Use of Brilliant Blue FCF during vein graft preparation inhibits intimal hyperplasia

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Background: **Intimal hyperplasia remains the primary cause of vein graft failure for the 1 million yearly bypass procedures performed using human saphenous vein (HSV) grafts. This response to injury is caused in part by the harvest and preparation of the conduit. The use of Brilliant Blue FCF (FCF) restores injury-induced loss of function in vascular tissues possibly via inhibition of purinergic receptor signaling. This study investigated whether pretreatment of the vein graft with FCF prevents intimal hyperplasia.**
Methods: **Cultured rat aortic smooth muscle cells (A7r5) were used to determine the effect of FCF on platelet-derived growth factor-mediated migration and proliferation, cellular processes that contribute to intimal hyperplasia. The effectiveness of FCF treatment during the time of explantation on preventing intimal hyperplasia was evaluated in a rabbit jugular-carotid interposition model and in an organ culture model using HSV.**
Results: **FCF inhibited platelet-derived growth factor-induced migration and proliferation of A7r5 cells. Treatment with FCF at the time of vein graft explantation inhibited the subsequent development of intimal thickening in the rabbit model. Pretreatment with FCF also prevented intimal thickening of HSV in organ culture.**
Conclusions: **Incorporation of FCF as a component of vein graft preparation at the time of explantation represents a potential therapeutic approach to mitigate intimal hyperplasia, reduce vein graft failure, and improve outcome of the autologous transplantation of HSV. (J Vasc Surg 2016;64:471-8.)**

Clinical Relevance: **Saphenous veins remain the most commonly used conduits for bypass procedures. Current surgical harvest and vein graft preparation induces injury to the conduits and promotes development of intimal hyperplasia, arguing for less injurious means to preserve vein graft function during the explantation period. Brilliant Blue FCF presents a potential therapeutic to be included as part of the vein graft preparation.**

Human saphenous vein (HSV) grafts used in coronary artery or peripheral bypass procedures often develop intimal hyperplasia, leading to narrowing of the lumen and vein graft failure. The leading cause of vein graft failure is intimal hyperplasia, a process characterized by pathologic narrowing of the lumen, graft stenosis, and ultimately, graft failure.[1] Intimal hyperplasia results from a cascade of molecular and cellular events that are triggered by injury, remains a significant limitation of vascular bypass procedures, and results in substantial morbidity, reintervention, limb loss, myocardial infarction, and death. Unfortunately, there is no effective strategy to prevent this poor outcome, and this clinical need remains unmet by an effective preventive strategy or therapeutic agent.

The period during which the vein graft is explanted provides a therapeutic window of opportunity during which exposure of therapeutics to the conduit is maximized and systemic exposure is limited. Although gene therapy and drug-eluting stents have been widely explored, there are few data regarding the ability of a single dose of a therapeutic at the time of explantation to reduce development of intimal hyperplasia. Pretreatment of grafts with thrombin inhibitors (argatroban and lepirudin)[2]; desferrioxamine manganese, a cell-permeable free radical scavenger and a superoxide dismutase mimetic[3]; vascular endothelial growth factor, an angiogenic and endothelial-protective growth factor[2]; recombinant inhibitor of the tissue factor pathway[2]; or inhibitors of the mitogen-activated

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protein kinase pathways that regulates migration and inflammatory responses (a MK2 kinase inhibitory peptide[4] and the extracellular signal-regulated kinase 1/2 inhibitor U0126),[5] resulted in reduction in intimal thickness in small-animal bypass grafting models. These data suggest that intervention at the time of explantation and before implantation of the transplanted vein graft may significantly affect the subsequent outcome of the bypass procedure. However, no treatment has affected vein graft failure in the diverse milieu of human tissue in clinical studies.

We previously demonstrated that the surgical vein harvest preparation of HSV leads to impairment in physiologic function, increased oxidative stress, and enhanced neointimal thickening.[6] The common practice of marking the vein graft with dye (typically with off-label use of surgical skin marking pens) for orientation purposes is particularly injurious.[7] We recently identified Brilliant Blue FCF (FCF), a food coloring dye, as a nontoxic alternative to surgical skin marker.[8] We showed that FCF did not impair physiologic function, enhanced endothelial-dependent relaxation, and reduced intimal thickening in vitro. Additionally, we demonstrated that FCF is an antagonist of the purinergic $P2X_7$ receptor.[9]

Extracellular purines (adenosine, adenosine 5′-diphosphate, and adenosine 5′-triphosphate [ATP]) are important signaling molecules exerting numerous biologic effects by activation of cell-surface receptors known as purinergic P2X receptors. The $P2X_7$ receptor isoform is expressed in vascular smooth muscle cells[10,11] and mediates numerous effects, including apoptosis, production of inflammatory mediators, and platelet activation.[10,11] The $P2X_7$ receptor has been described as a "death receptor" because its activation by extracellular ATP mediates the production of proinflammatory cytokines,[12] increased activity of caspase-1, -3, and -8,[13] increased p38 mitogen-activated protein kinase activity,[14] apoptosis,[14,15] oxidative stress,[16] and cell membrane pore formation that allows passage of large molecules up to 900 Da and causes cytolysis under conditions of prolonged stimulation.[17] The $P2X_7$ receptor has been implicated in a diverse array of pathologic states and cardiovascular diseases such as atherosclerosis[18] and perivascular inflammation.[19] Purinergic receptors are gaining increasing attention as possible therapeutic targets for treatment of a variety of diseases; for example, $P2X_7$ inhibition has been demonstrated to improve outcomes after spinal cord injury.[20]

On the basis of the well-established role the $P2X_7$ receptor plays in pathophysiologic processes in a variety of tissues, we hypothesized that this receptor mediates cellular dysfunction in vascular tissue and that treating the graft with FCF during the time of explantation would attenuate the development of intimal hyperplasia. In this study, we examined the effect of FCF on migration and proliferation of vascular smooth muscle cells, two hallmark events that are important in the complex pathophysiology of intimal hyperplasia. We then evaluated the effectiveness of acute pretreatment with FCF in preventing the subsequent development of neointimal thickening in a rabbit interposition graft model and in an organ culture model using HSV.

METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo), unless specified otherwise.

Measurement of vascular smooth muscle cell migration. Smooth muscle cell (A7r5; American Type Culture Collection, Manassas, Va) migration was determined using a scratch assay, as previously described.[21] A7r5 rat aortic smooth muscle cells were used based on the well-validated nature of this model system for use in the study of neointimal hyperplasia in vein grafts.[22] Cells were cultured in six-well dishes, allowed to grow to 80% confluence in Dulbecco Modified Eagle's Medium supplemented with 10% fetal bovine serum, and then serum starved for 24 hours. A sterile pipet tip was used to scrape a straight line down the well, and cells were left untreated or were pretreated with FCF (25 and 50 μM) for 2 hours in the serum-deprived medium. Previous studies have shown that FCF at a dose of 50 μM is effective at enhancing endothelial function and inhibiting a 2′-& 3′-O-(4-benzoyl-benzoyl)-ATP (Bz-ATP)-induced contraction in HSV in a muscle bath.[10,11] A 2-hour interval for the treatment arm with FCF was used as an approximation of the duration of time vein grafts would be ex vivo before reconstruction. Cells were treated with platelet-derived growth factor (PDGF, 20 ng/mL; Life Technologies, Grand Island, NY) in serum-free medium for 48 hours. Three photographs were taken per well at 0, 24, and 48 hours on a Axiovert 200M (Carl Zeiss Co, Oberkochen, Germany) epifluorescence microscope at original magnification ×40, and the number of cells that invaded the scratch was determined.

Measurement of vascular smooth muscle cell proliferation. A7r5 cells were cultured in 96-well plates and allowed to grow to 60% confluence as previously described.[23] After serum starvation for 24 hours, cells were left untreated or were pretreated with FCF (25-100 μM) for 1 hour before PDGF treatment (5 ng/mL) for 24 hours. Previous studies have shown FCF (50 uM) treatment for 2 hours is effective at enhancing endothelial function.[10] However, subsequent work demonstrated that 1 hour was equally efficacious. Therefore, 1 hour for the treatment arm with FCF was used in these studies. Proliferation was measured by the MTT assay. In this assay, the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is converted to insoluble purple formazan by viable cells that is measured by a spectrophotometer. Relative absorbance at 595 nm compared with untreated cells was reported. Each data point represents an average of at least six wells and averaged for each assay ($n = 6$).

In vivo rabbit carotid interposition model. Vein bypass grafts were constructed and interposed into the common carotid arteries with an anastomotic cuff technique as previously described.[24] Briefly, external jugular veins (EJVs) were harvested (3.0-4.0 cm in length) from male New Zealand White rabbits (3.0-3.5 kg) for creation

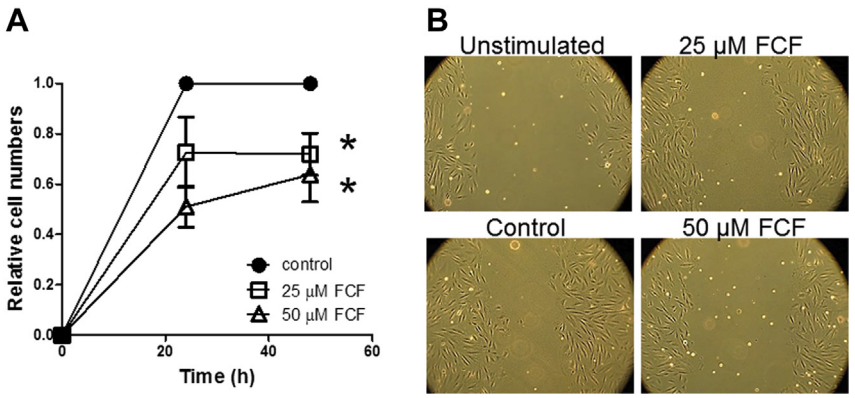

Fig 1. Brilliant Blue FCF (*FCF*) inhibited platelet-derived growth factor (PDGF)-mediated migration in rat vascular smooth muscle cells. Migration of A7r5 cells was measured in a scratch assay in six-well plates. Cells were pretreated without (control) or with FCF (25 and 50 μM) before stimulation with PDGF. **A,** Cells invading the scratch were counted after 24 and 48 hours. Results are reported as relative increase in cell number compared with the control ($n \geq 4$). The *range bars* show the standard error of the mean. *$P < .05$. **B,** Photomicrographs of representative experiment at 0 (*unstimulated*) and 48 hours (original magnification ×40).

of a reversed interposition graft into the common carotid artery. EJV ends were passed through polymer cuffs (Terumo Medical, Elkton, Md), everted, and fixed with 6-0 silk. The EJV grafts were stored in heparinized PlasmaLyte (HP; 10 U heparin/mL PlasmaLyte; control) or HP containing FCF (50 μM) for 60 minutes. An intravenous heparin bolus (250 U/kg) was administered 3 minutes before carotid cross-clamp and arteriotomy. Vein graft cuffs were inserted and secured into the artery with 2-0 silk.

Consistent with prior studies using this model system,[24] vein grafts were harvested at 28 days after implantation. The grafts were systemically perfusion fixed in situ with 10% neutral-buffered formalin, excised, divided into four segments, embedded in paraffin, sectioned, and stained using the Verhoeff-Van Gieson stain for morphometric analysis. Measurements of intimal and medial thickness were made on transverse sections of each vessel using a Axiovert 200M microscope (Carl Zeiss) with a computerized image analysis system using Zeiss software and Photoshop (Adobe, San Jose, Calif), as described previously.[25]

HSV procurement. HSV were obtained after approval from the Institutional Review Boards of Vanderbilt University Medical Center and the Tennessee Valley Veterans Affairs Medical Center from patients undergoing coronary artery bypass procedures. The method of vein harvest (open or endoscopic) was performed at the discretion of the surgical team, and the vein segments used were otherwise not manipulated by the surgical team. HSV segments were transported to the laboratory in HP for experimentation ≤30 minutes of collection. Areas showing visible signs of injury were not used in experimentation.

HSV organ culture. HSV organ culture was used because it is demonstrated to be a representative model in reproduction of cellular events occurring in the intimal hyperplastic lesion.[26] Rings (1-2 mm in width) were cut from HSV segments. Two rings were placed in 10% neutral-buffered formalin to measure basal (preculture) intimal thickness. Additional rings were left untreated or were treated with FCF (50 μM) in organ culture medium (Roswell Park Memorial Institute 1640 medium supplemented with 30% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin) for 2 hours. The rings were maintained in organ culture medium in the absence of FCF for 14 days at 37°C in an atmosphere of 5% CO_2 in air. After 14 days, the rings were fixed in 10% formalin, embedded in paraffin, sectioned, and stained using Verhoeff-Van Gieson stain for morphometric analysis as described above. Data were presented as the percentage change in the cultured rings compared with the preculture rings from each patient.

Statistical analysis. Data are reported as mean ± standard error of the mean. Paired *t*-tests were conducted to determine the significance (*P* value) of each experiment. $P < .05$ was considered statistically significant.

RESULTS

FCF inhibited rat vascular smooth muscle cell migration. Migration and proliferation are hallmark cellular events associated with intimal hyperplasia. To determine the mechanism of action of FCF, the effects of FCF pretreatment on migration in a smooth muscle cell line, A7r5, were examined. In a scratch assay, a trend of reduction in migration was observed after 24 hours of FCF pretreatment ($P = .18$; Fig 1). After 48 hours, FCF pretreatment significantly reduced PDGF-induced migration by 28% ± 8% at 25 μM and 36% ± 11% at 50 μM, compared with cells stimulated with PDGF alone (25 μM [$n = 5$], $P = .04$; 50 μM [$n = 7$], $P = .03$; Fig 1).

FCF inhibited rat vascular smooth muscle cell proliferation. PDGF induced proliferation of A7r5 cells by 20.3% ± 0.03% relative to untreated controls

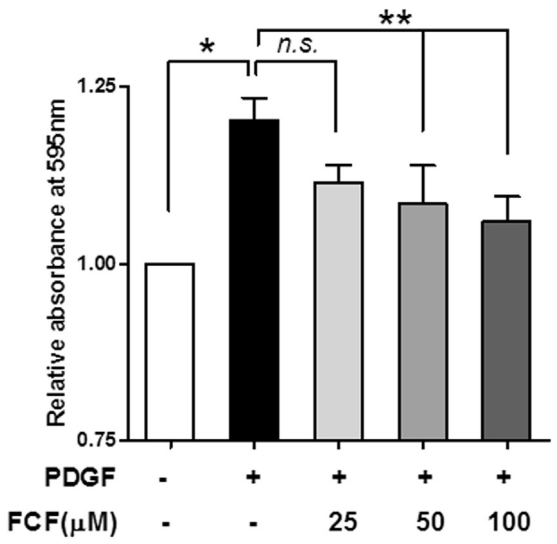

Fig 2. Brilliant Blue FCF (*FCF*) inhibited platelet-derived growth factor (*PDGF*)-mediated proliferation in rat vascular smooth muscle cells. Proliferation of A7r5 cells was measured using a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 96-well plates. Cells were pretreated without (control) or with different concentrations of FCF before PDGF stimulation. Each treatment was performed in at least six wells and averaged for each assay. Results are reported as relative increase in absorbance compared with the unstimulated control (n = 6). The range bars show the standard error of the mean. *$P < .05$, **$P < .005$, *n.s.,* nonsignificant.

($P = .001$). FCF pretreatment significantly reduced PDGF-induced proliferation of A7r5 cells in a dose-dependent fashion by 65% ± 21% at 50 μM and 72% ± 14% at 100 μM compared with PDGF-stimulated cells as measured by the MTT assay (n= 6, $P = .03$ and $P = .01$, respectively; Fig 2).

FCF reduced development of intimal hyperplasia in a rabbit carotid interposition model. Because FCF inhibits migration and proliferation of smooth muscle cells (Figs 1, *A* and 2), we hypothesized that treating vein grafts with FCF would reduce intimal hyperplasia in vivo. We treated the grafts at the time of explantation because this is therapeutically possible in the operating room. Bilateral carotid interposition grafts were constructed in a rabbit model using EJV conduits (Fig 3). The development of intimal thickening was significantly reduced in grafts stored in FCF (50 μM)-containing HP during a 60-minute explantation period compared with control grafts (56.66 ± 7.05 μm vs 119.2 ± 30.25 μm [n = 13]; $P = .03$; Fig 3, *B*). The difference in the medial thickness between the two groups was not significant (96.6 ± 26.4 μm vs 131.4 ± 60.9 μm; $P = .09$). The intimal-to-medial ratio was significantly lower in grafts treated with FCF than in control grafts (0.54 ± 0.08 vs 0.79 ± 0.14; $P = .02$; Fig 3, *C*).

FCF pretreatment reduced intimal thickening in cultured HSV. Because pretreatment with FCF reduced intimal thickness in the rabbit interposition graft model (Fig 4), we next determined if pretreatment with FCF would be effective in attenuating the development of HSV using an organ culture model. HSV segments were left untreated (control) or were pretreated with FCF (50 μM) for 2 hours before placement in organ culture. After culturing for 14 days in the absence of FCF, intimal thickening was significantly reduced in segments of HSV pretreated with FCF compared with untreated tissues (15.8% ± 13.6% vs 67.1% ± 24.6%; $P < .05$; Fig 4), suggesting that treatment with FCF at the time of vein graft explantation may have an inhibitory effect on neointimal thickening ex vivo in HSV tissues.

DISCUSSION

The per-patient vein graft failure rate in the PRoject or Ex-vivo Vein graft ENgineering via Transfection (PREVENT) III (peripheral) and PREVENT IV (coronary bypass) studies was 39% and 45%, respectively, at 12 to 18 months.[27,28] The most common cause of vein graft failure is intimal hyperplasia, a process leading to progressive stenosis or occlusion. No pharmacologic or genetic therapeutic interventions have been translated into successful therapeutic agents, despite the efforts of numerous clinical trials. Recent reports analyzing the PREVENT IV trial data demonstrated that lower vein graft failure rates were associated with HSV stored in buffered solution compared with nonbuffered, acidic, normal saline, highlighting the importance of intraoperative surgical preparation techniques.[29] It follows that there is a therapeutic window of opportunity at the time of explantation to prevent the early activation of pathways that instigate the development of intimal hyperplasia.

Comparing freshly isolated, unmanipulated HSV with cognate segments obtained after intraoperative preparation, we previously demonstrated significant impairment in functional responses and increases in oxidative stress that correlated with enhanced intimal thickening after organ culture in HSV that were subjected to current preparation techniques. These findings argue that the technical elements of current vein graft preparation techniques remain injurious. Vein graft marking with ink remains a nearly universal practice used with the intention of maintaining graft orientation by preventing kinking, thereby preventing technical obstruction of flow after implantation into the arterial circulation.[30] The off-label use of surgical skin markers gained popularity, probably in part due to their availability as ready-to-use sterile pens and safety for use on skin. However, gentian violet, the dye contained in surgical skin markers, has been known to be clastogenic, damaging chromosomes.[31] Shoemaker et al[32] subsequently showed that gentian violet reduces sodium nitroprusside-induced relaxation in HSV. In addition, surgical skin markers contain 50% isopropanol as a solvent, a chemical fixative that is converted to acetone in vascular tissue by an endogenous alcohol dehydrogenase.[33] Cell exposure to alcohol or acetone fixation results in loss of integrity of cell membrane and intracellular structures.[34]

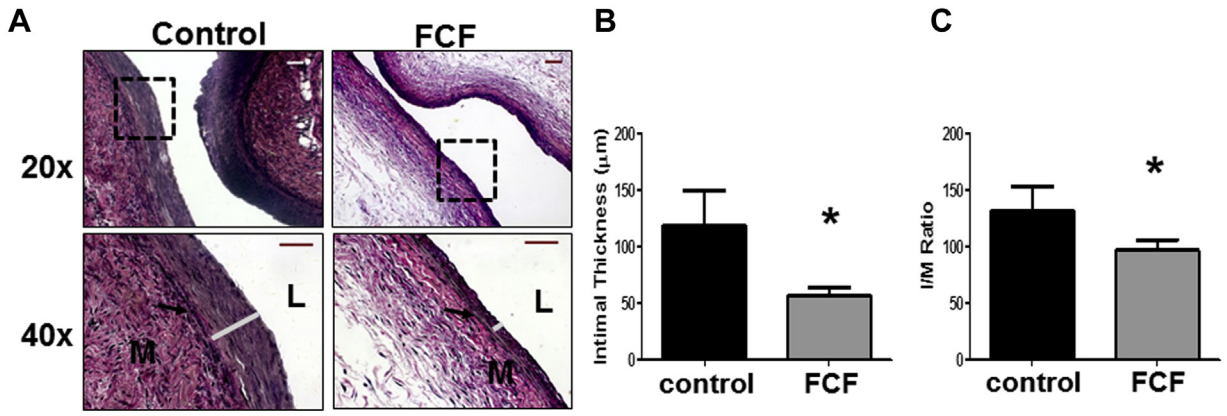

Fig 3. Brilliant Blue FCF (*FCF*) treatment during graft explantation reduced intimal hyperplasia in the rabbit carotid interposition graft model. The external jugular veins (EJVs) from rabbits were stored in heparinized PlasmaLyte (HP) without (control) or supplemented with 50 μM FCF during a 60-minute explantation period before implantation into the carotid artery. Grafts were harvested after 28 days and stained to visualize the elastic lamina. Intimal and medial thickness were measured. **A,** Representative grafts stained with Verhoeff-Van Gieson in control or FCF-treated animals. The *boxed areas* in the top row (original magnification ×20) are shown in the bottom row (original magnification ×40). Scale bar = 50 μm. *L,* Lumen; *M,* media. The *arrows* indicate internal elastic lamina, and the *white lines* indicate the intimal thickness. **B,** Intimal thickness and **(C)** intimal-to-medial thickness (*I/M*) ratio were determined (n = 13). The range bars show the standard error of the mean. **P* < .05.

Collectively, use of surgical skin marker results in chemical fixation of the conduit, causing extensive cell death.

FCF is a nontoxic dye that can be used for vein graft marking.[10] In this investigation, we demonstrated that FCF pretreatment reduced PDGF-induced migration and proliferation in the rat aortic smooth muscle cells, A7r5, (Figs 1 and 2) and prevented neointima formation in vivo and in vitro in two different models of intimal hyperplasia (Figs 3 and 4), suggesting that FCF modulates pathways central to the pathogenesis of intimal hyperplasia. We previously demonstrated that FCF is nontoxic to vascular smooth muscle and endothelium based on preserved smooth muscle contractility and improvement in endothelial-dependent relaxation when used at the same dose.[10,11] FCF has also been shown to enhance endothelial function and restore contractile function in injured HSV and porcine saphenous veins.[11] Thus, FCF is not only nontoxic but has pharmacologic properties that may be beneficial to the vein graft.

Pathophysiologic activation of the $P2X_7$ receptor in vascular smooth muscle is a plausible mechanistic mediator of vein graft dysfunction and intimal hyperplasia. Compared with other P2X receptor subtypes, significantly higher ATP concentrations are required for $P2X_7$ receptor activation.[35,36] Although such high ATP concentrations are typically not produced under physiologic conditions, moderate levels of extracellular ATP may play a role in mechanotransduction in a variety of tissues.[36] Moreover, significant release of ATP occurs from stressed, ischemic, hypoxic, or traumatized tissues.[35,36] The $P2X_7$ receptor is expressed on HSV smooth muscle.[12] Vascular endothelium has been shown to be an important source for ATP production, which may act on vascular smooth muscle cells in a paracrine fashion,[37] particularly in response to inflammation, hypoxia, and alterations in shear.[38,39] Therefore, extracellular ATP is produced by multiple sources in blood vessels during physiologic and pathophysiologic conditions. Only brief exposure to high concentrations of extracellular ATP is necessary for irreversible cell death to occur.[35] The process of HSV autotransplantation favors production of extracellular ATP via several of the mechanisms described above.

This investigation suggests that early vein graft injury occurring during graft preparation represents a sufficient stimulus for the subsequent development intimal hyperplasia and that therapeutic intervention with a 1-hour duration of FCF treatment at the time of explantation mitigates the subsequent development of intimal hyperplasia. This work represents a previously unexplored area of investigation into a therapeutic target for the prevention of intimal hyperplasia. We have demonstrated the efficacy of FCF treatment in a highly reproducible well-described in vivo model system using rabbit carotid interposition grafts. Moreover, we have demonstrated that FCF prevents intimal hyperplasia in our whole-tissue organ culture model using heterogeneous HSV samples obtained from patients at risk for peripheral artery disease, thereby adding further evidence that this compound holds promise as a therapeutic agent in human subjects.

Although the mechanisms of action of FCF in vascular tissue are not completely known, it is conceivable FCF modulates the $P2X_7$ receptor signaling pathway, which may play a previously undescribed role in the early

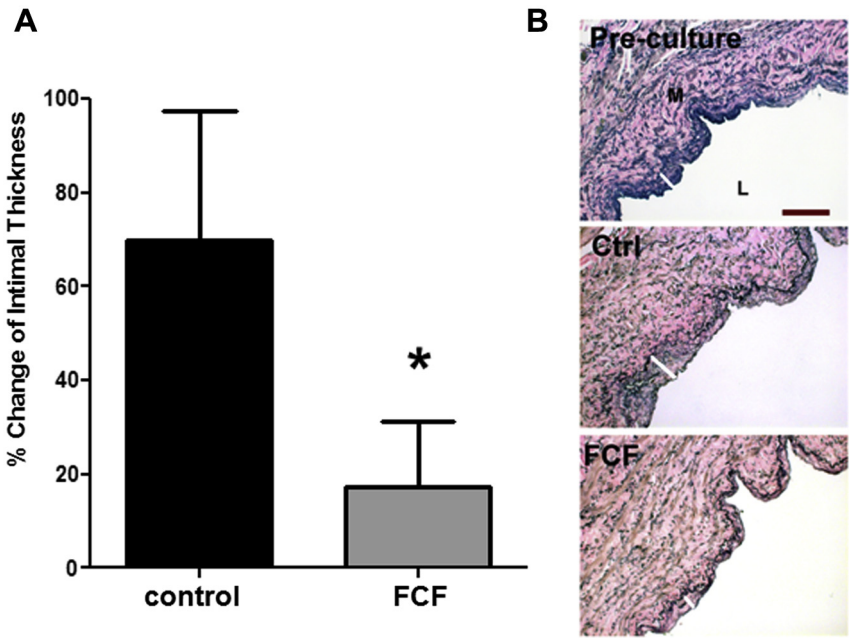

Fig 4. Brilliant blue FCF (*FCF*) pretreatment reduced the intimal thickness of human saphenous vein (HSV) in organ culture. HSV rings were left untreated (control) or pretreated with FCF (50 μM) for 2 hours before culture in Roswell Park Memorial Institute medium supplemented with 30% fetal bovine serum for 14 days. Veins were stained to visualize the elastic lamina, and intimal layer thickening was measured. **A,** Results are presented as the relative change in intimal thickness compared with preculture rings (n = 8). The *range bars* indicate the standard error of the mean. **P* = .016. **B,** Representative Verhoeff-Van Gieson stained HSV. The *white lines* indicate the intimal thickness. Scale bar = 500 μm. *L,* Lumen; *M,* media.

responses to preparation-induced injury of vein graft. $P2X_7$ receptor inhibition during the process of vein graft preparation shows promise as a therapeutic strategy in the prevention of vein graft failure. The use of a nontoxic, water-soluble dye may in part mitigate preparation induced injury, and the FCF dye may have salutary pharmacologic effects.

This study has some potential limitations. The exact molecular mechanism by which FCF reduces migration and proliferation of vascular smooth muscle cells remains to be elucidated. Although migration and proliferation of smooth muscle cells are key events in the formation of neointima, mechanisms involving other cell types pertinent to the intimal hyperplastic response warrant further study, including endothelial cells, adventitial fibroblasts, and inflammatory cells. The culturing medium for HSV contains serum to induce intimal hyperplasia; however, the organ culture model lacks in vivo elements (pressure, flow, and exposure to blood components) that also contribute to neointimal thickening.

Further studies are also needed to determine the dose ranging efficacy of FCF in preventing intimal hyperplasia and the ideal timing of administration. The efficacy of FCF should be evaluated in large-animal models that have similar vessel caliber to that of HSV, which will allow recapitulation of typical intraoperative manipulation used for humans.

CONCLUSIONS

FCF represents a nontoxic alternative for intraoperative vein graft marking for conduit orientation and appears to have beneficial pharmacologic effects in inhibiting intimal hyperplasia in vivo and in vitro. Future investigation of the mechanisms by which FCF reduces intimal hyperplasia will further our understanding of the complex pathophysiology of the process of intimal hyperplasia and its prevention. Our findings suggest that $P2X_7$ receptor signaling may represent an important and previously unrecognized component of vein graft failure.

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AUTHOR CONTRIBUTIONS

Conception and design: MO, KS, KH, CB, JC
Analysis and interpretation: KH, MO, JS, PK, CB, JC
Data collection: KH, MO, IV, KS, JS, PK
Writing the article: MO, CB, JC
Critical revision of the article: KH, MO, KS, IV, JS, PK, CB, JC

Final approval of the article: KH, MO, KS, IV, JS, PK, CB, JC
Statistical analysis: KH, JS, JC
Obtained funding: MO, IV, CB, JC
Overall responsibility: JC

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