Shear stress-stimulated endothelial cells induce smooth muscle cell chemotaxis via platelet-derived growth factor-BB and interleukin-1α

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Objective: **Vascular smooth muscle cell (SMC) migration is critical to the development of atherosclerosis and neointimal hyperplasia. Hemodynamic forces such as shear stress and cyclic strain stimulate endothelial cell signal-transduction pathways, resulting in the secretion of several factors, including SMC chemoattractants such as platelet-derived growth factor (PDGF). We hypothesized that mechanical forces stimulate endothelial cells to secrete SMC chemoattractants to induce migration via the mitogen-activated protein kinase (MAPK) pathway.**

Methods: **Bovine aortic endothelial cells were exposed to shear stress, cyclic strain, or static conditions for 16 hours. The resulting conditioned medium was used as a SMC chemoattractant in a Boyden chamber. Activation of SMC extracellular signal-regulated protein kinase 1/2 (ERK1/2) was assessed by Western blot analysis. Pathways were inhibited with anti-PDGF-BB or anti-interleukin-1α (IL-1α) antibodies, or the ERK1/2 upstream pathway inhibitor PD98059.**

Results: **Conditioned medium from endothelial cells exposed to shear stress corresponding to arterial levels of shear stress stimulated SMC migration but lower levels of shear stress or cyclic strain did not. Both PDGF-BB and IL-1α were secreted into the conditioned medium by endothelial cells stimulated with shear stress. Both PDGF-BB and IL-1α stimulated SMC chemotaxis but were not synergistic, and both stimulated SMC ERK1/2 phosphorylation. Inhibition of PDGF-BB or IL-1α inhibited SMC chemotaxis and ERK1/2 phosphorylation.**

Conclusion: **Shear stress stimulates endothelial cells to secrete several SMC chemoattractants, including PDGF-BB and IL-1α; both PDGF-BB and IL-1α stimulate SMC chemotaxis via the ERK1/2 signal-transduction pathway. These results suggest that the response to vascular injury may have a common pathway amenable to pharmacologic manipulation. (J Vasc Surg 2005;41:321-31.)**

Clinical Relevance: **One difficulty in the pharmacologic treatment of atherosclerosis or neointimal hyperplasia leading to restenosis is the multiplicity of activated pathways and thus potential treatment targets. This study demonstrates that shear stress, a hemodynamic force that may be a biologically relevant stimulus to induce vascular pathology, stimulates endothelial cells to secrete PDGF-BB and IL-1α. Both of these mediators stimulate the SMC ERK1/2 pathway to induce migration, a critical event in the pathogenesis of atherosclerosis and neointimal hyperplasia. Therefore, this study suggests a relevant common target pathway in SMC that is amenable to manipulation for clinical treatment.**

Vascular smooth muscle cell (SMC) proliferation and migration from the media to the intima are critical events in the development of atherosclerosis and neointimal hyperplasia. SMC proliferation and migration are regulated by several factors, including cytokines that may be released by cells within the vessel wall or from cells circulating within the blood.[1-3]

Platelet-derived growth factor (PDGF) is a strong chemoattractant for SMC migration and is produced by endothelial cells, SMC, platelets, or monocytes.[4-5] PDGF has been shown to be required for accumulation of SMC in the neointima in the rat balloon-injury model[6-7] and may stimulate SMC migration by inducing phosphorylation and activation of SMC mitogen-activated protein kinase (MAPK) intracellular signaling pathways.[8-10]

Although the entire blood vessel is subjected to hemodynamic forces such as blood pressure and cyclic strain, only endothelial cells are ordinarily exposed to shear stress, the lateral frictional force of the flowing blood on the endothelial cell apical membrane. Hemodynamic forces induce endothelial cell proliferation, cytoskeletal reorganization, growth factor production, MAPK signal transduction, phosphatidylinositol-3′-kinase (PI3K) signal transduction, as well as activation of other endothelial cell intracellular pathways.[11-15] The endothelial cell response to hemodynamic forces may result in the synthesis and secretion of SMC mitogens and chemoattractants.[16-19] In particular, several studies have demonstrated that exposure of endothelial cells to laminar shear stress or cyclic strain induces PDGF-A and -B chain mRNA expression in vitro.[12,20-22]

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Shear stress stimulates additional endothelial cell pathways, such as the mammalian target of rapamycin pathway, leading to expression of pp70s6 kinase, the protein kinase B/Akt pathway, and the Sma- and Mad-related protein pathway, as well as endothelial cell production of nitric oxide and integrins.[23-25] We hypothesized that shear stress stimulates endothelial cells to synthesize and secrete several proteins, including and in addition to PDGF, that stimulate SMC chemotaxis. We examined interleukin-1α (IL-1α) because it is secreted by endothelial cells in response to shear stress, stimulates SMC migration, and stimulates the SMC extracellular signal-regulated protein kinase (ERK) 1/2 pathway, as does PDGF-BB. We report that endothelial cells secrete PDGF-BB and IL-1α in response to shear stress, and that both PDGF-BB and IL-1α secreted by endothelial cells in response to shear stress stimulate SMC chemotaxis by the ERK1/2 pathway.

MATERIALS AND METHODS

Cell culture. Bovine aortic endothelial cells and SMC were obtained from freshly killed calves at a local slaughterhouse, as previously described.[26] In brief, endothelial cells were obtained by gently scraping the intimal surface of the thoracic aorta. SMC were obtained by cleaning the aorta of intima and adventitia, mincing it into small pieces, and culturing in 10% fetal bovine serum (FBS) until the SMC migrated away from the tissue.

Cells were maintained in Dulbecco modified Eagle medium F-12 (GIBCO BRL, Gaithersburg, Md), supplemented with 10% FBS (Hyclone Laboratories, Logan, Utah), 5 μg/mL deoxycytidine (Sigma Chemical, St. Louis, Mo), 5 μg/mL thymidine (Sigma Chemical), and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL; and amphotericin B, 250 ng/mL, GIBCO BRL). Cells were grown to confluence at 37°C in a humidified incubator with 5% carbon dioxide.

Endothelial cells were identified by their typical cobblestone appearance and indirect immunofluorescence staining for factor VIII antigen and lack of SMC α-actin staining. SMC were identified by their typical hill and valley morphology and by indirect immunofluorescent staining for α-actin and lack of endothelial cell factor VIII staining.

Cell populations were more than 99% pure for all samples. Cells used in this study were between passages 3 to 9. Cells were synchronized prior to hemodynamic force exposure by being incubated in serum-free media (SFM) for 48 hours.

Shear stress and cyclic strain application. Endothelial cells were seeded on 6-well plates coated with collagen I, without any SMC, and treated with 30 or 210 rpm oscillatory shear stress for 1 to 16 hours by using an orbital shaker (DS-500, VWR International, West Chester, Penn), as previously described.[23,27-28] A frequency of oscillation of 30 rpm produces approximately 0.5 dyne/cm^2 shear stress, and a frequency of 210 rpm produces approximately 9.8 dyne/cm^2 shear stress at the bottom of a single well in a standard 6-well culture plate[23,27]; measurement of these values with optical velocimetry confirmed that frequencies of oscillation of 30 or 210 rpm produce less than 0.1 dyne/cm^2 or 11.1 dyne/cm^2, respectively, in our orbital shaker (unpublished data). These measurements correspond to published reports that a frequency of oscillation of 200 rpm corresponds to arterial levels (11.5 dynes/cm^2) of shear stress.[23]

Some endothelial cells were seeded on flexible silicone membranes coated with collagen I (Flexcell International Corp, McKeesport, Penn). Cyclic strain was applied by using a Flexercell Strain Unit (Flexcell FX-2000; Flexcell International Corp) with 150 mm Hg of vacuum, which produces an average strain of 10% at a frequency of 60 cycles/min, for 16 hours at 37°C.[11]

Conditioned medium (CM) was collected after 1 to 16 hours of exposure to shear stress or cyclic strain, or from control cells exposed to static conditions, and used as a chemoattractant for SMC migration. In some experiments, actinomycin D (10 μg/mL) was included in the medium overlying the endothelial cells for the 4 hours prior to CM collection to determine whether shear stress stimulated endothelial cell protein synthesis.

SMC migration assay. A modified Boyden 48-well microchemotaxis chamber with a polycarbonate polyvinylpyrrolidone-free membrane with 8-micrometer pores (NeuroProbe Inc, Gaithersburg, Md) was used as previously described.[29] In the lower chamber, 30 μL of CM or control media (10% FBS, SFM, or SFM containing PDGF-AA, PDGF-BB, IL-1α, or IL-1ra [human recombinant; Calbiochem, La Jolla, Calif]) was placed as a chemoattractant. The cell suspension (50,000 SMC in 50 μL SFM) was placed in the top chamber, and the Boyden chamber was incubated at 37°C for 4 hours. The membrane was removed, fixed in 70% ethyl alcohol, stained with hematoxylin, and mounted on a slide. The cells on the upper surface were mechanically removed, and the cell nuclei remaining on the undersurface of the filter were counted under high power magnification (×400) for five fields per well.

In migration inhibition experiments, neutralizing antibodies against PDGF-AA, PDGF-BB, IL-1α, IL-1 receptor, insulin like growth factor-1 (IGF-1), heparin-binding epidermal growth factor-like growth factor (HB-EGF), transforming growth factor-β (TGF-β) (Calbiochem), or antiphospho ERK1/2 (Cell Signaling, Beverly, Mass) were added to the chemoattractant at various concentrations.

For MAPK inhibition studies, PD98059 or dimethyl sulfoxide (DMSO) (Calbiochem) was diluted in SFM (10 μmol/L)[10,30] and preincubated with SMC for 30 minutes at 37°C prior to placement in the Boyden chamber.

PDGF-BB, IL-1α measurement. The concentration of PDGF-BB or IL-1α was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn) according to the manufacture's instructions. The optical density at 405 nm was measured by using a microplate spectrophotometer (Dynatech Laboratories Inc, Indianapolis, Ind). Four samples were measured in each group.

ERK1/2 determination. SMC were exposed to SFM, CM, PDGF-BB (0.5 ng/mL), or IL-1α (0.1 pg/mL) for 30 minutes. For the MAPK inhibition studies, PD98059 or DMSO was diluted in SFM (10 μmol/L) and preincubated with SMC for 1 hour at 37°C. After stimulation, SMC were

washed twice with ice-cold phosphate-buffered saline solution and scraped in lysis buffer containing 50 mmol/L HEPES (pH 7.4), 150mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride.

The cell extracts were centrifuged at 15,000*g* for 10 minutes. The supernatant was collected and protein content measured by the Bradford method. Laemmli sample buffer was added to equivalent protein amounts of each sample, followed by boiling for 5 minutes. Samples were resolved by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blots were incubated in 5% nonfat dry milk with antibodies to antiphospho ERK1/2 or anti-total ERK1/2 (Cell Signaling), prior to detection of immunoreactivity by enhanced chemiluminescence (Amersham Life Science Inc, Arlington Heights, Ill).

Statistical analysis. Data were calculated for each group as mean ± SEM and were compared by analysis of variance; selected subgroups were compared by using the Scheffé post hoc test. The data were computed with the StatView 5.0.1 software (SAS Institute Inc, Cary, NC); $P \leq .05$ was considered significant.

RESULTS

CM stimulates SMC migration. To determine whether shear stress stimulates endothelial cell secretion of SMC chemoattractants, endothelial cells were exposed to shear stress for 16 hours in SFM and then the CM was collected and used as a chemoattractant for SMC migration. SMC migration was stimulated by medium conditioned by endothelial cells exposed to shear stress, corresponding to arterial levels of shear stress (CM 210 rpm), to a similar degree as 10% FBS or PDGF-BB (Fig 1, *A*). Medium conditioned by endothelial cells not exposed to shear stress (CM static), low levels of shear stress (CM 30 rpm), cyclic strain (CM cyclic strain), or medium containing PDGF-AA alone, did not significantly stimulate SMC migration (Fig 1, *A*).

Medium conditioned by shear stress, corresponding to arterial levels of shear stress (CM 210 rpm), that was either boiled or freeze-thawed lost the ability to stimulate SMC migration (data not shown), suggesting that proteins secreted into the CM were responsible for attracting SMC migration. In addition, CM that was passed through a 0.2-micrometer pore filter had the same ability to stimulate SMC migration as unfiltered CM (data not shown), suggesting that proteins were secreted into the CM and were not attached to potentially broken pieces of cell membranes, and thus the shear stress treatment did not damage the endothelial cells.

To determine the time course of protein secretion, CM was collected after 1 to 16 hours to determine its ability to attract SMC migration; SMC migration was stimulated to a greater extent by endothelial cells exposed to shear stress (SS-CM), compared with endothelial cells exposed to static conditions (static-CM), by as early as 1 hour (Figure 1, *B*). However, maximal SMC attraction was achieved by 16 hours, suggesting that endothelial cells secrete proteins into the CM in response to shear stress, both upon initial exposure as well as several hours later.

To determine whether shear stress stimulated endothelial cell protein synthesis, endothelial cells were preincubated with actinomycin D (10 μg/mL) for 4 hours prior to medium collection after 4 or 16 hours of shear stress exposure. The ability of CM to attract SMC migration was unchanged at 4 hours in the presence of actinomycin D, whereas this ability was decreased at 16 hours (Fig 1, *C*), suggesting that shear stress stimulates endothelial cell protein synthesis and secretion of proteins into the CM by 16 hours, whereas secretion of protein at earlier time points may be due to release of previously synthesized proteins.

PDGF-BB stimulates SMC migration. Because shear stress stimulates endothelial cells to secrete PDGF-BB, and PDGF-BB is a potent SMC chemoattractant, we determined whether SMC migration induced by shear stress-CM was due to PDGF-BB secreted into the CM by endothelial cells exposed to shear stress. An ELISA assay was used to directly measure PDGF-BB secreted into the medium; endothelial cells exposed to shear stress, corresponding to arterial levels of shear stress (210 rpm), secreted more PDGF-BB into the CM compared with endothelial cells exposed to static conditions (0 rpm), low levels of shear stress (30 rpm), or cyclic strain (Figure 2, *A*). To determine whether PDGF-BB in the CM was physiologically active, neutralizing antibodies to PDGF-BB were added to medium conditioned by endothelial cells exposed to shear stress and SMC migration determined. Anti-PDGF-BB antibody inhibited SMC migration in a dose-dependent fashion, whereas anti-PDGF-AA did not; anti-PDGF-BB (10 μg/mL) inhibited SMC migration induced by CM by 37% and inhibited SMC migration induced by PDGF-BB (5 ng/mL) by 82% (Fig 2, *B*).

IL-1α stimulates SMC migration. Because anti-PDGF-BB inhibited CM-induced SMC migration by only 37% compared with 82% inhibition of PDGF-BB–induced SMC migration, it was possible that the CM contained additional factors that stimulated SMC migration other than PDGF-BB. To determine whether additional SMC chemoattractants were present in CM, an excess amount of neutralizing antibodies directed against other cytokines known to be secreted by endothelial cells in response to shear stress and that stimulate SMC migration were also added individually to the CM. Antibodies against IL-1α inhibited SMC migration; however, excess amounts of antibodies against IGF-1, HB-EGF, or TGF-β (10 μg/mL) did not inhibit SMC migration (data not shown). The inhibition of CM-induced SMC migration by anti-IL-1α was dose-dependent, with maximal inhibition (62%) occurring with 10 ng/mL anti-IL-1α (Fig 2, *C*).

Since IL-1α is known to be biologically active at very low (picomolar) concentrations, and low concentrations of anti-IL-1α inhibited SMC migration induced by CM, low concentrations of IL-1α were tested as a chemoattractant for SMC migration in the absence of serum or conditioned medium. IL-1α stimulated SMC migration in a dose-dependent fashion, with maximal stimulation of migration at

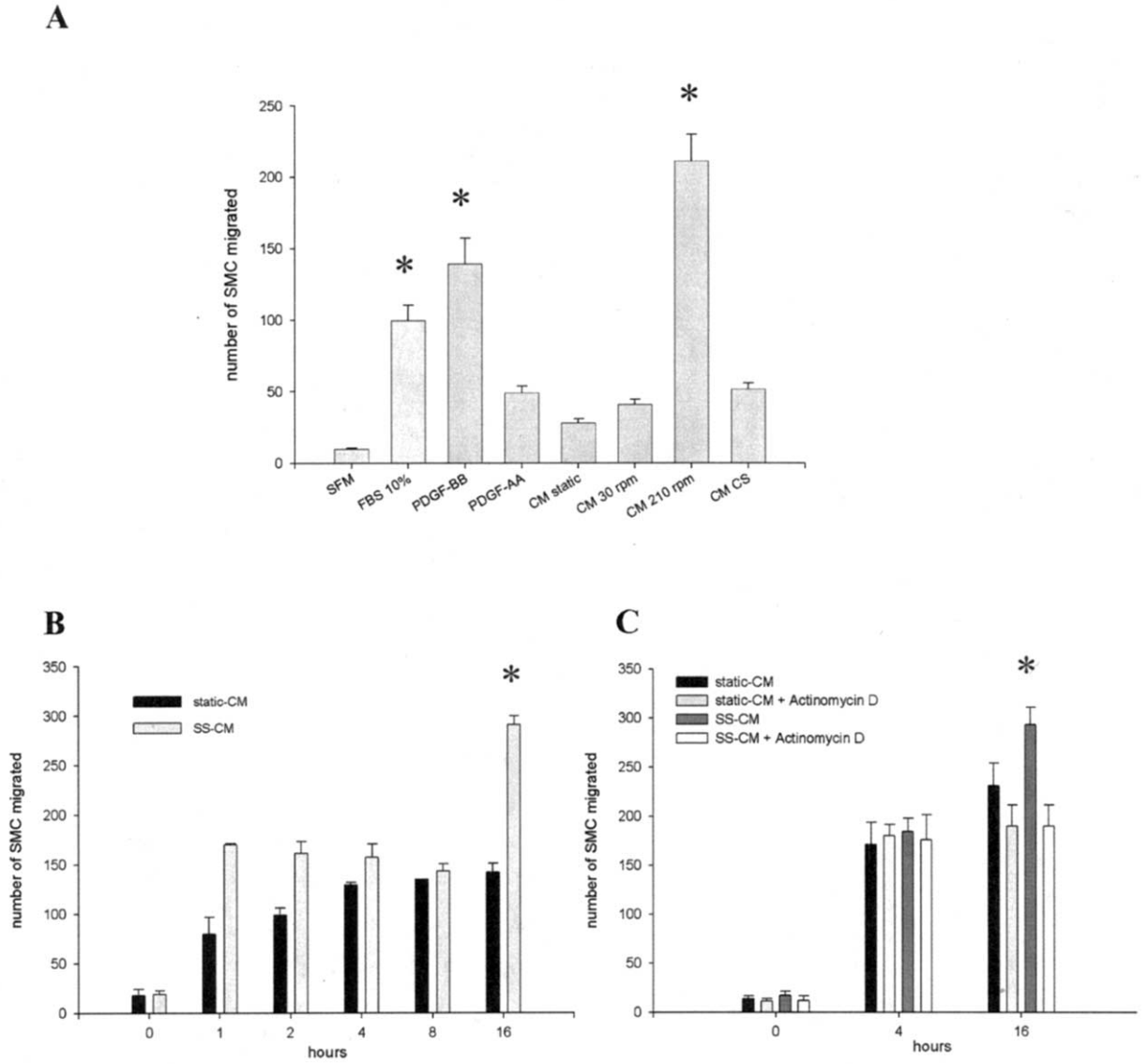

Fig 1. Shear stress-stimulated endothelial cell-derived conditioned medium (CM) stimulates smooth muscle cell (SMC) chemotaxis. **A,** Stimulation of SMC migration in response to CM. The increase in SMC migration in response to medium conditioned by shear stress (SS), corresponding to arterial levels of SS (CM 210 rpm), or positive controls (FBS 10%, PDGF-BB) was significant compared with static conditions (SFM or CM static) or PDGF-AA (n=3, $*P <$ .05); medium conditioned by low levels of SS (CM 30 rpm) or cyclic strain (CM CS) did not stimulate SMC migration. PDGF-BB and PDGF-AA were 0.5 ng/mL, in SFM (SFM, serum-free medium; FBS, fetal bovine serum; PDGF, platelet-derived growth factor). **B,** SMC migration in response to CM is time-dependent. The difference in migration due to CM obtained under static (0 rpm, static-CM) and SS conditions (210 rpm, SS-CM) is significant (n = 7, $P <$.0001, ANOVA) as is the difference over time ($P <$ .0001). **C,** SMC migration in response to CM depends on SMC protein synthesis after 16 but not 4 hours. The difference in SMC migration due to SS-CM, at 16 hours, between groups receiving actinomycin D or not, is significant (time 16 hours, last 2 bars; n = 7; $*P =$ .003, ANOVA).

0.1 pg/mL, achieving approximately 65% of the SMC migration attracted by CM (Fig 3, *A*). Identical concentrations of IL-1ra, an inactive form of IL-1α, did not stimulate SMC migration (Fig 3, *A*). As this concentration of IL-1α was below the limit of detection of an IL-1α ELISA, the concentration of IL-1α used to attract SMC migration was confirmed by ELISA prior to dilution (data not shown). Antibodies to IL-1α inhibited the SMC migration stimulated by IL-1α, whereas a similar isotype but unrelated antibody did not inhibit IL-1α–induced SMC migration (Fig 3, *B*). Similarly, an antibody to the IL-1 receptor also inhibited IL-1α–induced SMC migration (Fig 3, *B*).

Because IL-1α stimulated SMC migration to a slightly lesser extent than PDGF-BB, the response to both stimuli

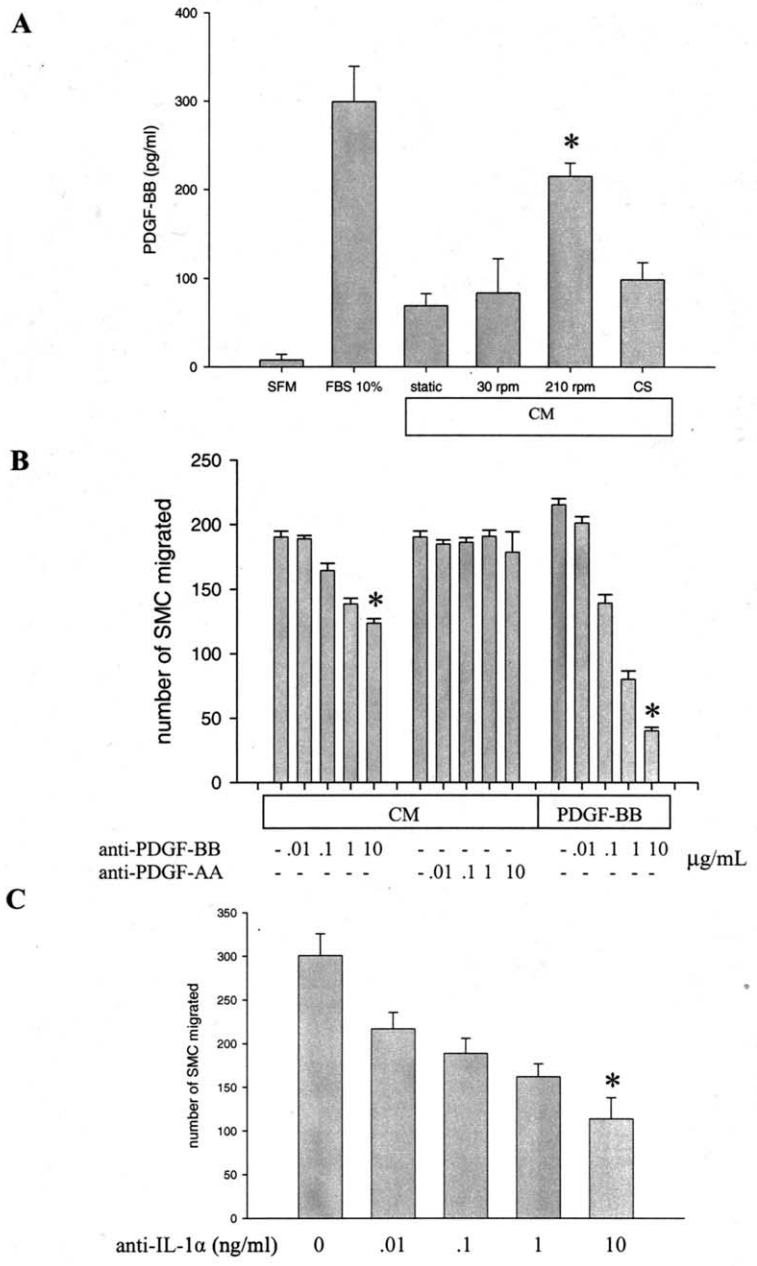

Fig 2. Conditioned medium stimulates smooth muscle cell (SMC) migration via platelet-derived growth factor-BB (PDGF-BB) and interleukin-1α (IL-1α). **A,** Enzyme-linked immunoabsorbent assay (ELISA) determination of the PDGF-BB concentration (pg/mL) in serum-free medium (SFM), fetal bovine serum (FBS) (10%), or conditioned medium (CM) under static conditions (0 rpm), 30 rpm SS, 210 rpm shear stress (SS), or cyclic strain (CS), after 16 hours. The difference between the amount of PDGF-BB in CM 210 rpm and the other groups of CM is significant (n = 4; **P* = .02, ANOVA). **B,** Dose-dependent inhibition of SMC migration with anti-PDGF-BB but not anti-PDGF-AA. Inclusion of anti-PDGF-BB (10 µg/mL) decreased SMC migration 37% in response to CM and 82% in response to PDGF-BB (5 ng/mL; n = 3; **P* < .0001, ANOVA). **C,** Dose-dependent inhibition of SMC migration with neutralizing antibodies (0 to 10 ng/mL) directed against IL-1α. Maximal inhibition (62%) occurred with 10 ng/mL anti-IL-1α (n = 4, **P* = .0006, ANOVA).

were tested simultaneously; exposure to both PDGF-BB and IL-1α did not stimulate SMC migration beyond the response to either one alone (Fig 3, *C*). Similarly, inhibition of SMC migration induced by CM was inhibited slightly less by antibodies to IL-1α than by antibodies to PDGF-BB, and simultaneous use of both antibodies did not result in a further decrease in SMC migration (Fig 3, *C*). These results suggest lack of synergy between the ability of

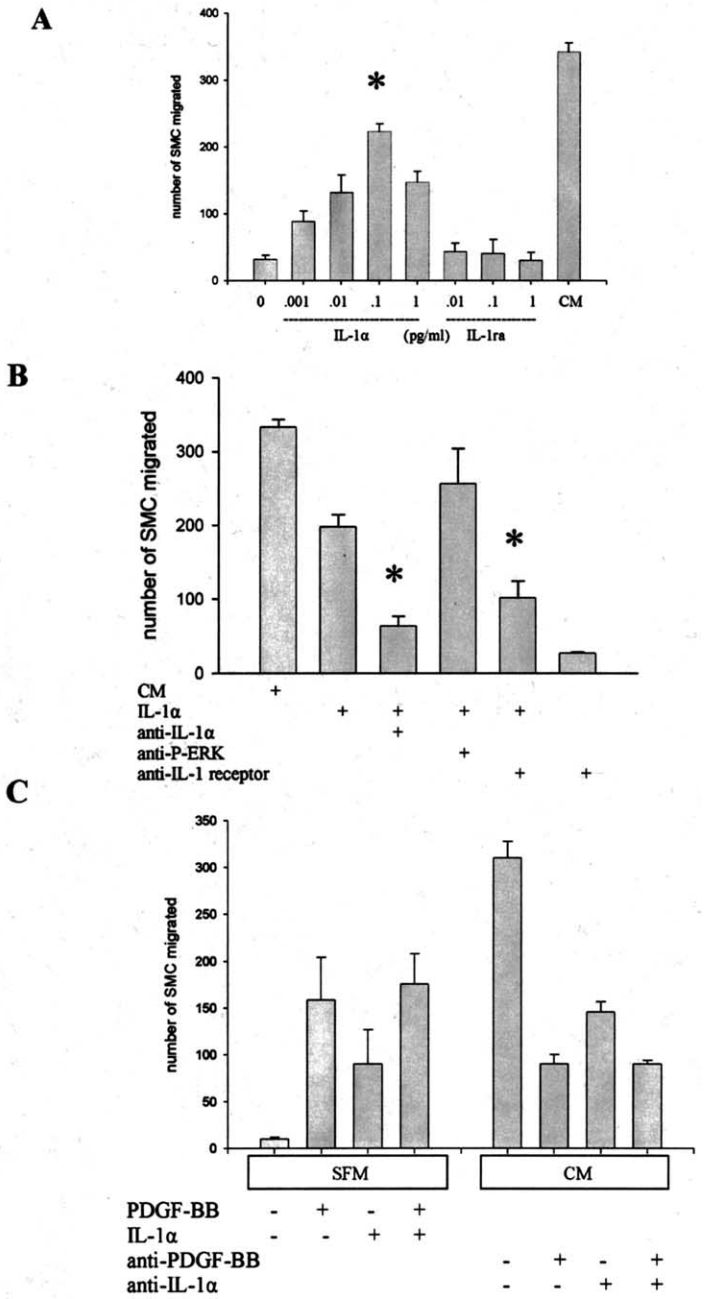

Fig 3. Interleukin-1α (IL-1α) stimulates smooth muscle cell (SMC) migration. **A,** Dose-dependent stimulation of SMC migration with IL-1α. Maximal SMC migration occurred in response to 0.1 pg/mL (n = 5, $P < .0001$,* ANOVA); identical concentration of IL-1ra produced no migration (n = 4, $P = .023$, ANOVA). **B,** Specificity of IL-1α induced SMC migration. IL-1α–induced SMC migration was inhibited in the presence of anti-IL-1α (10 ng/mL, n = 4, $P = .0006$, ANOVA) but not in the presence of a similar isotype antibody (anti-P-ERK, 10 ng/mL; p=.34). IL-1α induced SMC migration was also inhibited in the presence of anti-IL-1α receptor (p=.005, ANOVA) (anti-P ERK, antiphospho extracellular signal-regulated protein kinase) . **C,** Lack of synergy of PDGF-BB and IL-1α to stimulate or inhibit SMC migration. The inclusion of IL-1α (0.1 pg/mL) produced no additional SMC migration beyond that induced by PDGF-BB (0.5 ng/mL) alone (11% increase; n = 3, $P = .99$, ANOVA). The inclusion of anti-IL-1α (10 ng/mL) produced no additional inhibition of SMC migration due to CM beyond that inhibited by anti-PDGF-BB (10 ng/mL) alone (1% decrease; n = 5, $P = .99$).

PDGF-BB and IL-1α to stimulate SMC migration and, therefore, PDGF-BB and IL-1α may stimulate identical intracellular signaling pathways leading to SMC migration.

CM, PDGF-BB, and IL-1α stimulate SMC chemotaxis via ERK1/2. To determine whether the SMC ERK1/2 pathway is the mechanism for SMC migration

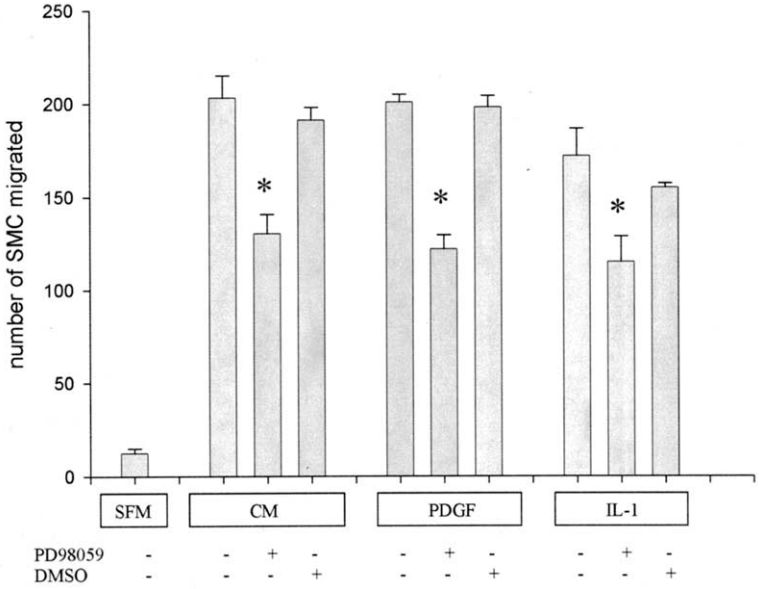

Fig 4. Inhibition of smooth muscle cell (SMC) chemotaxis by extracellular signal-regulated protein kinase (ERK1/2) upstream pathway-inhibitor PD98059. Inclusion of PD98059 (10 μmol/L) inhibited SMC migration stimulated by conditioned medium (CM) (n = 3, $P = .004$, ANOVA), platelet-derived growth factor-BB (PDGF-BB) (0.5 ng/mL; $P = .002$), or IL-1α (0.1 pg/mL; $P = .01$).

induced by CM, PDGF-BB, or IL-1α, SMC were preincubated with PD98059, an upstream inhibitor of the MAPK ERK1/2 pathway, or vehicle alone, prior to attraction of migration. CM-stimulated SMC migration was inhibited 36% in the presence of PD98059 but not in the presence of vehicle alone (Fig 4). Similarly, PDGF-BB–stimulated SMC migration was inhibited 39% and IL-1α–stimulated SMC migration was inhibited 33% in the presence of PD98059 (Fig 4). These results suggest that the ERK1/2 pathway mediates SMC migration induced by CM, PDGF-BB, or IL-1α.

SMC ERK1/2 phosphorylation was directly assessed by Western blot analysis after exposure to PDGF-BB, IL-1α, or CM. PDGF-BB, IL-1α, and CM (210 rpm) all stimulated SMC phosphorylation of ERK1/2 (Fig 5), but medium conditioned with low levels of shear stress (30 rpm) or conditioned with cyclic strain did not stimulate ERK1/2 phosphorylation (data not shown). Phosphorylation due to PDGF-BB was inhibited in the presence of antibodies to PDGF-BB but not PDGF-AA, and phosphorylation due to IL-1α was inhibited in the presence of antibody to IL-1α (Fig 5). Phosphorylation of SMC ERK1/2 that was stimulated by CM (210 rpm) was reduced in the presence of antibodies to either PDGF-BB or to IL-1α (Fig 5). These results suggest that PDGF-BB and IL-1α in the CM stimulate the SMC ERK1/2 pathway.

DISCUSSION

We demonstrate that shear stress stimulates endothelial cells to synthesize and secrete PDGF-BB and IL-1α, both of which stimulate SMC chemotaxis via the MAPK ERK1/2 pathway. Neither low levels of shear nor cyclic strain stimulate endothelial cells to secrete these chemoattractants. These data suggest that hemodynamic forces can stimulate endothelial cells to secrete soluble factors that may act in a paracrine fashion on SMC to stimulate chemotaxis.

Hemodynamic forces are known to play a role in the pathogenesis of atherosclerosis and neointimal hyperplasia. Under normal arterial conditions, laminar shear stress is one of the factors responsible for maintenance of the quiescent endothelial cell phenotype in vivo. Conversely, alterations in shear stress, such as disturbed or turbulent flow induced by endothelial injury or loss or by a physiologic or iatrogenic repair process, activate endothelial cells and lead to the common response characterized by atherosclerosis and neointimal hyperplasia.[15]

Mechanisms of how shear stress affects endothelial cell signal transduction are becoming increasingly understood. In vitro studies have demonstrated activation of the MAPK signaling pathways in endothelial cells in response to shear stress.[25,26,31] Shear stress also activates the transcription factor egr-1 in endothelial cells[27,32-33] that stimulates additional gene transcription and may lead to secretion of soluble factors such as PDGF-B, fibroblast growth factor-2 (FGF-2), and TGF-β1.[12,20,21,35-36]

PDGF is a family of signaling molecules important for cell growth and motility and consists of disulfide-linked dimers of polypeptides A, B, C, and D; five isoforms have been identified so far (AA, AB, BB, CC, and DD).[2] PDGF isoforms exert their effects on target cells by binding and activating two structurally related but differing affinity protein tyrosine kinase receptors (α and β); PDGF-AA binds to the PDGF-αα receptor alone, whereas PDGF-BB binds to the PDGF-αα, -αβ, and -ββ receptors.[2,37] Vascular SMC

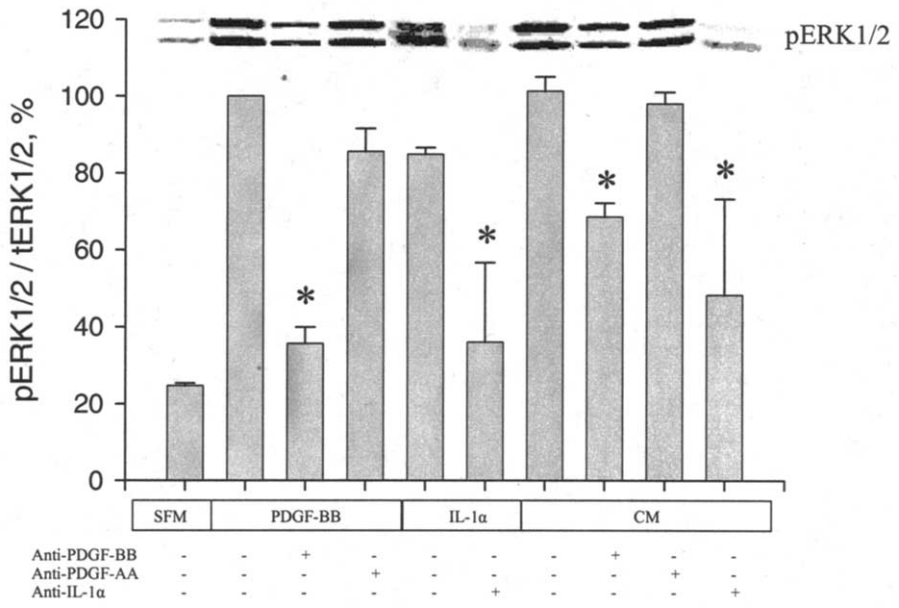

Fig 5. Smooth muscle cell (SMC) extracellular signal-regulated protein kinase (ERK1/2) phosphorylation is stimulated by endothelial cell conditioned medium, platelet-derived growth factor-BB (PDGF-BB), and interleukin-1α (IL-1α). The bar graph represents mean of phosphorylated ERK1/2 normalized to total ERK1/2 bands (n = 3). * $P < .05$ compared with control conditions. The photomicrograph shows a representative experiment of phosphorylated ERK1/2 bands on Western blot; total ERK1/2 bands demonstrated equivalent loading (data not shown). Shear stress-stimulated SMC ERK1/2 phosphorylation was also inhibited in the presence of PD98059 (10 μM, data not shown). PDGF-BB was 0.5 ng/mL; IL-1α was 0.1 pg/mL. Anti-PDGF-BB, anti-PDGF-AA, and anti-IL-1α were 10 ng/mL.

migration is stimulated by PDGF-BB but inhibited by PDGF-AA.[5,8,37-39] We confirm PDGF-BB, but not PDGF-AA, stimulates SMC chemotaxis in our model, and we detect slightly lower, but similar, levels of PDGF-BB secreted into the CM as reported using a similar model.[39] We extend these observations and report that shear stress, but not cyclic strain, stimulates endothelial cells to release PDGF-BB, confirming the differential effects of these hemodynamic forces on endothelial cells.[40]

Additional factors can modulate PDGF-BB–stimulated SMC chemotaxis; for example, PDGF-AA and FGF-2 each inhibit PDGF-BB–stimulated chemotaxis.[5,41] Our finding that anti-PDGF-BB antibody only partially inhibited SMC chemotaxis towards CM, compared with almost complete inhibition of chemotaxis towards PDGF-BB (Fig 2, *B*), suggests that additional factors may be present in the CM that modulate the effect of PDGF-BB. Other factors, such as IGF-1, HB-EGF, TGF-β, or IL-1α, are known to be secreted by endothelial cells and stimulate SMC chemotaxis[42,43]; of these factors, only IL-1α activity was detected in the CM. However, it is possible that these antibodies did not recognize bovine isoforms of these substances, so their lack of reactivity does not exclude their presence in the CM. In addition, other factors may also be present in the CM that affect SMC migration, such as plasminogen activator inhibitor-1.[44] Vascular endothelial growth factor activity may also be present in the CM (data not shown).

IL-1α and IL-1β, the two isoforms of the proinflammatory cytokine IL-1, have identical biologic effects in most studies.[45,46] IL-1β is regulated independently of IL-1α but is thought to be the major form that is biologically active as its precursor form is cleaved by caspase-1 (IL-1–converting enzyme) to be secreted in its active form, whereas IL-1α typically remains cell-associated or membrane-associated. Both IL-1α and IL-1β exert their biologic effects by binding to the type I IL-1 receptor, a member of the Toll-like receptor superfamily.[47,48] Binding of IL-1 to the type I IL-1 receptor results in the formation of a complex with the IL-1 receptor accessory protein,and ultimately initiates signal transduction. However, binding of IL-1 to the type II receptor (which lacks a cytoplasmic domain), binding to the soluble IL-1 receptor, or competitive inhibition from IL-1ra (a naturally secreted but inactive form of IL-1) inhibit IL-1 signaling and may serve to regulate the pathway in vivo.[45] IL-1 receptor signaling stimulates several intracellular pathways, including the MAPK ERK1/2 pathway that is necessary for activation of the iNOS and NF-κB pathways.[49,50]

IL-1 is mitogenic for vascular SMC, although not during the short time course of the experiments reported here[51]; the effect of IL-1 on SMC proliferation may be due to modulation of PDGF-BB activity and signaling.[52] In addition, PDGF can regulate IL-1 receptor levels and enhances, through an ERK1/2 dependent mechanism, IL-1–induced activation of NF-κB and iNOS expression.[53]

ERK1/2 is a member of the MAPK signaling family that is known to play a role in vascular biology.[26,54-56] Although the ERK1/2 pathway was once suggested to play a minor role

in SMC migration,[57] other reports have confirmed its activity in SMC migration.[10,58] Our results confirm the activity of this pathway in migration stimulated by PDGF-BB, IL-1α, or CM, but do not exclude the presence of additional factors in the CM that may stimulate ERK1/2 or modulate PDGF or IL-1α activity (Fig 4). In addition, PDGF or IL-1α may stimulate other intracellular pathways that stimulate chemotaxis, such as the MAPK p38 or jnk pathways, or the PI3K, Akt, and phospholipase C-γ pathways.[3,39,59]

We examined the ERK1/2 pathway as this pathway is activated by both PDGF-BB and IL-1α and induces SMC migration.[10] We used PD98059, a small cell-permeable molecule that is a specific inhibitor of MAPK kinase-1 (MEK)[30] as PD98059 does not inhibit p70 S6 kinase, phospholipase C, Raf-kinase, cAMP-dependent kinase, protein kinase C, v-Src, EGF receptor kinase, PDGF receptor kinase, or PI3K at the concentrations used in this study.[10,60] Although the use of chemical inhibitors is less specific than some techniques used to inhibit signal transduction, such as the use of dominant negative mutations, PD98059 has similar specificity to antisense oligonucleotides[10]; nevertheless, it is possible that PD98059 inhibits other intracellular signal transduction pathways.

We used the orbital shaker to generate oscillatory shear stress on the endothelial cell monolayer,[23,27,28] because it allows collection of CM more easily than do other methods of generating shear stress such as the parallel plate apparatus. The small volume of medium in a culture well allows detection of low concentrations of secreted chemoattractants such as IL-1α.

Palumbo et al[39] used a cone-and-plate apparatus to expose endothelial cells to laminar shear stress; the resulting CM produced maximal SMC migration at 5 dyne/cm^2, with less migration at 15 dyne/cm^2. We report maximal SMC migration at 210 rpm, which may reflect differences in oscillatory and laminar shear stress effect on endothelial cells, including release of other factors in addition to PDGF-BB.

Although the orbital shaker does not produce uniform laminar shear stress on the endothelial cells, most of the cells are exposed to near maximal oscillatory shear stress (τ_{max}), calculated as

$$\tau_{max} = a\sqrt{\rho\eta(2\pi f)^3}$$

where a is the radius of orbital rotation (0.95 cm), ρ is the culture medium density (0.997 g/mL), η is the medium viscosity (0.0101 poise), and f is the frequency of rotation (rotations/sec).[23] At 210 rpm, the frequency of rotation used in most of these experiments, calculated τ_{max} is 9.8 dyne/cm^2, which is similar to the value that we measured (11.1 dyne/cm^2) as well as the values reported by others (11.5 dyne/cm^2) to be equivalent to arterial levels of shear stress in vivo.[23]

This model cannot account for the presence of other cell types, such as platelets, monocytes, and macrophages that may affect SMC migration in vivo. In addition, the role of cell-cell contact between endothelial cells and SMC is not assessed by use of CM, which may be important for the mechanism of action of IL-1α in vivo.[61] Our finding that low magnitudes of shear stress conditioning produce less SMC chemotaxis than higher levels of shear stress (Fig 1, *A*) may be a limitation of this model, especially since the temporal variation in shear stress in this model may not reflect that of the cardiac cycle in vivo.[62] However, our data support a role for hemodynamic forces such as shear stress inducing SMC migration, at physiologically relevant levels, at least by affecting the overlying endothelial monolayer.

Hemodynamic forces such as shear stress may stimulate endothelial cells to secrete SMC chemoattractants such as PDGF-BB and IL-1α, which act on deeper layers of the vessel wall in a paracrine or juxtacrine fashion to subsequently stimulate SMC signal transduction and cell migration. Since signal transduction pathways stimulated by shear stress may converge on common pathways, pharmacologic manipulation of a common pathway may allow control of the response to vascular injury.

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CORRECTION

In: "Prevalence, anatomic patterns, valvular competence, and clinical significance of the Giacomini vein" (Delis KT, Knaggs AL, and Khodabakhsh P. J Vasc Surg 2004;40:1174-83).

On page 1176, Fig 1 is incorrect. The following is the correct figure:

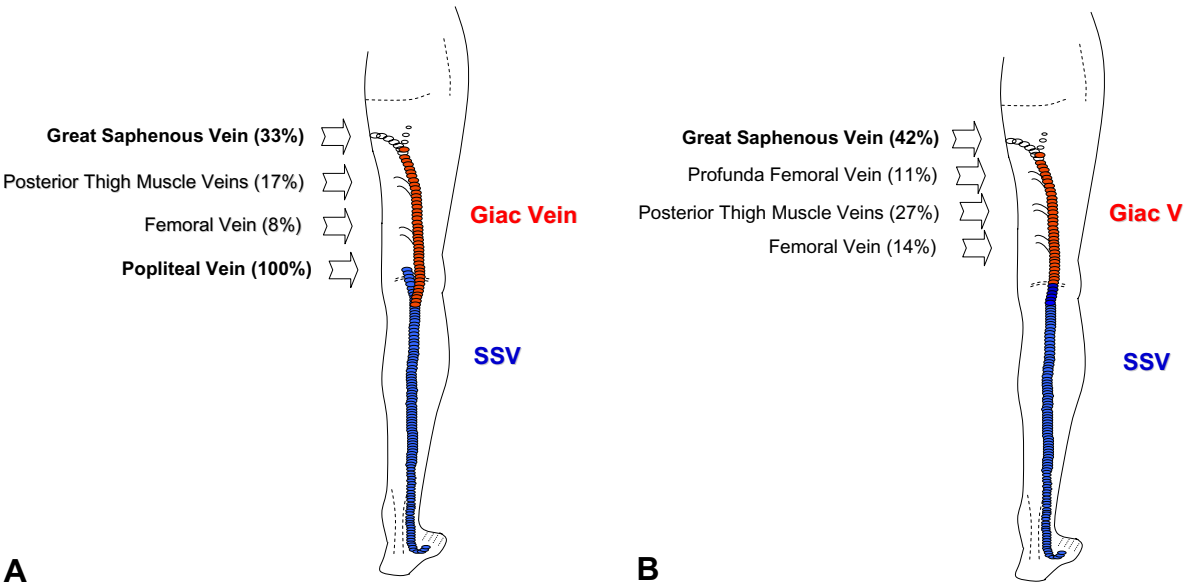

Fig 1. Schematic representation of thigh extension (Giacomini vein) of small saphenous vein (SSV) in limbs with a typical saphenopopliteal junction (**A**) and in limbs with a high or a very high SSV termination (**B**). See Methods for definitions. Epitomized are the single or multiple endings (see Table III) of Giacomini vein. Percentage attached to named veins represents cumulative occurrence in limbs with (**A**) a typical saphenopopliteal junction alone (236 of 301; great saphenous vein, 33% [78 of 236]; posterior thigh muscle veins, 17% [40 of 236]; femoral vein, 8% [19 of 236]; popliteal vein, 100% [236 of 236]) and (**B**) with a high or a very high SSV termination alone (65 of 301; great saphenous vein, 42% [27 of 65]; profunda femoral vein, 11% [7 of 65]; posterior thigh muscle veins, 27% [18 of 65]; femoral vein, 14% [9 of 65]). Differences in cumulative proportions not statistically significant. (Please note the possible extension of Giacomini vein at the top of the thigh.) *Giac V*; Giacomini vein.