Brilliant blue FCF is a nontoxic dye for saphenous vein graft marking that abrogates response to injury

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Background: **Injury to saphenous vein grafts during surgical preparation may contribute to the subsequent development of intimal hyperplasia, the primary cause of graft failure. Surgical skin markers currently used for vascular marking contain gentian violet and isopropanol, which damage tissue and impair physiologic functions. Brilliant blue FCF (FCF) is a nontoxic dye alternative that may also ameliorate preparation-induced injury.**

Methods: **Porcine saphenous vein (PSV) was used to evaluate the effect of FCF on physiologic responses in a muscle bath. Cytotoxicity of FCF was measured using human umbilical venous smooth muscle cells. Effect of FCF on the development of intimal hyperplasia was evaluated in organ culture using PSV. Intracellular calcium fluxes and contractile responses were measured in response to agonists and inhibitors in rat aorta and human saphenous vein.**

Results: **Marking with FCF did not impair smooth muscle contractile responses and restored stretch injury-induced loss in smooth muscle contractility of PSV. Gentian violet has cytotoxic effects on human umbilical venous smooth muscle cells, whereas FCF is nontoxic. FCF inhibited intimal thickening in PSV in organ culture. Contraction induced by 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate and intracellular calcium flux were inhibited by FCF, oxidized adenosine triphosphate, KN-62, and brilliant blue G, suggesting that FCF may inhibit the purinergic receptor $P2X_7$.**

Conclusions: **Our studies indicated that FCF is a nontoxic marking dye for vein grafts that ameliorates vein graft injury and prevents intimal thickening, possibly due to $P2X_7$ receptor inhibition. FCF represents a nontoxic alternative for vein graft marking and a potentially therapeutic approach to enhance outcome in autologous transplantation of human saphenous vein into the coronary and peripheral arterial circulation. (J Vasc Surg 2016;64:210-8.)**

Clinical Relevance: **Saphenous veins remain the most commonly used conduits for bypass procedures. Current use of surgical skin markers to orient conduits impairs physiologic functions of the vein grafts. Brilliant blue FCF represents an alternative marking dye that has pharmacologic properties as well as a potential therapeutic approach to prevent vein graft injury and to enhance outcome.**

Approximately 1,000,000 aortocoronary and peripheral vascular bypass procedures are performed annually using human saphenous vein (HSV). However, outcomes from these procedures remain limited by high rates of vein graft failure, with a per patient rate of 39% and 45% at 1 year in the Project of Ex-Vivo vein graft Engineering via Transfection (PREVENT) III and IV trials of infrainguinal and aortocoronary bypass,[1,2] respectively. The leading cause of vein graft failure is intimal hyperplasia,[3] a process characterized by pathologic narrowing of the lumen, graft stenosis, and ultimately graft failure,[4] leading to substantial morbidity, reintervention, limb loss, myocardial infarction, and death. Despite significant efforts to prevent intimal hyperplasia, no therapeutics, techniques, or devices have been demonstrated to prevent this process in humans.

HSV undergoes a series of surgical manipulations during the time of explantation to be prepared for implantation into the arterial circulation. Common intraoperative vein graft preparation includes warm ischemia times in nonbuffered, nonphysiologic storage solutions (eg, normal saline)[5]; marking with a surgical skin marker for orientation; and mechanical injury, such as stretching and traction during harvest and pressure distention. These current means of vein graft preparation are injurious to the conduits, resulting in cellular dysfunction and increased oxidative stress, and promote the development of intimal hyperplasia of HSV.[6-9] Collectively, less injurious means of preparing HSV before autologous transplantation may improve outcomes of the procedures.

The off-label use of surgical skin markers to prevent twisting and kinking on implantation critically impairs smooth muscle and endothelial function in HSV.[8] These markers generally contain 10% gentian violet (GV) with 50% isopropanol (IPA) as the solvent. These dyes have been shown to reduce vascular function[10,11]; however, they continue to be used for marking conduits. We previously demonstrated that the food dye brilliant blue FCF

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This study was supported by NIH R01HL70715-09 and a VA Biomedical Laboratory Research and Development Grant (C.B.) and NIH R01HL105731-01A1 (J.C.-F.).

Author conflict of interest: C.B and J.C.-F. disclose that they have a financial relationship with VasoPrep Surgical.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214

http://dx.doi.org/10.1016/j.jvs.2014.12.059

(FCF) enhances endothelium-dependent relaxation in HSV and restores contractility in HSVs that were otherwise considered functionally nonviable, possibly by inhibiting the purinergic receptor $P2X_7$ ($P2X_7R$).[12] FCF, a highly water soluble dye, is structurally related to brilliant blue G (BBG), which has been shown to ameliorate stretch-induced injury in the spinal cord through $P2X_7R$ antagonism.[13] In this current investigation, we hypothesized that FCF can be used as a nontoxic alternative for vein graft marking and restores stretch-induced loss of physiologic function in a porcine model. We compared the biocompatibility of FCF and other marking dyes on physiologic function as well as the development of intimal hyperplasia in an organ culture model. The findings of these studies suggest that FCF not only is nontoxic but also may be a beneficial component of the vein graft preparation that mitigates detrimental effects of stretch-induced injury during bypass procedures.

METHODS

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

HSV procurement. HSVs 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 grafting procedures. The method of vein harvest (open or endoscopic) and graft preparation (including hydrostatic distention, marking with a surgical skin marker, and placement in storage solution) was at the discretion of the surgical team. Surgical remnant segments were transported to the laboratory in heparinized PlasmaLyte (HP; 10 units heparin/1 mL PlasmaLyte [Baxter International, Deerfield, Ill]) for experimentation within 30 minutes of collection. Areas showing visible signs of injury were not used in experimentation.

Animal procedures. Animal procedures followed study protocols approved by the Vanderbilt Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines for care and use of laboratory animals.

Porcine saphenous vein (PSV) procurement. Immediately after euthanasia, great saphenous veins ($n = 6$) were procured from adult Yorkshire pigs (Oak Hill Genetics, Ewing, Ill) by an open harvest method and transported in HP to the laboratory for immediate experimentation.

Rat aorta procurement. Immediately after euthanasia, aortae were gently dissected from adult female Sprague-Dawley rats ($n = 8$), collected in HP at room temperature, and used immediately for experimentation.

Mechanical stretch injury of PSV. After harvest, unmanipulated PSV segments were reserved as control vessels. Additional segments were stretched to 200% the resting length as previously described.[7] This amount of stretch is equivalent to the extent of passive stretch that PSV segments would tolerate. Stretched tissues were then subjected to different treatments before measurement of physiologic responses.

Measurement of physiologic responses. To determine the effect of surgical skin marker or dyes on contractile responses, untreated or stretched PSV segments were either left untreated, painted on the extravascular surface with a surgical skin marker (Aspen Surgical Products, Caledonia, Mich) or a cotton swab saturated with a solution of FCF (2.6 mM), or incubated in HP containing methylene blue (MB; 1%), IPA (50%), BBG (a structural analogue of FCF; 50 μM), or allura red (another food coloring dye; 50 μM) for 15 minutes. Endothelium was denuded to assess smooth muscle function before suspension in the muscle bath.

Rings from PSV segments were suspended in a muscle bath containing a bicarbonate buffer (120 mM sodium chloride, 4.7 mM potassium chloride, 1.0 mM magnesium sulfate, 1.0 mM monosodium phosphate, 10 mM glucose, 1.5 mM calcium chloride, and 25 mM sodium bicarbonate, pH 7.4) equilibrated with 95% O_2/5% CO_2 at 37°C for 1 hour at a resting tension of 1 g, manually stretched to three to four times the resting tension, and maintained at resting tension for an additional 1 hour. This produced the maximal force-tension relationship as previously described.[12] Force measurements were obtained using the Radnoti force transducer (model 159901A; Radnoti LLC, Monrovia, Calif) interfaced with a PowerLab data acquisition system and Chart software (ADInstruments, Colorado Springs, Colo). After equilibration, the rings were contracted with 110 mM potassium chloride (with equimolar replacement of sodium chloride in bicarbonate buffer) to determine smooth muscle functional viability. Contractile functions were compared with the unmarked, the nonstretched, or the stretched segments from the same vessel of the same animal.

Measurement of dye cytotoxicity. Primary human umbilical venous smooth muscle cells (HUVSMCs; ScienCell, Carlsbad, Calif) were cultured per the manufacturer's instructions and treated with reagents or dye at indicated concentrations diluted in medium. Stock solution of GV was prepared as 10% with 50% IPA as solvent. For phase contrast imaging, cells seeded in six-well plates were treated for 10 minutes. Cells were then washed to remove dyes, and images were taken before and after trypan blue (0.4%) staining. For cytotoxicity assay, cells seeded in 96-well plates were treated 2 to 12 hours. Cytotoxicity of the dyes was evaluated by measuring the release of dead-cell protease using the CytoTox-Glo assay kit (Promega, San Luis Obispo, Calif). The percentage of dead cells was determined. Each data point represents the average of triplicate wells of each treatment for each independent experiment. Cytotoxicity was determined by comparison to the untreated cells.

Organ culture. Rings (1-2 mm) were cut from PSV segments. Two rings were fixed in 10% neutral buffered formalin to measure basal (preculture) intimal thickness. Additional rings were cultured either without any dye (control) or in the presence of FCF (50 μM), BBG (50 μM), or allura red (50 μM) in RPMI 1640 medium supplemented with 30% fetal bovine serum, 1%

L-glutamine, and 1% penicillin/streptomycin for 14 days at 37°C in an atmosphere of 5% CO_2 in air. Tissues were fixed, paraffin embedded, sectioned, and stained with Verhoeff-van Gieson stain to allow the visualization of the internal elastic lamina. Measurements of intimal thickness were made on transverse sections of each vessel using a Zeiss Axiovert 200M microscope (Carl Zeiss, Jena, Germany) with a computerized image analysis system (Zeiss software and Adobe Photoshop) as described previously.[9] Intimal thicknesses of treated rings were compared with the control, cultured rings of the same vessel from the same animal.

Measurement of purinergic receptor-induced contraction and cytosolic calcium ion flux. Rat aorta was dissected free of fat and connective tissue, sectioned into 1-mm rings, and suspended in a FluoroPlex Tissue Bath Fluorometry System (IonOptix LLC, Milton, Mass, and Radnoti LLC), which enables fluorescence ion recording in parallel with force measurement. Rings were loaded with 10 μM Fura-2 acetoxymethyl ester (Invitrogen, Carlsbad, Calif) and 0.01% Pluronic F-127 (Invitrogen) in the bicarbonate buffer, as described previously,[14] and then either left untreated or treated with FCF (50 μM), the $P2X_7R$ antagonists adenosine 5′-triphosphate periodate oxidized sodium salt (oATP; 50 μM), KN-62 (10 μM), or BBG (50 μM) for 30 minutes before contraction with the $P2X_7R$ agonist 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP; 100 μM). Force and calcium fluorescence were measured continuously for 15 minutes after the addition of BzATP. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were determined as previously described.[14] Contractile responses and $[Ca^{2+}]_i$ were compared with the untreated ring from the same vessel of the same animal.

Rings from HSV segments were suspended and equilibrated in the muscle bath and contracted with 110 mM KCl as described before to determine smooth muscle viability. Tissues produced stress of $<0.025 \times 10^5$ N/m^2 were considered nonviable and were not used in further studies.[7] Viable rings were then either left untreated or treated with 50 μM FCF for 30 minutes before contraction with 100 μM BzATP. BzATP-induced contraction was expressed as the percentage of maximum KCl-induced contraction. Contractile responses were compared with the untreated rings from the same vessel of the same patient.

Immunohistochemistry of HSV. Antigen retrieval of formalin-fixed tissue sections was performed with citrate buffer (pH 6) at 95°C for 12 minutes. After preincubation with 5% goat serum to block nonspecific sites, sections were incubated with primary antibodies against $P2X_7R$ (Alomone Labs, Jerusalem, Israel) overnight at 4°C. The sections were then incubated with Alexa 568-tagged anti-rabbit antibodies (Invitrogen) for 1 hour. Controls were performed by preabsorbing the primary antibody with the immunogen peptide. Immunostaining was examined under a fluorescence microscope.

Statistical analysis. Contractile responses were defined by stress, calculated using force generated by tissues as follows: Stress ($\times 10^5$ N/m^2) = Force (g) × 0.0987/area, where area = wet weight (mg)/at maximal length (mm)]/1.055. Sample size (n) was determined with $\alpha = .05$ and power = 0.9 using PS program version 3.0.43 (http://biostat.mc.vanderbilt.edu/PowerSampleSize)[15] based on prior studies.[7,8,12,16] Data were reported as mean responses ± standard deviation of the mean. The statistical significance (*P* value) and achieved power of each experiment were determined using GraphPad Prizm version 5.0 and G*Power version 3.1.9.2 (www.gpower.hhu.de/en.html), respectively. Paired *t*-tests were used for experiments with dependent (matched-pairs) samples (ie, different techniques used on samples of the same tissue from the same patient/animal). One-way analysis of variance test followed by Tukey multiple comparison post-tests was used for experiments with independent groups. A *P* value < .05 and power ≥ 0.9 were considered statistically significant.

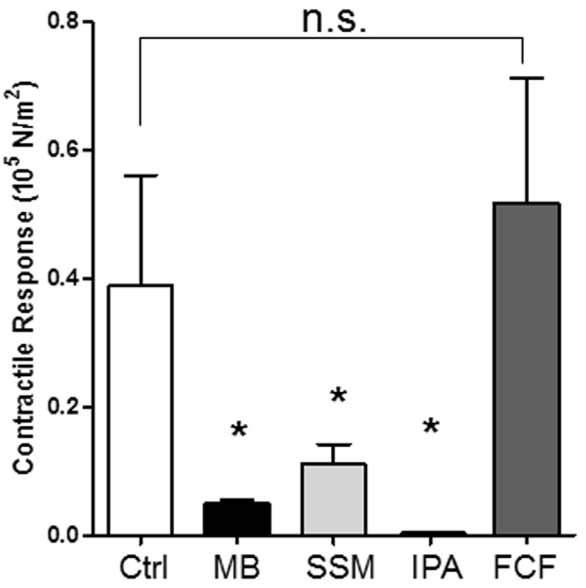

Fig 1. Brilliant blue FCF (*FCF*) does not impair contractility in porcine saphenous veins (PSVs). Segments of PSVs (n = 3-15) were either left untreated (*Ctrl*), marked with a surgical skin marker (*SSM*) or a solution of FCF (2.6 mM, in 5% propylene glycol and water) with a cotton swab in a longitudinal line and incubated at room temperature in PlasmaLyte for 15 minutes, or treated with 50% isopropanol (*IPA*) or 1% methylene blue (*MB*) for 15 minutes. Treated segments were then incubated, cut into rings, suspended in a muscle bath, and treated with KCl (110 mM). Force generated was converted to stress. The *error bars* show the standard deviation; *n.s.*, not significant. *$P < .05$ in paired *t*-tests.

RESULTS

FCF did not impair functional responses in PSV. Smooth muscle functional viability (contraction in response to a depolarizing KCl stimulus) was significantly reduced in PSV marked with MB ($0.051 \pm 0.005 \times 10^5$ N/m^2 vs $0.220 \pm 0.066 \times 10^5$ N/m^2 in control; $P = .04$; n = 3; Fig 1) and surgical skin marker ($0.111 \pm 0.031 \times 10^5$ N/m^2 vs $0.255 \pm 0.095 \times 10^5$ N/m^2 in control;

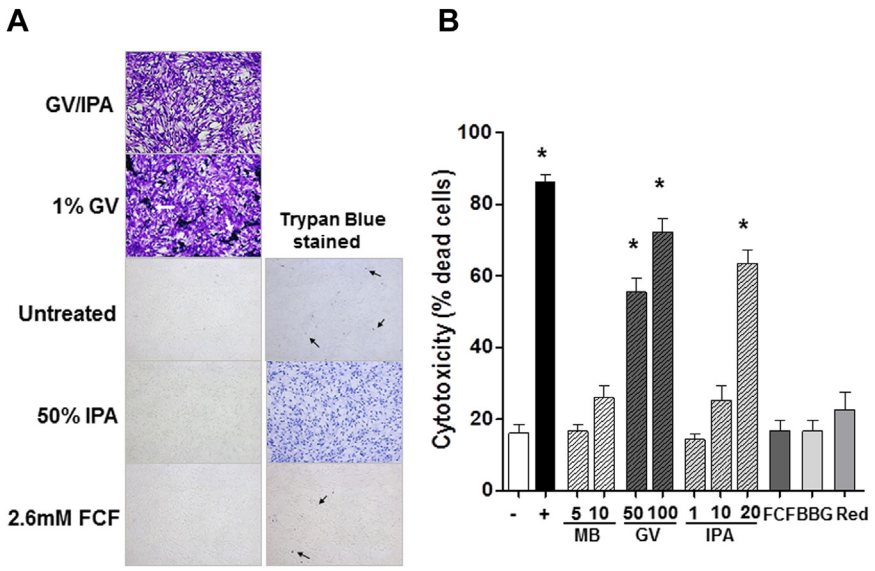

Fig 2. Cytotoxicity of vein graft marking dyes on human umbilical venous smooth muscle cells (HUVSMCs). **A,** Phase contrast images of HUVSMCs either left untreated or treated with gentian violet (1%) dissolved in 50% isopropanol (*GV/IPA*), 50% isopropanol (*IPA*), 1% gentian violet (*GV*), or 2.6 mM brilliant blue FCF (*FCF*) for 10 minutes (*left panels*) and then stained with trypan blue (*right panels*). Minimal dead cells were detected in untreated and FCF-treated cells (*black arrows*), whereas all cells took up trypan blue after brief exposure to 50% IPA. GV alone also chemically fixed the cells and could not be washed off. Because the cells were already stained, no trypan blue staining was performed on these cells. The *white arrows* indicate precipitation of GV due to low organic solvent content. **B,** HUVSMCs seeded in 96-well plates were left untreated (−) or treated for 12 hours with staurosporine (+; 800 nM), methylene blue (*MB*; 5 and 10 mM), GV (50 and 100 μM), IPA (1%- 20%), FCF (50 μM), brilliant blue G (*BBG*; 50 μM), and allura red (*Red*; 50 μM). Cytotoxicity of the treatments was evaluated by measuring release of protease in the medium using a dead-cell protease assay and expressed as percentage of dead cells in each well. Each data point represents an average of triplicate wells for each treatment in each independent experiment. Data represent mean ± standard deviation; n = 5-7; *$P < .05$ in Tukey multiple comparison tests.

$P = .03$; n = 5; Fig 1) or treated with 50% IPA (0.0002 ± 0.00002 × 10^5 N/m^2 vs 255 ± 0.095 × 10^5 N/m^2 in control; $P = .03$; n = 3). Topically applied FCF had no effect on smooth muscle physiologic responses in PSV (0.564 ± 0.19 × 10^5 N/m^2 vs 453 ± 0.167 × 10^5 N/m^2 in control); $P = .12$; n = 10; Fig 1), suggesting that FCF is nontoxic to the tissue.

Cytotoxicity of marking dyes and FCF. Upon exposure to 1% GV (25 mM, a concentration that is 10 times lower than surgical skin markers) and 50% IPA for 5 minutes, either alone or combined, HUVSMCs essentially underwent cytologic fixation and preservation and adhered to the bottom of the wells (Fig 2, *A*). Trypan blue dye exclusion test on IPA-treated cells suggested that cells are rendered nonviable by the chemical constituents of surgical skin markers (Fig 2, *A*). Likewise, application of 1% (or 30 mM) MB immediately lysed cells. The intense blue staining precluded the ability to perform trypan blue dye exclusion test or cytotoxicity assay (data not shown). Solubility of GV is poor in nonorganic solvent as evidenced by the precipitation of dye in wells in which cells were treated with 1% GV (with effective concentration of IPA at 5%; Fig 2, *A*). Exposure to 2.6 mM FCF (concentration used for extravascular marking of PSV) for 5 minutes did not result in cell death compared with untreated cells (Fig 2, *A*). A cytotoxic effect of a considerably low concentration of GV (0.002% or 50 μM), FCF (50 μM), BBG (50 μM), or allura red (50 μM) was not observed after short-term (2-hour) treatment (data not shown). After 12-hour treatment (n = 7), 10 mM MB (a concentration that is three times lower than clinical use) did not result in significant cell death (26.0% ± 8.5% vs 16.1 ± 6.3%; $P > .05$). Both GV (55.4% ± 10.1% for 50 μM and 72.1% ± 10.6% for 100 μM) and 20% IPA (63.5% ± 9.1%) were cytotoxic to HUVSMCs (Fig 2, *B*; $P < .05$ vs untreated cells). The cytotoxicity of GV was not due to residual IPA (0.01%) in the diluted dye as 1% IPA was not toxic to the cells (14.3% ± 4.3% vs 16.1% ± 6.3% in untreated cells; $P > .05$). Prolonged treatment with 50 μM FCF (16.77% ± 8.0%), BBG (22.6% ± 13.1%), or allura red (16.62% ± 8.2%) remained nontoxic to the cells (Fig 2, *B*; $P > .05$ vs untreated cells).

FCF restored functional responses in PSV impaired by mechanical stretch injury. We previously showed that longitudinal stretch impairs functional viability in PSV.[14] We used this model of stretch injury to determine the

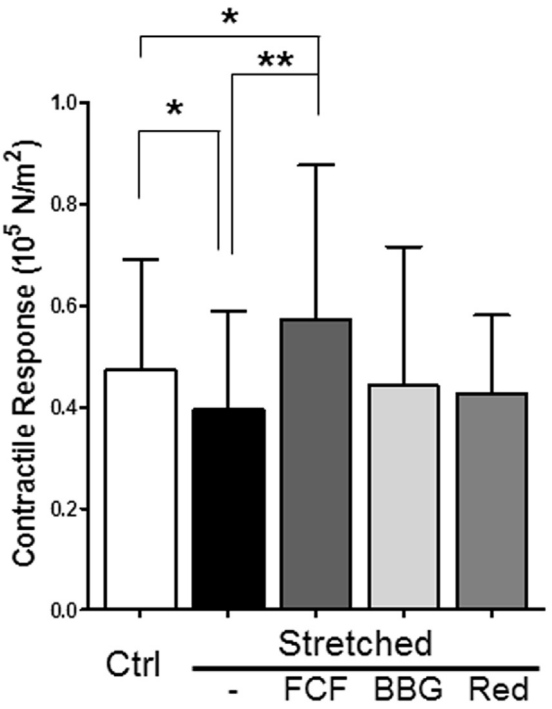

Fig 3. Brilliant blue FCF (*FCF*) restores functional viability after stretch injury in porcine saphenous vein (PSV). Pig saphenous veins were either untreated (*Ctrl*; n = 5-10) or stretched to twice their resting length (*Stretched*; n = 10). Stretched segments were then either left untreated, marked with a solution of FCF (2.6 mM, in 5% propylene glycol and water) with a cotton swab in a longitudinal line, or treated in 50 μM brilliant blue G (*BBG*) or 50 μM allura red (*Red*). The segments were then incubated at room temperature for 15 minutes in PlasmaLyte and cut into rings, suspended in a muscle bath, and treated with KCl (110 mM). Force generated was converted to stress. Results are presented as mean ± standard deviation. *$P < .05$, **$P < .005$ in paired *t*-tests.

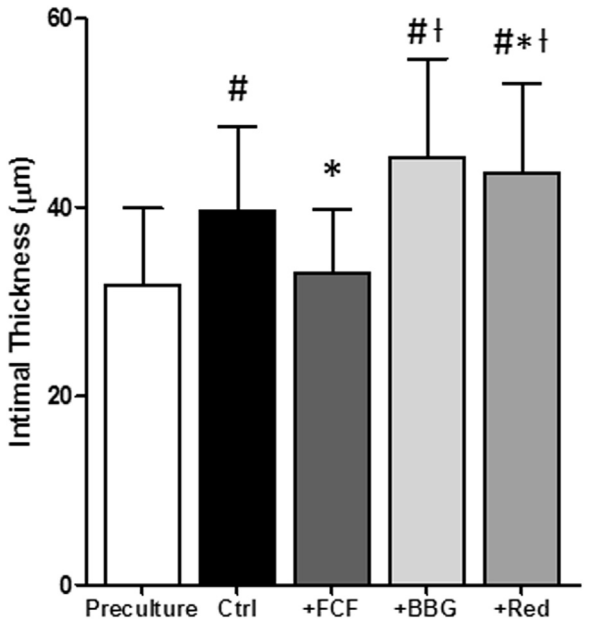

Fig 4. Brilliant blue FCF (*FCF*) inhibits intimal thickening in porcine saphenous veins (PSVs) in organ culture. Rings from pig saphenous veins were either left untreated (*Ctrl*) or treated in the presence of FCF (50 μM), brilliant blue G (*BBG*; 50 μM), and allura red (*Red*; 50 μM) in organ culture for 14 days. Veins were stained with Verhoeff-van Gieson stain, and intimal layer thickening was measured. Results are presented as mean ± standard deviation. #$P < .05$ vs preculture; *$P < .05$ vs control; †$P < .05$ vs FCF in paired *t*-tests.

effect of FCF on restoring smooth muscle contractile responses. Compared with control segments, PSV subjected to stretch injury generated significantly less contractile force in response to 110 mM KCl (0.473 ± 0.22 × 10^5 N/m^2 vs 0.396 ± 0.19 × 10^5 N/m^2 in control; $P = .02$; n = 10; Fig 3). Treatment with a topical application of FCF restored the contractile response of stretch-injured PSV (0.573 ± 0.101 × 10^5 N/m^2 vs 0.389 ± 0.2 × 10^5 N/m^2 in stretched; $P = .005$; n = 9; Fig 3). Treatment with BBG (0.442 ± 0.101 × 10^5 N/m^2 vs 0.351 ± 0.21 × 10^5 N/m^2 in stretched; $P = .09$; n = 7) or allura red (0.427 ± 0.069 × 10^5 N/m^2 vs 0.417 ± 0.19 × 10^5 N/m^2 in stretched; $P = .8$; n = 5) did not restore smooth muscle contractile response (Fig 3).

FCF reduced intimal thickening in cultured PSV. Because injury leads to the development of intimal hyperplasia, the effect of FCF on the development of intimal hyperplasia was determined in an organ culture model. PSVs were either left untreated (control) or treated in the continuous presence of dyes in organ culture. After 14 days in organ culture, intimal thickness increased 8.4% ± 6.8% from 31.7 ± 8.2 μm to 39.6 ± 8.9 μm ($P < .0001$; n = 22) in untreated (control) rings. Intimal thickening was significantly reduced in segments of FCF-treated PSV compared with untreated tissues (32.4 ± 6.5 μm vs 39.8 ± 9.4 μm; $P < .0001$; n = 21; Fig 4) with a 1.2% ± 4.9% increase over the precultured rings, suggesting that treatment with FCF has an inhibitory effect on neointimal thickening ex vivo. The presence of BBG (44.7 ± 10.6 μm vs 39.4 ± 9.2 μm; $P = .03$; n = 19) or allura red (44.8 ± 9.2 μm vs 40.4 ± 9.5 μm; $P = .02$; n = 18) during organ culture led to increased intimal thickness compared with the untreated PSV (Fig 4), implicating that these dyes did not prevent intimal hyperplasia and accelerated the processes that contributed to intimal thickening.

FCF inhibited P2X$_7$ purinergic receptor-induced contraction and cytosolic Ca^{2+} fluxes in rat aorta. Spinal cord stretch injury has been shown to be ameliorated by treatment with BBG, and the mechanism is thought to be through P2X$_7$R antagonism.[13] We hypothesized that FCF restores stretch-injured PSV by targeting the P2X$_7$R. In addition, P2X$_7$R activation leads to increases in $[Ca^{2+}]_i$.[17] We therefore measured force generation and $[Ca^{2+}]_i$ concurrently in response to BzATP, a P2X$_7$R agonist that is known to elicit a contraction.[16,18] We chose to use rat aorta because of the thin nature of the arterial wall that allows penetration of the fluorochrome and reproducibility

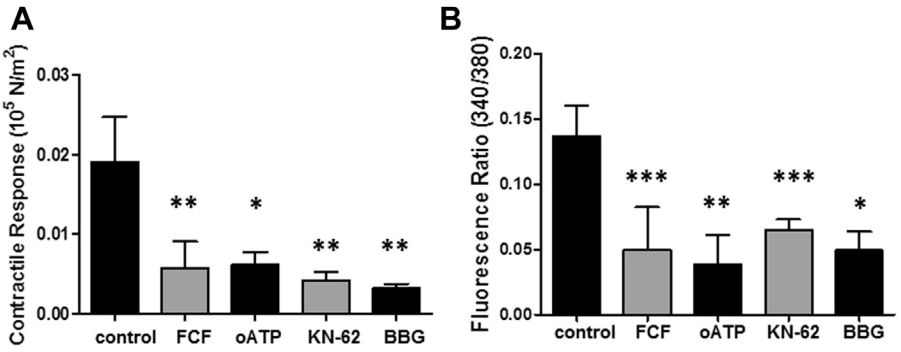

Fig 5. Brilliant blue FCF (*FCF*) inhibits P2X$_7$R-mediated cytosolic Ca^{2+} fluxes in rat aorta. Rat aortic rings (n = 4-8) were suspended in the FluoroPlex muscle bath, either left untreated (*control*) or treated with FCF or the other P2X$_7$R antagonists adenosine 5′-triphosphate periodate oxidized sodium salt (*oATP*), KN-62, or brilliant blue G (*BBG*) before contraction with 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP). Concurrent force generation **(A)** and cytosolic Ca^{2+} flux **(B)** were measured. Results are presented as mean ± standard deviation. *$P < .0$, **$P < .001$. ***$P < .0001$ vs control in paired *t*-tests.

of the results. Pretreatment with FCF blocked BzATP-induced contraction ($0.006 \pm 0.003 \times 10^5$ N/m^2 vs $0.019 \pm 0.006 \times 10^5$ N/m^2 in control; $P = .002$; n = 8; Fig 5, *A*). In addition, FCF blocked BzATP-induced calcium ion flux (0.50 ± 0.032 A.U. vs 0.016 ± 0.023 A.U. in control; $P = .0004$; n = 8; Fig 5, *B*). FCF inhibition of BzATP-induced contraction and Ca^{2+} fluxes was comparable to that mediated by known P2X$_7$R antagonists oATP ($0.006 \pm 0.007 \times 10^5$ N/m^2 vs $0.019 \pm 0.007 \times 10^5$ N/m^2 in control; $P = .005$; n = 4; and 0.039 ± 0.022 A.U. vs 0.135 ± 0.021 A.U. in control; $P = .004$; n = 4), KN-62 ($0.004 \pm 0.002 \times 10^5$ N/m^2 vs $0.043 \pm 0.05 \times 10^5$ N/m^2 in control; $P = .005$; n = 4; and 0.066 ± 0.008 A.U. vs 0.128 ± 0.01 A.U. in control; $P < .001$; n = 4), and BBG ($0.003 \pm 0.0006 \times 10^5$ N/m^2 vs $0.042 \pm 0.05 \times 10^5$ N/m^2 in control; $P = .005$; n = 4; and 0.05 ± 0.014 A.U. vs 0.15 ± 0.03 A.U. in control; $P = .02$; n = 4), inferring that FCF is likely an antagonist of the P2X$_7$R in vascular tissues (Fig 5, *A* and *B*).

FCF inhibited P2X$_7$ purinergic receptor-induced contraction in HSV. To determine whether FCF interferes with P2X$_7$R in HSV, BzATP-induced contraction was measured. Preincubation with FCF significantly reduced contraction to BzATP compared with the control rings (7.7% ± 0.9% vs 11.8% ± 2.9% of maximum KCl-induced contraction in control; $P = .006$; n = 6; Fig 6, *A* and *B*), suggesting that FCF inhibits P2X$_7$R activation in HSV. Expression of P2X$_7$R in HSV was confirmed by immunohistochemistry (Fig 6, *C* and *D*).

DISCUSSION

Intimal hyperplasia is the leading cause of vein graft failure. Although the specific inciting events remain uncertain, there is general agreement that this process is a "response to injury" initiated by vein graft harvest, surgical preparation, implantation, reperfusion, and exposure to arterial hemodynamics.[19,20] Surgical dissection of the vein graft causes significant mechanical stretch injury, and subsequent preparation of the vein graft is deleterious to vein grafts; optimization of these techniques may reduce intimal hyperplasia.[6]

Surgical skin markers, commonly used to mark vein grafts, are not approved by the Food and Drug Administration for use on vein tissue. We have previously showed that these marking pens reduce viability and impair physiologic function of the HSV, and such detrimental effects are attributable to the GV dye and IPA.[10] We sought an alternative to the off-label use of surgical skin marker and identified FCF as a nontoxic dye that improves endothelial and smooth muscle functional responses in HSV, suggesting that the FCF dye has pharmacologic properties.[12] In the current study, we demonstrated further that FCF can be used to mark vein graft and may have additional benefits in restoring stretch injury resulting from vein harvest and preparation.

Similar to our earlier study using HSV,[8] surgical skin marker essentially abolished contractile responses in PSV (Fig 1). Exposure to another common dye, MB, and 50% IPA, the solvent found in surgical skin markers, resulted in 60% to 100% loss in contractile responses (Fig 1). In contrast, extravascular marking with FCF did not affect the contractile responses in PSV (Fig 1). Because contractile response to 110 mM KCl correlates with cellular viability in vascular tissues,[7] cell death likely occurs within the vein graft soon after vein graft marking. GV is widely used for medicinal purposes and is commonly used as a staining dye for biologic samples. However, it is a clastogenic dye that induces chromosomal aberrations and mitotic anomalies in various types of cultured mammalian cells at a low concentration of 5 μM.[21] Likewise, IPA is commonly used as a cell and tissue fixative in cytohistologic preparations and is converted to acetone by an endogenous enzyme.[22] Prolonged exposure to alcohols and acetone disrupts plasma membrane and damages intracellular structures.[23] Hence, exposing the graft to IPA is essentially fixing the conduits. This is analogous to cryopreservation

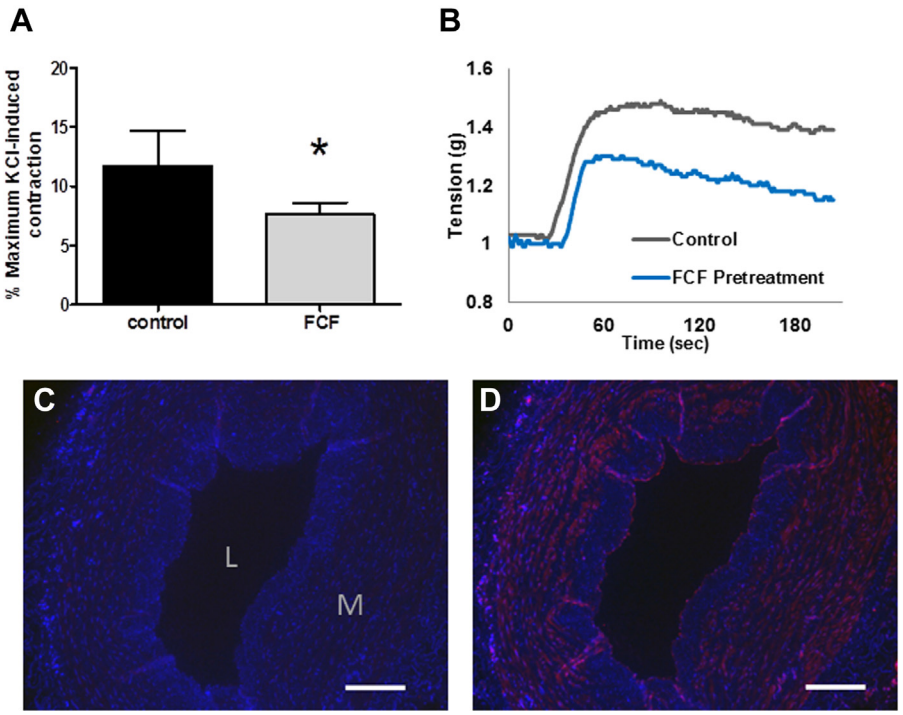

Fig 6. Brilliant blue FCF (*FCF*) inhibits $P2X_7R$-mediated contraction in human saphenous vein (HSV). **A,** Rings of HSV (n = 6) were suspended in the muscle bath, either left untreated (*control*) or treated with 50 μM FCF for 30 minutes before contraction with 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP). **B,** Representative muscle bath force tracings of BzATP-induced contraction in HSV. Results are presented as mean ± standard deviation. **P* = .006 vs control in a paired *t*-test. **C** and **D,** Expression of $P2X_7R$ in HSV as detected by immunohistochemistry using preabsorbed **(C)** or normal $P2X_7R$-specific antibody **(D)**. $P2X_7Rs$ were stained red. *Scale bar* = 200 μm. *L*, Lumen; *M*, medial.

of saphenous vein used in bypass procedures and causes substantial damages. The patency of cryopreserved veins is dismal at 27% and 17% at 1 and 3 years.[24] In this study, exposure to 1% GV or 50% IPA chemically fixed the HUVSMCs with complete loss of viability as evidenced by the trypan blue dye exclusion test (Fig 2, *A*). When HUVSMCs were treated with 2.6 mM FCF, a concentration that was used for extravascular application on PSV, no cell death was observed (Fig 2, *A*). Cytotoxicity of GV on HUVSMCs was further confirmed by treating cells with 0.002% or 50 μM GV, a concentration that is 5000 times lower than that found in surgical skin markers (Fig 2, *B*). Exposure to GV caused >50% cell death in HUVSMCs, levels that are comparable to staurosporine, an inducer of apoptosis that was used as a positive control for the assay (Fig 2, *B*). Conversely, HUVSMCs treated with a comparable concentration (50 μM) of FCF, BBG, or allura red demonstrated no cytotoxicity (Fig 2, *B*). Taken together, these data suggest that off-label use of the dye GV is particularly toxic to the conduit and that other nontoxic dyes are available for vein graft marking.

In a previous study, we showed that mechanical stretching of PSV reduced contractility of the vein. In the current study, the topical application of FCF restored functional viability after stretch injury in PSV (Fig 3). This was not observed with allura red or BBG (Fig 3), suggesting that the pharmacologic properties are unique to FCF. Moreover, FCF prevented neointimal thickening, whereas BBG or allura red promoted the development of intimal hyperplasia in organ-cultured PSV (Fig 4). Whereas BBG and allura red are biocompatible as marking dyes, they lack the pharmacologic properties that are unique to FCF and may promote intimal hyperplasia.

FCF inhibited the selective $P2X_7R$ agonist BzATP-induced smooth muscle force generation (Fig 5, *A*) and calcium ion flux (Fig 5, *B*) to levels comparable to other known antagonists of the receptor,[16,25,26] implying that FCF is an antagonist of the $P2X_7R$. In addition, the evidence that FCF inhibited BzATP-induced contraction and that $P2X_7R$ is expressed in HSV supports a potential role of $P2X_7R$ in the response to injury in saphenous veins (Fig 6).

Vein graft injury incurred during graft preparation is a sufficient stimulus for the development of intimal hyperplasia in HSV ex vivo, suggesting a crucial role for early injury in the cellular processes that contribute to the development of intimal hyperplasia.[6,12] Given that tissue damage, particularly that caused by stretching, leads to extracellular ATP release,[13,27-29] it can be envisioned that injury to the vein leads to release of ATP from damaged cells that activates the $P2X_7R$ in neighboring cells. Increases in intracellular

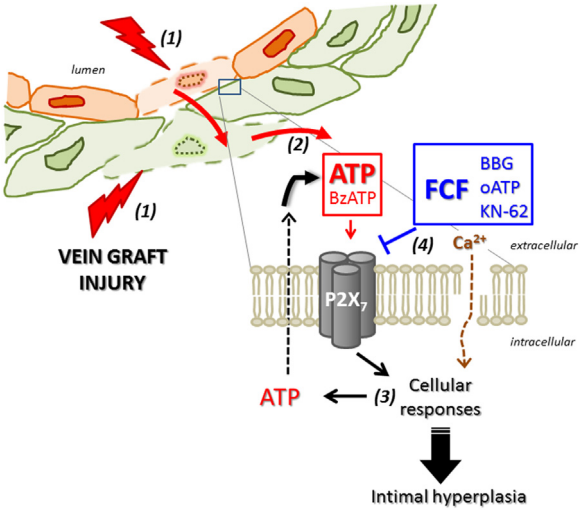

Fig 7. Model of $P2X_7R$ activation during vein graft preparation injury. Surgical harvest and preparation cause vein graft injury (*1*), leading to release of adenosine triphosphate (*ATP*, *2*). ATP activates the $P2X_7$ receptor on neighboring cells, propagating the response to injury (*3*). Brilliant blue FCF (*FCF*) may mitigate the effect of $P2X_7R$ activation (*4*) by inhibiting membrane pore formation, $[Ca^{2+}]_i$ flux, and additional release of extracellular ATP. Agonists, *red*; inhibitors, *blue*. *BBG*, Brilliant blue G; *BzATP*, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; *oATP*, adenosine 5′-triphosphate periodate oxidized sodium salt.

calcium ensue and result in further ATP release, thus propagating the injury response (Fig 7).[17] The findings of the current study offer evidence that deleterious effects of harvest-induced injury can be ameliorated by treatment with FCF. The mechanism for the pharmacologic properties of FCF may be due to inhibition of the $P2X_7$ purinergic receptor (Fig 7). Hence, it is conceivable that intervening $P2X_7R$ activation during the period of explantation is a clinically relevant approach to preventing intimal hyperplasia and vein graft failure.

Potential limitations. Whereas our stretch injury model of PSV recapitulated the potential injury to the vein grafts, these tissues came from healthy animals. The model system used for these experiments has the advantage of more homogeneity and greater reproducibility compared with HSV. In addition, large effect sizes were observed in studies that evaluated the toxicity of marking dyes on PSV as well as $P2X_7R$ blockade in rat aortae and achieved high statistical power ($\geq$0.9) with relatively small sample sizes, supporting that the findings were unlikely to be falsely negative or due to the lack of power. Moreover, the mechanistic links between $P2X_7R$ blockade and restoration of smooth muscle injury or reduction in intimal thickening require further evaluation in in vivo models. Aside from changes in calcium ion flux, it remains to be determined whether treatment with FCF affects downstream events elicited by $P2X_7R$ activation that have been characterized in other cell types. In addition, whereas the culturing medium for PSV contains serum, the organ culture model lacks in vivo elements (pressure, flow, and exposure to blood components).

CONCLUSIONS

We have demonstrated that FCF is a nontoxic alternative to the current off-label use of surgical skin marker for vein graft marking. FCF treatment during vein graft preparation inhibits the injury response in vascular tissues and inhibits intimal hyperplasia in vitro. Further work is needed to address the therapeutic properties of FCF in animal models to better characterize the mechanism of action of FCF. Treatment with FCF represents a new approach to vein graft marking and possibly a therapeutic clinical alternative to the off-label use of surgical skin markers during preparation of vein graft conduits.

This study was also supported in part with resources and materials from the VA Tennessee Valley Healthcare System.

AUTHOR CONTRIBUTIONS

Conception and design: KH, WL, PK, CB, JC
Analysis and interpretation: KH, WL, FL, PK, CB, JC
Data collection: KH, WL, FL, JC
Writing the article: CB, JC
Critical revision of the article: KH, WL, FL, PK, CB, JC
Final approval of the article: KH, WL, FL, PK, CB, JC
Statistical analysis: KH, WL, JC
Obtained funding: CB, JC
Overall responsibility: JC

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Submitted Jul 21, 2014; accepted Dec 19, 2014.