c-Kit signaling determines neointimal hyperplasia in arteriovenous fistulae

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Submitted 27 May 2014; accepted in final form 27 August 2014

The arteriovenous (A-V) fistula is the preferred type of vascular access for hemodialysis patients (29). It achieves higher patency rates, has fewer complications than synthetic grafts (12, 29, 31), and has a lower risk of infections than central venous catheters (17, 29). Despite its advantages, A-V fistulae frequently fail to mature or become dysfunctional after successful dialysis sessions, and this occurs primarily due to the development of neointimal hyperplasia (NIH) within the A-V fistula circuit (2, 5, 39), and has a lower risk of infections than central venous catheters (17, 29). Despite its advantages, A-V fistulae frequently fail to mature or become dysfunctional after successful dialysis sessions, and this occurs primarily due to the development of neointimal hyperplasia (NIH) within the A-V fistula circuit (2, 5, 39). Stenosed A-V fistulae can sometimes be salvaged through angioplasty or surgical interventions, but these attempts often result in restenosis or additional complications (32). Therefore, there is an unmet medical need for treatments that prevent NIH and improve A-V fistula function. To this end, it is necessary to better define the factors underlying the pathological remodeling of the A-V fistula wall.

A-V fistula maturation is a dynamic vascular process with multiple factors contributing to the development of NIH. These include vein configuration (22), hemodynamics stress (39), inflammation (20), and preexisting diseases like diabetes (40) and kidney failure (26). The above conditions induce de novo accumulation of growth factors such as VEGF (48), platelet-derived growth factor (41), fibroblast growth factor (42), and transforming growth factor-β (18) and their receptor tyrosine kinases (RTKs) in the outflow vein, which ultimately increase the proliferation, migration, and survival of neointimal cells and the subsequent thickening of the vessel wall. Recently, vascular expression of stem cell factor (SCF) and its receptor c-Kit have also been implicated in A-V fistula NIH (8). c-Kit is a RTK that regulates cancer and stem cell growth and maturation (4). Interestingly, Wang et al. (47) demonstrated that c-Kit and SCF attenuate vascular smooth muscle cell (VSMC) apoptosis and accelerate postinjury NIH in mouse arteries. However, whether c-Kit signaling plays a fundamental role in the adaptive response of the A-V fistula outflow vein to arterial circulation is still unknown.

In the present study, we investigated the role of c-Kit in the pathological remodeling of A-V fistulae. We show that activation of the c-Kit signaling pathway in adventitial and neointimal cells precedes arterialization of the fistula wall. Furthermore, we demonstrate for the first time that blockade of SCF expression and/or its receptor significantly reduces A-V fistula neointima formation in multiple experimental models. Altogether, our work underscores the critical role of c-Kit signaling in the development of NIH after fistula creation.

MATERIALS AND METHODS

Human A-V fistulae and veins. We studied c-Kit expression in 6 A-V fistulae and 6 veins from 12 hemodialysis patients undergoing surgery to receive a two-stage brachiobasilic fistula in the upper arm. The age of patients ranged from 53 to 69 yr old. Proximal basilic vein samples (1–2 mm) were collected at the time of fistula creation (first stage), and fistulae (1–2 mm) were collected 2 cm from the anastomosis site where the clamp was placed during transposition (second stage). This distance minimizes the effects of direct surgical trauma on the anastomosed end of the vein and allowed us to assess c-Kit expression in the juxtaposed segment, which is the area most vulnerable to NIH. M. Tabbara performed surgeries and sample collections. Tissues were rapidly formalin fixed and sectioned for immunohistochemistry. Tissues samples were collected with approval of the Institutional Review Board of the University of Miami and after written consent was obtained from patients.

A-V fistula experimental models. The rat aortocaval fistula was created in 66 male (2–4 mo old) Sprague-Dawley rats, as we have previously described (30). All surgeries were performed under isoflurane anesthesia (Webster Veterinary, Ocala, FL). The fistula was established by anastomosing the distal end of the renal vein to the abdominal aorta after unilateral nephrectomy. In this model, blood circulates from the aorta through the A-V fistula (renal vein) and to
the inferior vena cava. The increased hemodynamic stress resulting from exposure of the renal vein to the arterial circulation induces total venous remodeling (arterialization) as early as 30 days after surgery. To study the temporal expression of c-Kit in the A-V fistula wall, operated rats were euthanized 6 h or 7, 14, or 30 days after surgery (n = 4–8 rats/time point). In 25 control rats, the renal vein was ligated instead of being connected to the aorta after unilateral nephrectomy. In addition, the mouse A-V fistula was created by anastomosing the left common carotid artery to the nearby jugular vein, as previously described (45). These selected functional sequence for in vivo knockdown (1:100, DAKO) was added overnight at 4°C. The Envision G/2 System/AP (permanent red) kit (DAKO) was used to visualize places of c-Kit accumulation within the vascular wall. These sections were mounted in Faramount aqueous mounting medium (DAKO). For immunofluorescence, unspecific binding sites were minimized using Tris-borate saline buffer supplemented with 15% FBS for 20 min. Sections were then incubated overnight with a mix of primary antibodies that included rabbit anti-c-Kit polyclonal antibodies (1:100, Santa Cruz Biotechnology) and mouse anti-human smooth muscle actin clone 1A4 (1:50, Dako) in Tris-borate saline buffer supplemented with 10% FBS. After two washes with PBS for 3 min, bound antibodies were detected with Alexa fluor 488 goat anti-rabbit and Alexa fluor 488 goat anti-mouse (Invitrogen, Carlsbad, CA). Sections were mounted in Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and examined with a confocal scanning laser microscope (Zeiss LSM 510 META, Carl Zeiss Microimaging, Thornwood, NY) in an inverted configuration.

**Molecular analysis.** Total vascular RNA was purified using TRI reagent (Molecular Research Center, Cincinnati, OH). Briefly, rat A-V fistulae and veins were pulverized and mixed with 0.3 ml TRI reagent (Life Technologies). RNA was isolated from the aqueous phase after phenol-chloroform extraction and precipitated with 0.5 volumes of isopropanol and 10 μl of polyacryl carrier (Molecular Research Center). The pellet was washed twice with 70% ethanol. The total RNA yield was determined by spectrophotometry. Rat SCF, c-Kit, matrix metalloproteinase (MMP)-9, and β-actin mRNA were quantified using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI Prism 7500 Fast Real-Time PCR System (96-well plate). Relative gene expression was determined using the ΔΔCt method, where Ct equals threshold cycle (28).

For Western blot, VSMCs (2 × 10⁶ cells/well) were seeded in six-well plates and lysed in RIPA buffer (SantaCruz Biotechnology) supplemented with leupeptin (10 μg/ml), aprotinin (20 μU/ml), PMSF (10 μM), NaF (10 μM), and NaVO₄ (10 μM). Cell lysates were loaded onto QIAshredder homogenizer columns (Qiagen, Valencia, CA) and centrifuged at 9,000 g for 3 min. Total proteins were quantified with the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein extracts were diluted 1:1 (vol/vol) in Laemmli buffer, and 15 μg of protein were loaded on each lane of a 4–12% Tris-glycine gel (Invitrogen). The electrophoresed proteins were then transferred to Amersham Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Blots were incubated with either rabbit anti-SCF polyclonal antibody or mouse anti-β-actin (Sigma-Aldrich) and then developed using the WesternBreeze Chemiluminescent kit (Invitrogen).

**Statistical analysis.** Data are expressed as means ± SE of at least three independent experiments. Two-group comparisons were performed using either a t-test or Mann-Whitney U-test for nonparametrically distributed data. Multiple group statistical analysis was performed with the one-way ANOVA followed by the Bonferroni correction for multiple comparisons. Statistics were calculated with GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

**RESULTS**

*c-Kit* is expressed during the remodeling of human A-V fistulae. We assessed the presence of c-Kit in the venous segment of human A-V fistulae and compared it with veins obtained before anastomosis by immunohistochemistry (Fig. 1,
Sections from veins and A-V fistulae were obtained from 12 patients that underwent surgery for brachiobasilic fistula in the upper arm. Proximal basilic vein samples (control veins) were obtained at the time of fistula creation (n = 6), whereas fistulae were collected during transposition (n = 6). The histopathological analysis revealed increased NIH in A-V fistulae compared with control veins (data not shown). Consistent with this observation, higher numbers of c-Kit-positive cells were observed in the adventitia of A-V fistulae than in veins (Fig. 1E). In addition, the majority of neointimal cells in A-V fistulae but not in veins produced detectable levels of c-Kit, which suggests that differentiated VSMCs/myofibroblasts retained its expression at least during the period of maturation from the time of fistula creation to when samples were obtained. These results also suggest the participation of c-Kit in the remodeling of human A-V fistulae.

**c-Kit signaling in experimental A-V fistulae.** We used a rat model of A-V fistula to further investigate the role played by c-Kit in the development of NIH. This model consists of an aortocaval fistula created by anastomosing the left renal vein to the abdominal aorta after unilateral nephrectomy (30). The experimental controls were renal veins of sham-operated animals that were ligated instead of being connected to the aorta. With this model, we assessed the spatial and temporal pattern of c-Kit-expressing cells in the A-V fistula wall and control veins using immunofluorescence staining for c-Kit and SMA (n = 4–8 rats/time point). Figure 2A shows a representative section of a sham-operated vein demonstrating the absence of NIH and immunoreactivity against c-Kit in cells (in red). Figure 2, B–D, shows the increased c-Kit staining in the time interval from 6 h to 7 days after fistula creation, indicating not only a higher c-Kit-positive cell count but also an upregulation in gene expression. Interestingly, the increased expression of c-Kit in the adventitia correlated with the thickness of A-V fistulae (Fig. 2F) until day 30, when expression of this marker was no longer detected (Fig. 2E). No changes in c-Kit expres-
sion or morphometric measurements were detected over time in ligated veins from sham-operated animals (data not shown).

We further investigated if other players involved in the c-Kit activation pathway were upregulated during the remodeling of the A-V fistula vascular wall. To this end, we assessed the expression of c-Kit’s natural ligand, SCF, and MMP-9 in outflow veins of experimental fistulae using quantitative RT-PCR (Fig. 3A). SCF is expressed on the surface of VSMCs (16), where it becomes a substrate for proteolytic cleavage by MMP-9 (6). The latter protein is then essential for the mobilization of SCF into the extracellular space. Just like c-Kit, SCF and MMP-9 mRNAs were upregulated on day 14 in rat A-V fistulae compared with sham-operated veins (Fig. 3A). In addition, immunofluorescence staining for SCF and SMA in rat A-V fistulae indicated that SCF accumulation mostly colocalized with VSMCs/myofibroblasts in the neointima, although it
was also found at lower levels in the media and adventitia (Fig. 3, B and C). These results demonstrate the existence of active c-Kit signaling during arterialization of the A-V fistula wall.

**CKD increases c-Kit expression in the A-V fistula wall.** We then assessed the impact of CKD, one of the aggravating factors for NIH in A-V fistulae (23), on venous c-Kit expression. To this end, we created fistulae in healthy rats \( n = 8 \) and CKD rats \( n = 8 \) that had received an adenine-rich diet starting at 3 wk before surgery and until euthanization. The dietary delivery of adenine mixed in a casein-based diet caused

**Fig. 3. Stem cell factor (SCF) accumulation in the neointima of rat A-V fistulae.** A: increased expression of c-Kit, SCF, and matrix metalloproteinase (MMP)-9 mRNAs in rat A-V fistulae compared with sham-operated veins at 14 days after surgery. Bars are means ± SE; \( n = 4 \)/group. B and C: immunofluorescence staining for SCF (green) and SMA (red) in the outflow vein of a rat A-V fistula at 14 days postsurgery. Scale bars = 30 \( \mu \)m.

**Fig. 4. Increased c-Kit expression in A-V fistulae under chronic kidney disease (CKD) conditions.** A and B: immunofluorescence staining for c-Kit (yellow) in A-V fistulae from healthy (A) and adenine-induced CKD (B) rats harvested at 21 days postsurgery. Nuclei were counterstained with DAPI (blue). Scale bars = 100 \( \mu \)m. C: histogram showing the percentage of c-Kit-positive cells in the A-V fistula wall of healthy and CKD rats. Bars are means ± SE; \( n = 6–8 \). * \( P < 0.01 \).
tubulointerstitial lesions that led to CKD (23). Fistulae were collected from both healthy and CKD animals at 21 days after creation, when serum creatinine values in CKD rats were 3.25 times higher than in healthy animals (1.08 ± 0.57 vs. 0.33 ± 0.05). The analysis of c-Kit staining in fistula cross-sections revealed a significant elevation of c-Kit expression in outflow veins from CKD animals compared with those from healthy control rats \((P < 0.0001; \text{Fig. 4, A–C})\). These findings warrant further research to reveal the contribution of CKD drivings and worsening factors such as hypertension and uremia to the elevated c-Kit expression in the A-V fistula wall.

Blockade of either c-Kit or SCF in rat A-V fistulae effectively suppresses NIH. To determine the contribution of the c-Kit signaling pathway to the development of NIH, we performed inhibition experiments on the rat model of A-V fistula. We treated rats after A-V fistula creation with either vehicle \((n = 4)\) or 5 mg·kg\(^{-1}\)·day\(^{-1}\) imatinib mesylate orally for 7 days postsurgery \((n = 7; \text{Fig. 5A})\). Imatinib is a tyrosine kinase inhibitor that targets c-Kit, among other RTKs (19). As shown in Fig. 5, B–D, animals treated with imatinib mesylate developed significantly thinner neointimas compared with placebo-treated control animals \((P = 0.0026)\).

Next, we determined whether targeting of SCF using RNA interference before fistula creation in rats prevented NIH. To this end, we initially tested the efficiency of three shRNA-containing lentiviruses in reducing SCF mRNA and protein expression in cultured VSMCs by quantitative RT-PCR and Western blot analysis. shRNAs blunted SCF mRNA and protein levels in VSMCs at different levels compared with the scrambled shRNA control (Fig. 6, A and B). We then proved that VSMCs of renal veins could be efficiently transduced by means of a GFP-encoding lentivirus. This reporter virus was delivered into the lumen of the renal vein before fistula creation while a vascular clamp blocked venous outflow and the proximal end of the vein was temporally closed with a suture loop (Fig. 6C). Figure 6, D–F, shows the positive GFP signal in almost all smooth muscle actin-positive cells in the media of the newly created A-V fistula \((n = 4)\). As expected, the

![Fig. 5. Imatinib mesylate (Gleevec) attenuates neointima formation in rat A-V fistulae. A: experimental outline for the treatment of rats with Gleevec after fistula creation. Gleevec (5 mg·kg\(^{-1}\)·day\(^{-1}\)) was orally administered each day for 1 wk after fistula creation. B and C: representative microphotographs of A-V fistulae from placebo-treated (B) and Gleevec-treated (C) rats harvested at 21 days after surgery. The neointima is indicated by arrows. Scale bar = 100 \(\mu\)m. D: histogram showing the neointimal area corrected by the length of the lumen in placebo-treated \((n = 4)\) and Gleevec-treated \((n = 7)\) rats. Bars are means ± SE. \(*P = 0.0026\) using a two-tailed t-test.](image-url)
delivery of viral particles carrying anti-SCF shRNA to the experimental fistulae (n = 5) effectively prevented NIH development in the outflow vein compared with those treated with the scrambled shRNA control (n = 6; Fig. 6, G–I). These results demonstrate the feasibility of c-Kit inhibitory therapies to control NIH development in A-V fistulae.

Reduced c-Kit activity protects A-V fistulae from NIH. We finally confirmed the essential role of c-Kit signaling in NIH by creating A-V fistulae in c-Kit-deficient mice. The c-Kit<sup>W/W<su>vc</sup> mouse model is a naturally occurring mutant of c-Kit, which produces a protein with impaired tyrosine kinase activity (36). In this model, A-V fistulae were created by anastomosing the
left common carotid artery to the external jugular vein, as previously reported (21). Venous sections were obtained 30 days after surgery and submitted for histopathological analysis. As shown in Fig. 7, A–C, c-Kit-deficient mice developed significantly thinner neointimas compared with wild-type control mice ($P = 0.026$).

**DISCUSSION**

Even after the implementation of the Fistula First Initiative in 2003, our inability to prevent stenosis in A-V fistulae has contributed to a continued dependence on synthetic grafts and central venous catheters for hemodialysis, despite the higher risk of infection and mortality associated with these types of vascular accesses (29). Because of that and the persistently high rates of A-V fistula failures, the initiative has not reduced the costs of medical interventions to salvage stenosed fistulae as it was intended (29). In addition, anticoagulant- and anti-platelet-based pharmacological treatments to promote and maintain patency of this type of vascular access have limited impact (10, 37), in part because they do not target the formation of NIH, which is the underlying cause of fistula stenosis. This study shows for the first time that inhibition of c-Kit, a target for which there is an existing translatable platform for drug development, effectively prevents neointima formation in experimental A-V fistulae in rodents.

c-Kit is a type III RTK that controls the survival, migration, and proliferation of certain cells in response to SCF stimulation (13). In the vasculature, increased c-Kit activity protects cells from apoptosis, which favors neointima formation in injured vessels (47). Inhibition of c-Kit by pharmacological or genetic means has protected arteries from postinjury pathological remodeling (46, 47). In agreement with these studies, we have demonstrated the upregulation of c-Kit occurs during arterialization of the A-V fistula and that its levels are further augmented by experimental CKD conditions. We further showed that it is possible to suppress NIH in A-V fistulae by targeting c-Kit with imatinib mesylate, an RTK inhibitor, or by genetically suppressing this protein’s function in mutant mice. In our case, the timed inhibition of c-Kit in adventitial and neointimal cells from day 0 to day 7 in rats effectively diminished the accumulation of cells in the neointima of newly created fistulae, suggesting a role for this receptor in the differentiation and/or mobilization of myofibroblasts within the vascular wall. Adventitial fibroblasts/myofibroblasts are believed to be one of the main sources of neointimal cells in the A-V fistula wall (33). However, the mechanisms by which c-Kit signaling modifies vein remodeling and determines NIH require further investigation.

In agreement with previous results, we also demonstrated the accumulation of SCF in the neointima of A-V fistulas. SCF is mainly produced in the vasculature by endothelial cells and VSMCs (16, 34, 47), and its levels increase significantly in pathogenic stages (47). This ligand exists in both membrane-bound and soluble forms, and their production is regulated by...
alternative splicing mechanisms (27) or through the proteolytic action of MMP-9 (6, 16), which we and others (16) have also found to be upregulated in the fistula wall. Both forms of SCF play a role in the activation of c-Kit signaling, with potent effects on cell survival, migration, proliferation, and differentiation (27). Soluble SCF is able to act as a chemotactic, autocrine, and paracrine agent (14, 35), whereas the membrane-bound form can directly recruit cells through intercellular binding (11). Our experiments with anti-SCF shRNAs demonstrated not only the critical role of this factor in the activation of vascular c-Kit during arterialization of the A-V fistula but also showed the possibility of blunting SCF accumulation as a prophylactic strategy to prevent NIH and improve the fistula maturation process.

One of the most intriguing findings in our study was the presence of c-Kit in A-V fistula adventitial cells as early as 6 h after surgery. Expression of this receptor augmented in parallel with increased thickening of the neointima until NIH reached its maximum at day 30, which suggests the involvement of c-Kit-positive cells in the pathological evolution of the fistula wall. The presence of c-Kit may reflect progenitor cell activity in the vascular wall, as this RTK is considered a marker for stem cell identification. The existence of stem cells in the fistula wall was recently suggested by Caplice et al. (8), who described this type of cells as components of adventitial microvessels in the rat A-V fistula. A number of studies have proposed bone marrow-derived progenitor cells as the source of neointimal cells in stenotic blood vessels after arterial injury (38, 44, 46), but their contribution seems less pronounced in the case of venous hyperplasia, as clearly demonstrated in three independent studies (9, 21, 43). The present work highlights the critical role played by this pathway in A-V fistula remodeling. Therefore, finding the origin of neointimal VSMCs/myofibroblasts in A-V fistula NIH is an important factor to consider in the design of viable treatment options.

The limitations of this study include the small number of human A-V fistula and vein samples as well as the absence of important CKD-associated vascular stressors in our experimental models. For example, high flow rates imparted by the hemodialyzer, and the contact of blood with artificial surfaces in the extracorporeal circuit are all related with inflammation, hypercoagulability states, and negative vascular remodeling (15, 25). This study did not determine the contribution of either uremia and hypertension to the elevated c-Kit expression observed in A-V fistulae of CKD rats. Nevertheless, despite these limitations, our study demonstrates, using different molecular targets, that both systemic and local inhibitions of the c-Kit signaling pathway are effective approaches to prevent NIH in experimental A-V fistulae in rodents. We also show that these therapies are most feasible and needed at the time of surgery (for the local delivery of shRNAs) or during the active remodeling period after fistula creation.

In conclusion, despite the encouraging results of our study, further research is necessary to determine whether a c-Kit-targeted approach would be sufficient to prevent postoperative NIH and improve fistula maturation in humans. Both NIH and the degree of vasoconstriction (negative remodeling) contribute to postoperative stenosis, leading to primary and secondary fistula failure. Therefore, combinatorial therapies that block c-Kit activity and halt NIH while simultaneously increasing positive (outward) remodeling of the outflow vein may be important to improve the maturation process. Of note, these therapies will be limited to prevent postsurgical NIH and not preexisting neointimal lesions in the vein. The existence of preexisting NIH in veins of hemodialysis patients has been recently described, and its presence is not associated with A-V fistula outcomes (1, 3, 24).

GRANTS
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-098511 (to R. I. Vazquez-Padrón, F. Andreopoulos, and L. H. Salman).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


