Endothelial cell activation by hemodynamic shear stress derived from arteriovenous fistula for hemodialysis access

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Franzoni M, Cattaneo I, Longaretti L, Figliuzzi M, Ene-Iordache B, Remuzzi A. Endothelial cell activation by hemodynamic shear stress derived from arteriovenous fistula for hemodialysis access. Am J Physiol Heart Circ Physiol 310: H49–H59, 2016. First published October 23, 2015; doi:10.1152/ajpheart.00098.2015.—Intimal hyperplasia (IH) is the first cause of failure of an arteriovenous fistula (AVF). The aim of the present study was to investigate the effects on endothelial cells (ECs) of shear stress waveforms derived from AVF areas prone to develop IH. We used a cone-and-plate device to obtain real-time control of shear stress acting on EC cultures. We exposed human umbilical vein ECs for 48 h to different shear stimulations calculated in a side-to-end AVF model. Pulsatile unidirectional flow, representative of low-risk stenosis areas, induced alignment of ECs and actin fiber orientation with flow. Shear stress patterns of reciprocating flow, derived from high-risk stenosis areas, did not affect EC shape or cytoskeleton organization, which remained similar to static cultures. We also evaluated flow-induced EC expression of genes known to be involved in cytoskeletal remodeling and expression of cell adhesion molecules. Unidirectional flow induced a significant increase in Kruppel-like factor 2 mRNA expression, whereas it significantly reduced phospholipase D1, α1-integrin, and Ras p21 protein activator 1 mRNA expression. Reciprocating flow did not increase Kruppel-like factor 2 mRNA expression compared with static controls but significantly increased mRNA expression of phospholipase D1, α1-integrin, and Ras p21 protein activator 1. Reciprocating flow selectively increased monocyte chemotactic protein-1 and IL-8 production. Furthermore, culture medium conditioned by ECs exposed to reciprocating flows selectively increased smooth muscle cell proliferation compared with unidirectional flow. Our results indicate that protective vascular effects induced in ECs by unidirectional pulsatile flow are not induced by reciprocating shear forces, suggesting a mechanism by which oscillating flow conditions may induce the development of IH in AVF and vascular access dysfunction.

endothelial cells; wall shears stress; arteriovenous fistula; hemodialysis; intimal hyperplasia

NEW & NOTEWORTHY

The present investigation demonstrates that, in regions near the anastomosis of arteriovenous fistula for hemodialysis access, reciprocating flows acting on endothelial cells induce important changes in gene expression, cytokine production and cytoskeleton organization. These changes may be responsible for intimal hyperplasia and ultimately for vascular access failure.

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NATIVE ARTERIOVENOUS FISTULA (AVF) is recommended as the first choice to create vascular access for hemodialysis in patients with end-stage renal disease. Effective hemodialysis treatment requires a vascular access to provide a high blood flow rate (>400 ml/min) and a site resistant to repeated punctures for connection to the extracorporeal circulation (1, 45a). Although the AVF creation technique is widely used in clinical practice, its outcome remains unsatisfactory. Primary failure and nonmaturation affect 23–58% of AVF (3), and the primary patency of AVF ranges from 32% to 60% at 2 yr after surgery (11, 24). The main cause of AVF failure is vessel stenosis due to intimal hyperplasia (IH) (39), with the consequent lumen narrowing and vessel occlusion for thrombosis. IH develops preferentially in the juxtaanastomotic venous segment of the AVF, where the increased blood flow induces high-velocity gradients and shear forces acting on endothelial cells (ECs) (22, 65). These peculiar hemodynamic conditions play an important role in the mechanisms responsible for AVF failure. We and others have recently suggested that, despite the high flow rates present in AVF after maturation, local hemodynamic conditions near the vessel anastomosis are characterized by low and oscillating wall shear stress (WSS) in some focal areas (22, 53, 76). These oscillatory shear forces may be implicated in the development of IH.

Several investigations have shown that IH is related to EC activation, subendothelial infiltration of circulating inflammatory cells, and the production of chemokines and growth factors that induce the migration and proliferation of smooth muscle cells (SMCs) into the intimal layer with extracellular matrix remodeling (56, 59). SMC proliferation and deposition of the extracellular matrix lead to an increase in wall thickness and vessel stenosis (46, 58). Localized narrowing of the vessel lumen increases velocity gradients and secondary flows, inducing shear stress gradients in space and time on the vessel wall and sustaining a vicious cycle that can lead to vessel occlusion and AVF failure.

It is well known that WSS acting on ECs can modulate cell structure and function. Thus, WSS is relevant for normal vessel wall physiology and blood vessel remodeling (54, 68, 77). In addition, nonphysiological WSS patterns have been related to the pathogenesis of vascular diseases (15, 30, 31, 79). It has been well documented (12, 13, 41) that ECs exposed to pulsatile unidirectional WSS downregulate expression of pro-inflammatory molecules, whereas ECs exposed to oscillating WSS maintain sustained expression of transcription factors and proteins that induce SMC proliferation and migration as well as deposition of the extracellular matrix. Furthermore, frequency
components of WSS waveforms are involved in the inflammatory phenotype of vascular ECs (23).

The aim of the present study was thus to investigate the effects of exposure of ECs in vitro to shear forces similar to those that develop in the AVF. We studied changes in the structure and function of ECs exposed to oscillating WSS patterns that are present in areas prone to develop IH and compared them with those induced by pulsatile and unidirectional WSS patterns present in areas not prone to develop IH. For this, we considered WSS waveforms derived from computational fluid dynamic (CFD) analysis previously reported in idealized models of AVF (21), 1 day after the surgical procedure. We subjected human umbilical vein EC (HUVEC) monolayers to unidirectional and to reciprocating shear forces in vitro using a newly designed cone-and-plate device. This device allowed us to subject EC cultures to different WSS waveforms under controlled conditions. We investigated the effects of different flow conditions on EC morphology, cytoskeleton organization, gene expression of ECs (by RT2 gene expression analysis or gene expression array as well as by RT-PCR), and cytokine production. Furthermore, we evaluated the effect of medium conditioned by ECs exposed to different WSS waveforms on SMC proliferation in vitro.

MATERIALS AND METHODS

HLUVEC isolation. HUVECs were isolated from human umbilical cords collected by a local hospital (Azienda Ospedaliera Bolognini, Seriate, Bergamo). Before the tissue harvest, informed consent by the donor was collected. HUVECs were isolated from the umbilical vein by collagenase digestion (150 U/ml, Sigma-Aldrich, St. Louis, MO) according to the method of Jaffe et al. (32, 48). Freshly isolated HUVECs were plated on tissue culture dishes precoated with 1% bovine gelatin and cultured in medium 199 (Biowest, Nuaillé, France) supplemented with 10% newborn calf serum (Life Technologies Italia, Monza, Italy), 10% human serum, 1% penicillin-streptomycin (Life Technologies), 1% Fungizone (Life Technologies), 0.1% heparin (TEVA, Petah Tikva, Israel), and 0.2% EC growth supplement (Becton-Dickinson, Bedford, MA). HUVECs were used when they reached confluence and maintained in testing medium were used as controls.

Flow exposure experiments. HUVEC monolayers were exposed for 48 h to different WSS waveforms. As described above, we subjected HUVEC monolayers to three different WSS patterns derived from previous CFD analysis of an idealized side-to-end AVF model (21) with an anastomosis angle of 90°, as schematically shown in Fig. 2. The geometric model of the AVF and boundary conditions refer to the simulation of the postoperative condition (1 day after surgery) in which the changes in AVF blood flow rate are considered to have an important influence on EC mechanical load and the consequent vessel wall remodeling. Details on the CFD numeric analysis and WSS calculations have been previously reported in more detail (22). The first WSS waveform was derived from the cephalic vein in an area far from the anastomosis known to be protected from the development of IH. This WSS waveform was pulsatile and unidirectional (UND), with a peak value of 23 dyn/cm² at systole and a minimum value of 12 dyn/cm² at diastole (pulse cycle time-averaged shear stress of 16 dyn/cm²). Two reciprocating WSS waveforms (REC-1 and REC-2) were also selected from CFD results from sites near the anastomosis known to be prone to develop IH (see Fig. 2) (54). REC-1 was calculated at the anastomosis floor on the distal artery, whereas REC-2 was derived from the inner wall of the juxta-anastomotic vein. Both reciprocating WSS profiles have a high oscillatory shear index (0.49), as defined by Ku controller to accurately and rapidly change angular velocity as a function of time.

HUVEC monolayers were exposed for 48 h to different WSS stimuli, and the testing medium was completely changed, under sterile conditions, twice a day. The conditioned medium collected at 12, 24, 36, and 48 h of shear or static exposure (referred as T1, T2, T3, and T4) was then stored at −80°C. Phase-contrast microscopy images of HUVEC monolayers were obtained at the end of each flow exposure experiment. Cell monolayers were then processed for immunofluorescence microscopy or gene expression analysis. HUVEC monolayers maintained in static conditions within the cell incubator using the same testing medium were used as controls.

WSS waveforms. As described above, we subjected HUVEC monolayers to three different WSS patterns derived from previous CFD analysis of an idealized side-to-end AVF model (21) with an anastomosis angle of 90°, as schematically shown in Fig. 2. The geometric model of the AVF and boundary conditions refer to the simulation of the postoperative condition (1 day after surgery) in which the changes in AVF blood flow rate are considered to have an important influence on EC mechanical load and the consequent vessel wall remodeling. Details on the CFD numeric analysis and WSS calculations have been previously reported in more detail (22). The first WSS waveform was derived from the cephalic vein in an area far from the anastomosis known to be protected from the development of IH. This WSS waveform was pulsatile and unidirectional (UND), with a peak value of 23 dyn/cm² at systole and a minimum value of 12 dyn/cm² at diastole (pulse cycle time-averaged shear stress of 16 dyn/cm²). Two reciprocating WSS waveforms (REC-1 and REC-2) were also selected from CFD results from sites near the anastomosis known to be prone to develop IH (see Fig. 2) (54). REC-1 was calculated at the anastomosis floor on the distal artery, whereas REC-2 was derived from the inner wall of the juxta-anastomotic vein. Both reciprocating WSS profiles have a high oscillatory shear index (0.49), as defined by Ku

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only 8.5% of the cell monolayer, we can assume that most ECs experience a sustained and rather uniform WSS.

Due to the fact that we imposed fast variations in angular velocity of the cone, we wondered whether they actually deliver the corresponding changes in WSS over the plate, as calculated by Eq. 1. We then calculated the time of delay, in the worst condition, of a suddenly accelerating plane in a semi-infinite Newtonian fluid (9, 40, 67) to develop 99% of the velocity profile. The value of the time of delay changes along the plate radius and is proportional to the gap between the cone and plate surface. We calculated a maximum value of the time of delay of 0.037 s for a radius of 67 mm (outer position). This value is lower than the time of development of a single sinusoidal oscillation at 10 Hz, the maximum frequency we considered for the WSS waveforms. In line with previous investigations (6, 7), this guarantees that WSS values calculated as a function of the angular velocity by Eq. 1 are effectively acting on the EC surface.

Immunofluorescence staining. After exposure to shear or static conditions, cell monolayers were fixed in a 2% paraformaldehyde (Societá Italiana Chimici, Rome, Italy) and 4% sucrose (Sigma) solution for 10 min at room temperature. Cells were then permeabilized in 0.1% Triton X-100 (Sigma) and treated with 3% BSA (Sigma) for 30 min at room temperature. Tight junction (zonula occludens (ZO)-1) protein expression was visualized by a sequential incubation of ECs with rabbit anti-ZO-1 primary antibody (1 μg/ml, Sigma) overnight at 4°C and FITC-labeled goat anti-rabbit secondary antibody (1:25, Jackson ImmunoResearch, West Grove, PA) for 45 min at 37°C. We also characterized F-actin fiber organization by rhodamine-labeled phalloidin (1:40, Invitrogen, Paisley, UK) staining for 45 min at room temperature. Moreover, to evaluate the EC phenotype of cultured cells, we incubated the samples with rabbit anti-human vWF (1:100, Sigma) primary antibody for 2 h at room temperature and with FITC-labeled goat anti-rabbit secondary antibody (1:25, Jackson ImmunoResearch) for 45 min at 37°C. Counterstaining with 4’,6-diamidino-2-phenylindole (1 μg/ml, Sigma) for 10 min at 37°C was performed for cell nuclear staining. Samples were finally mounted with fluorescent mounting medium (Dako Cytomation, Carpinteria, CA), and immunofluorescence images were acquired by an ApoTome fluorescent microscope (Axio Imager.Z2, Carl Zeiss, Jena, Germany).

Image analysis. To quantify morphological changes in ECs, we performed manual postprocessing to correct mismatches of automatic cell recognition. To evaluate the orientation of F-actin stress fibers on rhodamine-labeled phalloidin staining images, we used the Orientation plugin (EPFL, Lausanne, Switzerland) of the image processing software ImageJ (National Institutes of Health, Bethesda, MD), as previously described (55).

PCR array. To study genes potentially expressed in a different way in HUVECs exposed to static or different WSS waveforms, we initially used the Human Cell Motility RT² Profiler PCR Array (PAHS-128ZC, SABioscience, Qiagen, Frederick, MD). In total, 84 genes encoding for mediators of Rho activity, cell projections, chemotaxis, cell adhesion, and integrin signaling were investigated. To this purpose, total RNA was extracted using TRizol (LifeTechnologies) and cleaned using the RNeasy MinElute Cleanup kit according to the manufacturer’s protocol (Qiagen). RNA from three experiments of each group was pooled and quantified spectrophotometrically (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE). First-strand cDNA (1 μg) was synthesized with the RT² First Strand Kit and analyzed by quantitative PCR. Data analysis was performed using online SABioscience software. We then identified genes differentially expressed by WSS waveforms as those affected by a twofold change (≥2 or <0.5) compared with the static control.
Real-time PCR. For three of the genes identified by the RT² Profiler PCR Array, more consistently differentially affected by UND and reciprocating WSS, mRNA expression was further evaluated with real-time PCR as previously described (4, 10). We also evaluated Kruppel-like factor (KLF2) mRNA expression by real-time PCR, a transcription factor well known to be expressed by ECs exposed to laminar flow. Briefly, total RNA was extracted from HUVECs after flow or static experiments using TRizol reagent (LifeTechnologies) according to the manufacturer’s instructions. Comcomitantly, genome DNA was removed by RNase-free DNase (Promega, Milan, Italy) for 1 h at 37°C. Two micrograms of purified RNA were reverse transcribed using a mix of random examers/oligoT and 200 units of SuperScript II RT (Invitrogen) for 1 h at 42°C. No enzyme was added for reverse transcriptase-negative controls. To amplify cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems, Monza, Italy) was used according to the manufacturer’s instructions and inventoried TaqMan assays of human KLF2 (Hs00360439_g1 FAM/MBG), human α5-integrin (ITGA4; Hs00168433_m1), human phospholipase D1 (PLD1; Hs00160118_m1), and human Ras p21 protein activator (GTPase activating protein) 1 (RASA1; Hs00963554_m1) probes together with an endogenous control (human hypoxanthine phosphoribosyltransferase 1, Hs99999909_m1 FAM/MBG) probe. PCR assay was performed on a 7300 Time PCR System (Applied Biosystems). After an initial hold for 2 min at 50°C and for 10 min at 95°C, samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s to reach the plateau. We used the ∆∆Ct technique (where C is threshold cycle) to calculate cDNA content in each sample using cDNA expression in static HUVECs as a calibrator.

Cytokine production. The production of monocyte chemotactic protein (MCP)-1, IL-8, IL-6, IL-10, and IL-1β by HUVECs in the static condition or exposed to flow were estimated by measuring their concentrations in samples of HUVEC medium using a Milliplex Map Human kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Briefly, HUVEC medium samples collected after exposure to static or flow conditions were incubated with specific antibody-coated beads overnight at 4°C and with a biotinylated detection antibody for 1 h at room temperature. The reaction mixture was finally incubated with streptavidin-phycocerythrin solution for 30 min at room temperature. A Bio-Plex 200 plate reader (Bio-Rad, Milan, Italy) was used to detect fluorescent signal strength. To confirm the Millipex results, and to evaluate the time course of cytokine production, we performed ELISA using samples of HUVEC medium collected at the end of the different periods of flow exposure. Cytokine concentrations were determined using a commercially available kit (Booster Technology, Fremont, CA, and Thermo Scientific) according to the manufacturer’s instructions. Infinite m200-pro (Tecan Group, Männedorf, Switzerland) was used to measure absorbance. All specimens were tested in duplicate. Cytokine concentrations were calculated from calibration curves using the recombinant protein as the standard. The production rate was calculated using medium concentration, medium volume, and incubation time (expressed in ng hr⁻¹, 10⁶ cells⁻¹).

Cell proliferation assay. Human aortic SMCs (HSMCs; Cell Applications, San Diego, CA) were grown in culture flasks in SMC growth medium specifically designed by the manufacturer (Cell Applications) to promote attachment, spreading, and proliferation. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. HSMCs at the fifth passage were seeded at a density of 8 × 10⁴ cells/well on 96-well plates and used for proliferation experiments when cells reached ~80% confluence. Cell proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were incubated for 24 h with 100 μl of T2 and T4 conditioned medium collected during different experiments of flow exposure on HUVECs, after which 20 μl MTS (1.9 mg/ml, Promega Italia) was added into each well and samples were incubated for 90 min at 37°C. HSMCs exposed to HUVEC testing medium, HSMC growth medium, and HSMC basal medium were used as controls. Four wells per treatment were used for the proliferation assay. The absorbance of the dye was measured at 490 nm using Infinite m200-pro (Tecan Group). Spectrophotometric readings were normalized using HSMC cell-free media as a blank. Results were normalized with respect to the absorbance of cells exposed to HUVEC medium maintained in the static condition.

Statistical analysis. Results are expressed as means ± SD. Data were analyzed using one-way ANOVA, and differences among groups were established using a post hoc Bonferroni’s multiple-comparisons test (Prism, GraphPad Software, La Jolla, CA). The statistical significance level was defined as P < 0.05.

RESULTS

The cone-and-plate device we developed allowed us to handle HUVEC monolayers under sterile conditions and to reproduce realistic WSS waveforms over the cell surface (as shown in Fig. 1). Standard culture conditions were maintained by the cell incubator to study the effects of long-term exposure of ECs to fluid forces. The WSS waveforms we tested are representative of shear forces acting in differentially IH-prone areas in a model of side-to-end AVF. As shown in Fig. 2, the UND waveform was not expected to be associated with IH, whereas REC-1 and REC-2 were characteristic of high-risk stenosis areas in the side-to-end configuration of the AVF.

Effect of WSS on EC shape and the cytoskeleton. As shown in Fig. 3, cell morphology was diversely affected by the three different shear stress waveforms. ECs exposed to the UND flow aligned and elongated in the flow direction compared with cells maintained in the static condition, which showed a randomly oriented cobblestone shape (see Fig. 3). In contrast, HUVECs exposed to reciprocating WSS (REC-1 and REC-2 profiles) did not elongate or aligned and maintained a cobblestone shape similar to cells in the static condition. As shown in Fig. 4, HUVEC monolayers maintained in the static condition showed a random distribution of F-actin fibers, mostly organized in dense peripheral actin bands, with a few fibers crossing the cellular body. HUVECs exposed to the UND waveform showed a loss of peripheral actin bands and a greatly increased number and thickness of F-actin stress fibers across the cell body. These fibers markedly oriented toward the flow direction. In HUVECs exposed to REC-1 and REC-2 waveforms, F-actin fibers remained mostly peripheral, with fibers not oriented in the flow direction, showing a pattern similar to that of cells maintained in the static condition (see Fig. 4). Using the cell morphology evaluation performed after staining of tight junction-associated ZO-1 protein (as shown in Fig. 4), we computed a mean ratio of the major to minor axis for cells exposed to the UND waveform of 5.10 ± 1.68, and this value was significantly (P < 0.01) higher than that calculated in cells exposed to REC-1 and REC-2 waveforms, in which the elongation ratio averaged only 1.64 ± 0.44 and 1.57 ± 0.38, respectively (see Fig. 5A). These values were comparable with those calculated for cells maintained in the static conditions (1.54 ± 0.37). We also quantified cell alignment by computation of the direction of the cell major axis. An average of ≥70% of ECs exposed to the UND waveform had major axes oriented between ±20° and −20° of the flow direction, whereas only 21% and 22% of REC-1- and REC-2-stimulated cells resulted aligned between ±20° and −20° of the flow direction, respectively. We also automatically detected the direction of F-actin fibers using digital image processing, as described above in METHODS. As shown in Fig. 5B, we
quantified that in HUVEC monolayers exposed to the UND waveform, 60% of F-actin fibers were parallel to the flow (within $\pm 15^\circ$ of the flow direction), with only 5% of fibers perpendicular to the flow (within $\pm 15^\circ$). At variance, in cells exposed to both REC-1 and REC-2 waveforms, F-actin fibers oriented between $15^\circ$ and $-15^\circ$ of the flow direction were, in average, only 18% and 10%, respectively.

Effect of shear stress on gene expression. The results of the PCR array used to investigate the expression of 84 genes involved in cytoskeletal remodeling, cell movement, and expression of adhesion molecules allowed us to identify 23 genes whose expression was affected by the flow condition by more than twofold change compared with static controls (as shown in Fig. 6). The remaining genes were either not affected by flow exposure or not detectable. Among the 23 genes differentially expressed, we selected three genes whose expression was most consistently affected by flow conditions (PLD1, ITGA4, and RASA1). The expression of these genes was further confirmed by real-time PCR.

The results of real-time PCR on the expression of the KLF2 gene and of the three previously identified genes are shown in Figs. 7 and 8. The mean KLF2 mRNA level was significantly increased in HUVECs exposed to the UND waveform compared with static HUVECs ($P < 0.01$). In contrast, KLF2 gene expression was not modified in HUVECs stimulated with REC-1 and REC-2 waveforms (see Fig. 7A) and was comparable with that of HUVECs maintained in the static condition. Three of the four selected genes were confirmed to be differentially regulated by the UND profile compared with mRNA expression induced by the static condition or both REC-1 and REC-2 waveforms. Thus, PLD1 mRNA expression was significantly downregulated by UND compared with static conditions and to REC-1 and REC-2 ($P < 0.05$ vs. the static control and $P < 0.01$ vs. REC-1 and REC-2, respectively). The PLD1 mRNA level induced by REC-2 was significantly higher than that in the static condition ($P < 0.05$; Fig. 7B). ITGA4 gene expression was significantly lower for the UND profile compared with REC-1 and REC-2 profiles ($P < 0.01$). Furthermore, ITGA4 mRNA expression by cells exposed to REC-1 was significantly upregulated compared with all other conditions.

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conditions ($P < 0.01$; Fig. 7C). Finally, RASA1 gene expression was downregulated by UND compared with static control conditions and with REC-1 and REC-2 ($P < 0.05$ vs. the static control and vs. REC-1 and $P < 0.01$ vs. REC-2; Fig. 7D).

**Cytokine production.** The results from the Milliplex kit showed increased MCP-1 and IL-8 production by HUVECs cultured under REC-1 and REC-2 waveforms compared with production induced by the UND waveform and the static condition (data not shown). Using the same assay, the production of IL-6 in HUVECs was not regulated by different flow conditions, whereas the production of IL-10 and IL-1β was lower than the detection level. The results of MCP-1 and IL-8 production by ELISA are shown in Fig. 8. MCP-1 production by HUVECs (Fig. 8A) exposed to the UND stimulus (during T1, T2, and T4 periods) was comparable with that of HUVECs maintained in the static condition. MCP-1 production by

| Gene name  | UND | REC-1 | REC-2 | Rho GTpase effectors | Cell adhesion molecules | Integrin receptors | Chemotaxis | Cellular projections | MCP-1 production
|------------|-----|-------|-------|----------------------|------------------------|-------------------|------------|---------------------|----------------
| ACTR2      | 0.66| 1.52  | 1.41  | X                    | X                      |                   |            |                     |                |
| ACTR3      | 0.55| 1.49  | 1.41  | X                    | X                      |                   |            |                     |                |
| ARHGDI     | 1.61| 1.84  | 1.79  | X                    | X                      |                   |            |                     |                |
| BAAP2      | 3.85| 1.71  | 1.69  | X                    | X                      | X                 |            |                     |                |
| CDC42      | 1.44| 3.32  | 1.31  | X                    | X                      |                   |            |                     |                |
| CFL1       | 0.84| 1.27  | 1.09  | X                    | X                      |                   |            |                     |                |
| CRK        | 0.59| 1.13  | 0.69  | X                    | X                      |                   |            |                     |                |
| MSN        | 1.01| 2.01  | 1.65  | X                    | X                      |                   |            |                     |                |
| PLCG1      | 2.97| 2.39  | 1.44  | X                    | X                      |                   |            |                     |                |
| LYM        | 0.41| 1.71  | 0.48  | X                    | X                      |                   |            |                     |                |
| MYL9       | 0.48| 0.95  | 0.56  | X                    | X                      |                   |            |                     |                |
| PAK1       | 0.34| 0.76  | 0.53  | X                    | X                      |                   |            |                     |                |
| PLD1       | 0.24| 1.33  | 1.04  | X X                  | X                      |                   |            |                     |                |
| PRKCA      | 1.84| 1.8   | 1.74  | X                    | X                      |                   |            |                     |                |
| PTK6       | 0.17| 0.52  | 0.42  | X                    | X                      |                   |            |                     |                |
| PTPN1      | 0.61| 1.52  | 1.42  | X                    | X                      |                   |            |                     |                |
| RAC1       | 0.83| 0.96  | 0.72  | X                    | X                      |                   |            |                     |                |
| ROA        | 0.86| 0.84  | 0.93  | X X                  | X                      |                   |            |                     |                |
| ROCK1      | 0.75| 1.71  | 0.93  | X X                  | X                      |                   |            |                     |                |
| STAT3      | 0.96| 1.36  | 1.12  | X                    | X                      |                   |            |                     |                |
| VIM        | 0.44| 0.49  | 0.56  | X                    | X                      |                   |            |                     |                |
| WASF1      | 0.21| 0.55  | 0.42  | X                    | X                      |                   |            |                     |                |
| WASF2      | 0.67| 0.6   | 0.85  | X X                  | X                      |                   |            |                     |                |
| WASL       | 1.44| 0.17  | 0.38  | X                    | X                      |                   |            |                     |                |
| ARF6       | 1.46| 1.97  | 1.38  | X                    | X                      |                   |            |                     |                |
| CAPN1      | 2.16| 1.54  | 0.84  | X                    | X                      |                   |            |                     |                |
| DPPO       | 1.51| 1.38  | 0.64  | X                    | X                      |                   |            |                     |                |

| Gene name  | UND | REC-1 | REC-2 | Rho GTpase effectors | Cell adhesion molecules | Integrin receptors | Chemotaxis | Cellular projections | MCP-1 production
|------------|-----|-------|-------|----------------------|------------------------|-------------------|------------|---------------------|----------------
| ENAH       | 0.59| 1.48  | 0.86  | X                    | X                      |                   |            |                     |                |
| EZR        | 2.85| 2.11  | 1.69  | X                    | X                      |                   |            |                     |                |
| DIAPH1     | 1.6  | 1.73  | 2.03  | X                    | X                      |                   |            |                     |                |
| MYH9       | 0.77| 1.47  | 1.13  | X X                  | X                      | X X X X           |            |                     |                |
| MYL10      | 0.21| 0.76  | 0.45  | X X                  | X                      | X X X X           |            |                     |                |
| MMP2       | 0.66| 0.98  | 0.85  | X                    | X                      |                   |            |                     |                |
| MMP14      | 0.63| 1.06  | 0.62  | X                    | X                      |                   |            |                     |                |
| MSN        | 1.01| 2.01  | 1.65  | X                    | X                      |                   |            |                     |                |
| PTK2       | 2.43| 1.73  | 1.3   | X X                  | X                      | X X X X           |            |                     |                |
| PXN        | 0.72| 1.08  | 0.62  | X                    | X                      |                   |            |                     |                |
| RASA1      | 0.43| 0.78  | 0.7   | X                    | X                      |                   |            |                     |                |
| RDX        | 0.59| 1.49  | 1.03  | X                    | X                      |                   |            |                     |                |
| TLN1       | 0.79| 1.67  | 1.16  | X                    | X                      |                   |            |                     |                |
| VCL        | 0.59| 1.36  | 0.81  | X                    | X                      |                   |            |                     |                |
| VEGFA      | 1.21| 0.82  | 0.88  | X X                  | X                      |                   |            |                     |                |
| WIPF1      | 1.20| 0.65  | 0.75  | X X                  | X                      |                   |            |                     |                |
| TGF81      | 0.78| 1.45  | 1.71  | X                    | X                      |                   |            |                     |                |
| CAPN2      | 1.73| 1.47  | 1.66  | X                    | X                      |                   |            |                     |                |
| CAV1       | 1.04| 1.85  | 1.04  | X                    | X                      |                   |            |                     |                |
| CSF1       | 0.58| 0.85  | 0.56  | X                    | X                      |                   |            |                     |                |
| ILK        | 0.98| 1.75  | 0.91  | X                    | X                      |                   |            |                     |                |
| ITGAA       | 0.22| 1.38  | 0.83  | X                    | X                      |                   |            |                     |                |
| ITGB1      | 0.34| 1.28  | 0.66  | X                    | X                      |                   |            |                     |                |
| ITGB3      | 0.72| 1.25  | 1.04  | X                    | X                      |                   |            |                     |                |
| MET        | 0.41| 2.61  | 1.47  | X                    | X                      |                   |            |                     |                |
| TIMP2      | 0.71| 0.57  | 0.82  | X                    | X                      |                   |            |                     |                |

Fig. 5. Morphological adaptations of HUVECs to different flow conditions. A: cell elongation expressed as the ratio of major to minor cell axis. B: distribution of F-actin fiber orientation in the flow direction. **$P < 0.01$, UND vs. all other groups.

Fig. 6. WSS induced differential mRNA expression. Genes that were downregulated ($<0.5$) are shown in green and genes that were upregulated ($>2$) are shown in red compared with the static condition. Three genes that were more consistently affected by UND and reciprocating WSS waveforms are highlighted in red. PLD1, phospholipase D1; RASA1, Ras GTpase activating protein 1; ITGA4, α4-integrin.
HUVECs exposed to reciprocating WSS was numerically higher compared with the UND waveform and the static condition. At T1, MCP-1 production with the REC-2 waveform was significant higher than the UND waveform or the static condition (P<0.01), and the increase in MCP-1 production with the REC-2 waveform was also significant during the T4 period compared with the UND waveform (P<0.05).

IL-8 production by HUVECs exposed to the static condition and the UND waveform was comparable (see Fig. 8B). At T2, IL-8 production by HUVECs exposed to the UND waveform was only numerically higher than in the static control condition. IL-8 production by cells exposed to REC-1 and REC-2 waveforms progressively increased with time and was significantly higher during the T4 period compared with the static control and the UND waveform (P<0.01 and P<0.05, respectively).

Cell proliferation assay. Medium collected during HUVEC flow exposure modulated HSMC growth. As shown in Fig. 8C, medium conditioned by HUVECs exposed to the UND waveform during the T2 period significantly lowered SMC proliferation compared with the static control condition (P<0.01, UND vs. all other groups; Fig. 8D).

**DISCUSSION**

The long-term patency of AVF remains an unsolved major clinical problem for chronic hemodialysis treatment (56). There is an increasing body of evidence that WSS plays a pivotal role not only in AVF maturation but also into the development of IH (25). However, the molecular mechanisms activated by WSS mechanotransduction in these extreme hemodynamic conditions remain to be precisely elucidated. The role of WSS in vascular disease has been investigated in numerous studies, with most of them addressing the localization and development of atherosclerotic plaques (15, 74). These studies indicated that low and oscillatory shear stress, acting on ECs, activates several molecular mechanisms related to cell activation, expression of adhesion molecules, adhesion of circulating cells, and deposition of the extracellular matrix and fatty material. Like atherosclerosis, the IH that develops in AVF is preferentially located where the endothelium experiences oscillating flows. In the present study, we wondered whether unsteady and oscillatory shear stresses that are present after surgical procedure in areas of the AVF prone to develop IH might be responsible for the changes in EC function and structure that may be linked to the development of IH.
investigations have specifically addressed the effects of the nonphysiological hemodynamic conditions that develop in AVF anastomosis on EC structure and function.

In our present investigation, we studied the structural and functional changes induced in vitro on ECs exposed to these specific WSS waveforms. For this, we selected a location of the AVF numeric model previously studied and characterized by unidirectional flow in the venous side of the AVF, far from the anastomosis, that is not expected to develop IH. WSS profiles were derived from a previously investigated AVF model (21). We compared the effects of mechanical stimulation derived from a relative protected area of AVF (UND) with those derived from two areas typically reported to be prone to develop IH (REC-1 and REC-2) (22, 65). The experimental device we used allowed us to accurately reproduce fast variations in time of the shear stress waveforms. In line with the well-known response of ECs to shear stress (13), we observed important structural and functional changes induced by UND flow that develop in the venous side of AVF, far from the anastomosis. Thus, after 48 h of exposure to this condition, cells were strongly aligned and elongated. The exposure to the UND waveform also induced EC reorganization of stress fibers within the cell body that strongly aligned with the flow direction. In contrast, HUVECs exposed to REC-1 and REC-2 waveforms did not show these structural changes at all and did not develop stress fiber organization in the flow direction.

In line with other investigations available in literature (13, 18, 72, 78), our present results confirmed that EC expression of mRNA coding for KLF2 was significantly increased by cell exposure to UND WSS. At the same time, this flow condition induced a decrease in the mRNA expression of PLD1, ITGA4, and RASA1. In contrast, cells exposed to REC-1 and REC-2 waveforms showed an opposite pattern of gene expression. Thus, reciprocating flow conditions did not increase KLF2 mRNA expression, which was comparable with static cultures, whereas a consistent increase in the mRNA expression of PLD1, ITGA4, and RASA1 genes was observed compared with the UND waveform and the static condition.

It has been previously demonstrated that KLF2 induces endothelial nitric oxide synthase expression, with consequent anti-inflammatory and anti-coagulant effects (14, 19, 26). KLF2 also inhibits the proinflammatory induction of adhesion molecules (VCAM-1 and E-selectin) that are involved in leukocyte adhesion and extravasation (64). In addition, it is known that sustained mRNA expression of KLF2 has several downstream effects, such as an antioxidant state, reduced cell proliferation, and the prevention of apoptosis (17, 27, 49). Our findings suggest that UND flow is necessary to maintain KLF2-related protective function in ECs and toward other cellular components of the vessel wall. In contrast, in regions where disturbed blood flow induces reciprocation of the WSS direction, as in the vein just-anastomotic site of AVF, the
The protective effects of KLF2 are lost and ECs may become proinflammatory and proadhesive toward circulating leukocytes.

The increase in the expression of PLD1, ITGA4, and RASA1 genes by ECs we have observed between unidirectional versus reciprocating flow would reinforce this hypothesis. PLD1 is known to mediate hydrolysis of phosphatidylcholine and cytoskeletal remodeling (61). PLD1 activation is also involved in vWF secretion by ECs, suggesting a critical role in the regulation of thrombosis (20). Furthermore, a number of studies have reported that vWF might stimulate noncanonical activities of SMCs (52), leading to intimal thickening (16, 43). Taken together, these findings indicate a potential role for PLD1 in the development of IH in areas of the vascular wall subjected to reciprocating flow where PLD1 is overexpressed, whereas unidirectional flow lowers PDL1 gene expression.

ITGA4 (CD49d) is a protein that forms the α1β1-integrin (very late antigen-4) dimer. Very late antigen-4 is known to be expressed in monocytes and causes monocyte adhesion to ECs and transendothelial migration (8, 60) CD49d, however, is also expressed in ECs, where it is involved in cell elongation. Localization of CD49d phosphorylation imposes a spatial restriction of Rac to the leading side of migrating cells (47). Upregulation of the ITGA4 gene, as we found in ECs exposed to REC-1 and REC-2 waveforms, may be related to a continuous attempt of the cell to establish a preferential direction for elongation and alignment that is impeded by reciprocating flows for continuous changes in the WSS direction.

RASA1 (p120-RasGAP) is a protein considered protective for the endothelium, since it inhibits Ras activity by hydrolysis of GTP to GDP (45) and interacts with p190-RhoGAP to form a membrane complex that blocks Rho activity (2, 37, 50). These two functions seem to be inconsistent with the detrimental effects induced by oscillating WSS in ECs. However, we may consider RASA1 upregulation as a consequence of nonstable cytoskeletal remodeling. A potentially negative effect in ECs causing RASA1-sustained upregulation could be related to HGF receptor (MET) signaling (70). Adaptor protein GRB2-associated-binding protein 1 (GAB1) phosphorylates upon association with MET and provides a binding site for other proteins, such as p120-RasGAP. The formation of this membrane complex prevents Ras activation. Tyrosine phosphatase Src homology protin 2 can, however, dephosphorylate GAB1, causing the detachment of p120-RasGAP and leading to Ras activation (42, 44). To our knowledge, this is the first time that differential expression of RASA1 by ECs has been related to WSS. Further studies are necessary to investigate the role of this gene in WSS-induced cytoskeletal remodeling.

In addition to important effects on EC structure and gene expression, we found that REC-1 and REC-2 waveforms induced increased production of MCP-1 and IL-8, a phenomenon not observed in ECs exposed to UND flow. These results are in line with previous investigations on the effect of oscillating flow in ECs (13, 71) and support the hypothesis that in selected locations of the AVF, local infiltration of inflammatory cells may be promoted by flow-induced EC activation, with consequent deleterious effects on IH (29, 34). Finally, we investigated the potential paracrine proliferative EC signaling induced by unidirectional and oscillating flows using medium conditioned during HUVEC flow exposure on the proliferation of HSMCs. The results showed a limited but statistical significantly higher proliferation of SMCs compared with that observed with medium conditioned by HUVECs exposed to the UND profile. Likely, the small effect quantified is due to the short time duration (24 h) of cell incubation. These findings represent evidence that ECs subjected to unidirectional blood flow have a protective function toward the other cellular components of the vessel wall, the SMCs. On the contrary, oscillating WSS may be responsible for the initiation of a cascade of events in ECs that induces SMC proliferation and the development of IH.

In uremic patients, even before AVF surgery, several factors may impair EC function, such as end-stage chronic kidney disease, age, cardiovascular disease, and diabetes. Actually, it has been reported that in patients with chronic kidney disease, preexisting IH is already present in venous samples obtained during vascular surgery for primary access placement (38, 75). This evidence suggests that EC function, which is dependent from physiological stimulation of blood flow, may be impaired in these patients and that local disturbed flow conditions may even more reduce the physiological function of ECs for protection of the vascular wall against inflammation, oxidative stress, cell proliferation, and even platelet adhesion and thrombosis (35, 73). Thus, the role of the hemodynamic effects of unsteady flow we have suggested on the basis of the present results could be even more important in the clinical setting in patients with chronic kidney disease. Actually, it is difficult to demonstrate this in the in vitro setting, since ECs from uremic patients or experimental animal models should be used. In addition, the in vitro setting may not be adequate to maintain the uremic milieu for ECs.

The unsteady flow conditions that develop in AVF cannot be easily modified or prevented. It would be important to limit secondary flows that induce reciprocating shear stresses. However, the shape of side-to-end anastomosis is rather generally established and it is characterized by the angle between the artery and vein. We have shown numerically that a narrow angle is the better strategy to minimize areas exposed to oscillatory shear stress (21). Very encouraging results were obtained in the clinical setting using a narrow-angle anastomosis technique (5). Another possibility to limit the deleterious effects of oscillatory shear on ECs would be to pharmacologically protect EC function, for example, with drugs such as statins, which are known to protect endothelial function (72) by preventing endogenous cholesterol synthesis (33). These molecules are known to induce KLF2 expression by ECs and to exert a protective action toward vascular cells (63). The protective effects of these treatments on AVF function, however, have not been proven so far (51, 57). In line with our results, studies on new ways to protect EC function are now worthy of being investigated.

In conclusion, our present results demonstrate that different hemodynamic forces importantly affect EC structure and function and, in particular, that UND shear forces are important for maintaining physiological structure and protective functions of the vessel wall. In contrast, reciprocating shear stresses induced by disturbed flow conditions on the venous side of the AVF alter EC functions and initiate a cascade of events that lead to EC activation, interaction of ECs with circulating cells, and the proliferation of underlying SMCs. These events may trigger and sustain the development of IH in AVF wall areas exposed to disturbed flow.
EFFECTS OF AVF-DERIVED SHEAR STRESS ON ENDOTHELIAL CELLS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M. Franzoni, I.C., L.L., and M. Figliuzzi performed experiments; M. Franzoni, I.C., and A.R. prepared figures; M. Franzoni, I.C., L.L., and A.R. edited and revised manuscript; A.R. approved final version of manuscript; I.C., L.L., B.E.-I., and A.R. drafted manuscript; M. Franzoni, I.C., L.L., M. Figliuzzi, B.E.-I., and A.R. analyzed data; L.L., M. Figliuzzi, and A.R. interpreted results of experiments; L.L., M. Figliuzzi, B.E.-I., and A.R. edited and revised manuscript; A.R. conception and design of research.

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