levels of apoptotic cell death were seen in the sunitinib-treated and untreated vein samples (fig. 8A, B), indicating that the inhibitory effect of sunitinib on NH formation was not the result of apoptosis. Sunitinib-treated vein samples had fewer proliferating cells than untreated vein samples (2 and 9% of Ki-67-positive nuclei in the representative images in sunitinib-treated and control veins, respectively), consistent with the notion that sunitinib prevents NH formation through inhibiting cell proliferation (fig. 8C, D). Expression of VEGF and PDGF-B in IJV tissues cultured under flow for 12 days without or

with sunitinib was assessed by immunohistochemistry and shown in figure 9. Sunitinib-treated vein samples expressed less VEGF and PDGF-B than untreated vein samples.

Discussion

The purpose of our investigation was to utilize a variety of model systems to assess the properties of sunitinib, which will allow us to better understand the underlying pathophysiology and explore potential pharmacotherapy to prevent NH. In this study, the mechanism of sunitinib's action was examined in cultured arterial and venous SMCs stimulated with PDGF, and in ECs stimulated with VEGF. The selection of sunitinib, an angiogenesis inhibitor, was motivated by the presence of neovessels (fig. 1) and the expression of the growth factors PDGF, VEGF and their respective receptors (fig. 2) in the juxta-anastomotic NH lesions in our porcine AVG model. A recent study by Ishii

Fig. 9. Sunitinib inhibits the enhanced expression of VEGF and PDGF in the veins cultured ex vivo. Expression of VEGF and PDGF-B in fresh (Day 0, D0) EJV (left column) and in the IJV tissues cultured under flow for 12 days (D12) without sunitinib (middle column) and with sunitinib (right column) was assessed by immunohistochemistry. A rust-brown color indicates positive staining for the indicated proteins. Blue structures are hematoxylin-stained nuclei (colors refer to the online version only). Sunitinib-treated vein samples had less VEGF and PDGF-B than untreated vein samples. L = lumen.

et al. [35] reported that sunitinib inhibited PDGF-induced DNA synthesis, proliferation and migration in cultured HASMCs, and inhibited NH formation in a rat model of balloon-injury of carotid arteries. We previously showed that there was a significant difference between cultured arterial and venous SMCs in their response to the PDGFR inhibitor imatinib [36]. Building on this knowledge, here, we investigated the effects of sunitinib on cultured HVSMCs, besides HASMCs, and on explanted porcine whole blood vessel segments cultured ex vivo under flow conditions. An important feature of the ex vivo culture system is that it incorporates a segment of vein, which is the most relevant vessel for studies on venous NH associated with AVG based on clinical observations and previous work in porcine models.

We demonstrated that sunitinib blocked signal transduction downstream of mitogenic RTKs by inhibiting the phosphorylation of key proteins in the MAPK and the PI3K/Akt pathways in cultured SMCs (fig. 5). These changes in signaling protein phosphorylation were accompanied by the reversal of changes in the expression of key cell-cycle regulatory proteins. The effects of sunitinib on the MAPK and PI3K/Akt pathways in vascular SMCs, and especially its effects on p70S6k and rpS6, have not been previously reported. Whether additional pro-NH intracellular pathways are activated in the presence of aberrant NH-promoting conditions will need to be examined in future studies.

It has been proposed that multiple pathogenic factors contribute to NH development after AVG placement [3]. However, the localized nature of NH with strong predis-

position at the venous anastomosis points toward abnormal blood flow as an important pathogenic factor because shunting of arterial blood flow exposes the vein to a highly aberrant hemodynamic environment. Previous biomechanics studies have shown that manipulation of certain hemodynamic parameters in the vein, such as hydrostatic pressure or WSS, can induce pathological vessel remodeling, including wall hypertrophy and NH development [26, 27]. In our study, we explored this concept using an ex vivo model system that reproduced the pathological WSS occurring at the venous anastomosis of AVG in vivo, and observed that aberrant WSS was indeed associated with NH formation in the explanted porcine vein segments (fig. 6). These ex vivo NH lesions resembled the lesions observed in vivo and were also comprised primarily of disorganized, proliferating cells that stained positive for smooth-muscle α -actin [6, 23]. Chronic exposure to low concentrations of sunitinib substantially attenuated NH formation in vein segments in this ex vivo perfused culture system. Of interest, past studies used human saphenous vein segments to demonstrate substantial NH formation under static culture conditions without perfusion [37, 38], which we were unable to reproduce in our porcine jugular veins cultured statically. These earlier studies suggested that mechanical forces may not be necessary stimuli for intimal growth, at least in culture conditions that were adopted using human veins [37, 38]. However, a number of factors including species differences, the anatomical source of the veins and differences in culture methods could have contributed to the discrepant findings. Our re-

sults agree with those reported by Gusic et al. [27] and Jodard et al. [28], in which minimal NH development was observed in porcine saphenous veins cultured under static culture conditions, in contrast to perfused veins.

In our ex vivo model, there was a loss of resident ECs in our cultured veins, which was similar to previous observations [26, 27] and occurred even in the absence of antiproliferative drugs. The EC loss was likely due to the tissue damage during vein explanting or the absence of critical factors that maintain EC viability in our culture system. In all of the static culture experiments that we conducted in parallel to the perfused experiments, none showed an intact endothelium (data not shown). Corroborating our observations, studies by Gusic et al. [27] reported the lack of an intact endothelium monolayer after culturing porcine vein segments for 7 days or longer under abnormal shear stress and/or pressure. Although we expect that improved explanting techniques may minimize this resident EC loss, it is also noteworthy that the role of damaged endothelium (and restoration of the endothelium) on NH pathogenesis is controversial in the current literature and has been the subject of persistent debate [39]. Future investigations are necessary to determine whether and how prevention of endothelium damage may affect the ex vivo culture model.

A notable advantage of the ex vivo perfused culture model is that it permits the investigation of explanted whole-vessel segments in well-defined hemodynamic environments that may cause NH in vivo. At the same time, the whole-vessel segment provides the opportunity for cell migration and cross-talk within the architectural framework of the native vessel that may be critical for vessel wall remodeling and NH development. Prominent features of NH were readily observed in these cultured vein segments

(fig. 6), highlighting the relevance of this model to in vivo events. The requirement for only a small number of animals to provide the necessary blood vessel segments for these ex vivo experiments greatly reduced the costs. An additional advantage of our ex vivo perfusion system is that it is well suited to study the effect of other isolated factors, such as uremia, on the pathogenesis of NH. The study of uremia is of particular interest, since NH has been reported to be present in blood vessels of uremic patients even prior to the creation of a vascular access [40], and uremia appears to potentiate NH development in mouse and rat AV fistula models [41–43]. For example, uremic plasma, specific uremic toxins or isolated blood vessels from uremic animals or patients could be used in this ex vivo model to investigate the effects of uremia on NH development and to study potential anti-NH agents.

In conclusion, sunitinib was demonstrated to be effective in inhibiting several cellular signaling events associated with NH development in AVGs. Furthermore, sunitinib attenuated NH formation induced by nonphysiological mechanical cues in an ex vivo perfused organ culture system. The long-term efficacy and safety of sunitinib therapy in vivo require further evaluation.

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Disclosure Statement

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