Shear stress inhibits smooth muscle cell migration via nitric oxide-mediated downregulation of matrix metalloproteinase-2 activity

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Garanich, Jeffrey S., Manolis Pahakis, and John M. Tarbell. Shear stress inhibits smooth muscle cell migration via nitric oxide-mediated downregulation of matrix metalloproteinase-2 activity. Am J Physiol Heart Circ Physiol 288: H2244–H2252, 2005. First published January 6, 2005; doi:10.1152/ajpheart.00428.2003.—Vascular smooth muscle cell (SMC) migration is a hallmark of intimal hyperplasia (IH), the progression of which is affected by hemodynamic conditions at the diseased site. The realization that SMCs are exposed to blood flow in both denuded vessels (direct blood flow) and intact vessels (interstitial blood flow) motivated this study of the effects of fluid flow shear stress (SS) on SMC migration. Rat aortic SMCs were seeded onto Matrigel-coated cell culture inserts, and their migratory activity toward PDGF-BB when exposed to SS in a rotating disk apparatus was quantified. Four hours of either 10 or 20 dyn/cm2 SS significantly inhibited SMC migration to the bottom side of the insert. This inhibition was associated with downregulation of SMC matrix metalloproteinase (MMP)-2 activity. Four hours of 10 dyn/cm2 SS also drastically increased SMC production of NO. A NO synthase inhibitor (N5-nitro-L-arginine methyl ester; 100 μM) abolished the shear-induced increase in SMC NO production as well as the inhibition of migration and MMP-2 activity. A NO donor (S-nitroso-N-acetyl-penicillamine; 500 μM) suppressed SMC migration via the reduction of both total and active MMP-2 levels. Addition of 10 μM MMP-2 inhibitor I to inserts significantly reduced SMC migration. Western blots showed no effect of 4 h of 20 dyn/cm2 SS on SMC production of PDGF-AA, another chemical known to suppress SMC migration. Thus it appears that SS acts to suppress SMC migration by upregulating the cellular production of NO, which in turn inhibits MMP-2 activity.

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As hallmarks of IH development. It has been shown in balloon catheter injury animal models (19) that intimal thickening in the absence of the inner endothelial cell (EC) lining is accelerated in low-flow (SS) environments, suggesting that SMCs respond directly to changes in blood flow. In that study (19), the intimal thickening response to decreased flow levels was a result of increased SMC migration compared with high-flow conditions.

In vitro studies of the effects of SS on vascular cells have focused primarily on ECs (see, e.g., Refs. 1, 6, 8, 16, 36) because, under normal physiological conditions, they are directly exposed to the SS of flowing blood, having a magnitude on the order of 10 dyn/cm2 (26). It had long been thought that the underlying SMCs were shielded from blood flow and were only subjected to SS when the EC layer was denuded, as in balloon angioplasty, directly exposing SMCs to blood flow SS. Recent modeling studies have shown, however, that even in the case of an intact endothelium, SMCs are subjected to SS through exposure to interstitial flow driven by the transmural pressure gradient (39, 43). Although the magnitude of the superficial velocity of this flow is low (order of 10−5–10−6 cm/s, roughly 4–8 orders of magnitude lower than average velocities throughout the circulation), the interstitial spaces are very small (on the order of nanometers), resulting in SS levels estimated to be the same order as those experienced by ECs due to direct blood flow.

Although the effects of SS on SMCs are not as well characterized in vitro as those on ECs, there have been several studies suggesting that elevated SS levels inhibit SMC proliferation rates (30, 38, 42), agreeing well with in vivo work (2, 11, 19, 20, 24). One study hypothesized that SS inhibition of SMC proliferation is due to altered SMC production of TGF-β1 in response to SS, an autocrine control mechanism (42). The direct effects of SS on SMC migration are not as well studied. In the only investigation we are aware of that examined the effects of SS on SMC migration, Palumbo et al. (29) used a cone-plate apparatus to subject bovine aortic SMCs to SS in culture. After SS exposure, the cells were trypsinized and incorporated in a variation of the Boyden chamber assay to determine their migratory activity. Fifteen hours of 12 dyn/cm2 SS inhibited SMC migration by decreasing matrix metalloproteinase (MMP)-2 activator, membrane-type MMP (MT-MMP; an activator of latent MMP-2 on the cell surface), and PDGF receptor-β levels. Although this study provided valuable insight into the role of SS on SMC migration, the cells were not sheared directly while migrating, and they were subjected to harsh, nonphysiological treatment (trypsinization) during the experiment. Therefore, it is important to conduct quantifiable experiments under conditions in which SMCs are subjected to SS directly while migrating through a representative extracellular matrix.

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The MMPs and PDGF, examined by Palumbo and colleagues (29), are generally considered to play a major role in SMC migration. MMPs are a family of endopeptidases with the ability to degrade extracellular matrix molecules (12). Zempo et al. (45) highlighted the importance of MMPs in SMC migration by using a chemical MMP inhibitor that effectively suppressed SMC migration through a representative extracellular matrix (Matrigel) in vitro. In particular, it has been shown that MMP-2, a gelatinase that is secreted in a proenzyme form and activated on the cell surface, is necessary for rat aortic SMC migration through Matrigel in vitro (33), and Kenagy et al. (18) demonstrated that MMP-2 plays a role in primate SMC migration through native matrix.

PDGF has long been recognized as a chemoattractant for SMCs, but the various PDGF isoforms appear to have different mitogenic properties. Whereas PDGF-BB is accepted to be chemoattractive to SMCs, PDGF-AA has been shown (22) to have very little, if any, chemoattractive activity for SMCs by itself and, in fact, appears to inhibit SMC migration when used in combination with PDGF-BB, compared with PDGF-BB used alone (21). The production of PDGF by SMCs is affected by alterations in flow. Both elevated (41) and reduced (23) flow (SS) in vivo have been associated with increased SMC PDGF-AA mRNA expression. Sterpetti et al. (37) reported increased SMC synthesis of PDGF in response to increased flow levels in vitro but did not specify which isoform was being produced. However, Barrett and Benditt (3) reported that SMCs produce PDGF-AA primarily, and other work (25) has shown that only a small percentage (1.6%) of SMCs cultured from normal medial tissue express PDGF-BB mRNA. So it is likely that the PDGF production reported by Sterpetti and colleagues was PDGF-AA. It is plausible, therefore, that SS acts to increase SMC PDGF-AA production, which acts to inhibit their migration in an autocrine fashion.

Another diffusible substance known to affect SMC function is NO. SMCs produce both NO (31) and inducible NO synthase (NOS) (14) in response to elevated SS levels, and other studies have demonstrated that NO inhibits SMC migration (13, 34). The intracellular concentration of NO in a vessel has also been linked to the activity of MMPs in that vessel (40). Therefore, it is plausible that, in response to SS, SMCs produce NO, which in turn affects their migratory response.

In the present study, we plated rat aortic SMCs on Matrigel-coated cell culture inserts and observed their direct migratory activity. Rat aortic SMCs, positively characterized by both their “hill and valley” growth pattern and staining for smooth muscle-specific α-actin (monoclonal anti-α-smooth muscle actin clone 1A4; Sigma), were obtained from male Sprague-Dawley rats weighing ~150 g via a primary culture procedure described elsewhere (44). This procedure was approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Cells were then expanded in 75-cm² culture flasks housed in a 37°C, 5% CO₂–95% air incubator with DMEM-F-12 (Sigma) supplemented with 10% FBS (HyClone) and 100 μg/ml streptomycin (1% P/S; Sigma) serving as the culture medium. Medium was changed every 2 days, and cells were split on reaching confluence (4–5 days) with a 0.07% trypsin-EDTA solution (Sigma). Passages 3–9 were used in experiments.

Migration insert preparation. Cell culture inserts (8.0-µm pore size, Becton Dickinson) housed in six-well companion plates (Becton Dickinson) were coated with 750 µl of growth-factor-reduced Matrigel matrix (GFR Matrigel; BD Biosciences) diluted in Dulbecco’s PBS (Mediatech) to a concentration of 476 µg/ml and then placed in a 37°C, 5% CO₂–95% air incubator for 3 h to provide sufficient time for the Matrigel proteins to gel. Plates containing inserts were then placed into a hood previously sterilized by exposure to ultraviolet light and allowed to air dry for 48 h. Once dry, plates were stored at 4–8°C until needed for experimentation. This protocol reproducibly yielded a 5- to 7-µm GFR Matrigel coating thickness near the center of the insert as determined by scanning electron microscopy. Uniformity of the Matrigel coating was assessed via the use of a Coomassie Brilliant Blue R250 (Sigma) staining procedure.

Migration experiments. A modification of the traditional Boyden chamber assay, used by others to investigate SMC migration in vitro (see, e.g., Refs. 21, 33), was used in these experiments. Inserts that had been coated as described above were first rehydrated in PBS for 1 h in a 37°C, 5% CO₂–95% air incubator. PDGF-BB (Sigma) was then diluted to a final concentration of 100 ng/ml (32) in DMEM-F-12 plus 1% P/S, and 2.5 ml of this solution was placed in the well of the companion plate to serve as the chemoattractant. SMCs were then trypsinized from culture flasks, centrifuged, and resuspended in DMEM-F-12 plus 10% FBS and 1% P/S at a concentration of 250,000 cells/ml. This concentration provided a confluent SMC monolayer on the GFR Matrigel coating. The PBS added previously was removed, and 2 ml of this cell solution was added to the insert. Depending on the experiment, the inserts were then incubated for 6–9 h. It is of note that cells seeded on GFR Matrigel-coated inserts in serum-free medium in the presence of 100 ng/ml PDGF-BB in the well of the companion plate did not migrate on timescales up to 24 h. In experiments conducted to determine the role of NO produced by SMCs on their migration and MMP-2 activation, a NOS inhibitor [Nω-nitro-L-arginine methyl ester (L-NAME), 100 μM; Sigma] was added to inserts 1 h after initial cell seeding. Inserts were then returned to the incubator and later exposed to SS as described below.

After incubation, inserts to be used in SS experiments were moved to a hood containing a rotating disk apparatus (see Ref. 36 for a thorough description) and were subjected to 1–20 dyn/cm² SS for 1–4 h. This SS was calculated with the equation

\[ \text{SS} = \frac{4 \times \pi \times r^2 \times \text{viscosity}}{h} \]

where \( r \) is the disk radius (11.12 mm), and \( h \) is the gap distance between the disk and the SMC surface (251 µm). All SS magnitudes presented here are minimum values, and magnitudes ranged from 0 dyn/cm² (center of insert) to a maximum at the edge of the insert, with the average value being two-thirds of the maximum. Inserts were maintained at 37°C in 5% CO₂–95% air during the entire SS exposure. There was no significant change in cell morphology due to exposure to SS. Total experimental time from cell seeding on the insert to completion of SS exposure was 10–11 h. For example, if an insert was incubated first for 6 h, it was then subjected to SS for 4 h. This time course was determined to be optimal through an initial set of experiments in which it was observed that after 6–7 h cells had attached to the GFR Matrigel coating firmly enough to withstand shear exposure but none had yet migrated to the underside of the insert (data not shown). Inserts used as controls were also housed in the experimental hood with a rotating disk in place, but the disk remained stationary throughout the duration of the experiment. Under control conditions,
2.1–3.4% of cells initially seeded migrated through the Matrigel to the underside of the insert in 10–11 h. After electrophoresis, proteins were transferred to protein concentrations were determined through the use of a commercial extraction buffer as outlined by DeMaio et al. (8). Lysate was purified in the incubator throughout the duration of the experiment along with their companion controls. SNAP was diluted in cell culture medium (DMEM-F-12 + 10% FBS and 1% P/S), and MMP-2 inhibitor I was diluted in DMSO [0.5% (vol/vol) of the final culture medium; Sigma].

After SS or chemical exposure, inserts were first examined under ×100 light microscopy to ensure that the SMC monolayer was still intact and had not been compromised by SS or chemical application. The conditioned medium from the top of the insert was then removed and stored at −20°C for future assays. In some cases, samples of this medium were used to determine the number of cells that became dissociated from the GFR Matrigel as a result of SS or chemical exposure. This number was found to be minimal (<4% of total cells seeded).

Cells that had migrated through the Matrigel to the underside of the insert were fixed and stained with DiffQuik staining solution (Dade Behring). Cells on the top that had not migrated and the GFR Matrigel coating were mechanically removed with a cotton swab. The fixed cells on the underside of the insert were then counted in five ×100 fields, one in the center of the insert and four around the edges. The average number of cells migrated in these fields was used to quantify SMC migration. In some experiments, after application of SS, conditioned medium was removed and cells remaining on the GFR Matrigel layer were lysed. This lysate was then stored at −80°C and used in subsequent Western blot analyses to determine the influence of SS on PDGF-AA production (described below).

**Determination of both total and active MMP-2 levels in SMC conditioned medium.** Total (latent and active) and active MMP-2 levels in SMC conditioned medium obtained from inserts containing cells exposed to either SS or SNAP were quantified with the use of a commercially available ELISA (Amersham Pharmacia Biotech) according to the manufacturer’s specifications.

**Determination of NO levels in SMC conditioned medium.** Transwell filter supports were attached to glass slides via the use of a silicone elastomer kit (Sylgard), and the slides were sterilized by exposure to ultraviolet light overnight. SMCs were then seeded onto the slides and grown in DMEM-F-12 + 10% FBS and 1% P/S. A 7- to 8-h incubation was performed to allow sufficient time for the SMCs to reach confluence. Two hours before an experiment, the medium in three slides was changed to 2 ml of DMEM-F-12 without phenol red + 1% BSA (Sigma) and 1% P/S. In one slide, 100 µM L-NAME was added to determine its effect on shear-induced SMC NO production. Control, shear, and shear + L-NAME slides were moved to the hood containing the rotating disk apparatus described above, and 10 dyn/cm² SS was applied to SMC monolayers for 4 h. For controls, the rotating disk was in place but remained stationary. After SS application, conditioned medium samples (500 µl) were collected from all three slides and stored at −20°C. Nitrite levels in samples were later determined through the use of a fluorometric assay described elsewhere (28).

**Western blot analysis of PDGF-AA.** After exposure to SS as described above, SMCs remaining on top of the Matrigel layer were washed twice in PBS with Ca²⁺ and then lysed with the SDS extraction buffer as outlined by DeMaio et al. (8). Lysate was purified from insoluble material by centrifugation at 14,000 g for 10 min, and protein concentrations were determined through the use of a commercially available protein assay (Bio-Rad). Equal protein levels were then loaded onto 10% SDS-polyacrylamide gels under nonreducing conditions (10). After electrophoresis, proteins were transferred to Immob-Blot polyvinylidene difluoride membranes (Bio-Rad) and were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. Membranes were then incubated with a polyclonal human anti-rabbit PDGF-A antibody (Ref: 10; Santa Cruz Biotechnology) for 2 h at room temperature at a dilution of 1:200. This was followed by a 1-h room temperature incubation in a horseradish peroxidase-labeled anti-rabbit secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:2,000. PDGF-AA content on the membrane was then detected through the use of a chemiluminescence ECL Western blotting analysis system (Amersham Pharmacia Biotech), a 2-min exposure to Kodak Biomax Light film, and Scion Image analysis software (Scion).

**Statistical analysis.** PDGF-AA content quantifications are normalized with respect to control. All data are presented as means ± SE. Data sets were analyzed for statistical significance by a Student’s t-test with a two-tailed distribution. P < 0.05 was considered statistically significant. The Bonferroni correction, which gives a conservative significance level of P*m*, where m is the number of comparisons to be performed, was used where appropriate. For example, when three groups were compared, 0.05 was replaced by 0.05/3 = 0.017 to determine significance.

**RESULTS**

**Effect of SS on SMC migration.** The SMC migratory responses to various SS magnitudes and durations are shown in Fig. 1. In Fig. 1A, SMCs that had been seeded onto Matrigel-coated cell culture inserts were incubated for 6–7 h and then exposed to 1, 10, or 20 dyn/cm² SS for 4 h. On completion of SS exposure, inserts were examined microscopically to ensure that a significant number of cells did not detach in response to SS, and then migration levels were quantified as described above. Because control conditions were the same in each experimental case (6- to 7-h incubation followed by 4-h exposure to stationary shear rod), all control values are presented together. In Fig. 1A, 83.0 ± 13.01 cells migrated per field examined under control conditions (n = 19). SMC exposure to both 10 dyn/cm² (30.07 ± 11.99 cells migrated per field; n = 7; P < 0.05) and 20 dyn/cm² (9.69 ± 6.22 cells migrated per field; n = 6; P < 0.01) SS for 4 h resulted in a significant inhibition of SMC migration. There was also significantly less migration in inserts exposed to 4 h of 20 dyn/cm² SS compared with those subjected to 1 dyn/cm² SS (56.5 ± 10.71 cells migrated per field; n = 6; P < 0.05) for 4 h.

In Fig. 1B, SMCs grown on Matrigel-coated inserts were subjected to 20 dyn/cm² SS for 1, 2, 3, or 4 h to determine the duration of SS exposure required for significant inhibition of migration. Total running time for each group of experiments was 10–11 h. For example, if SS was to be applied for 4 h, the cells were first incubated for 6–7 h, a 7- to 8-h incubation preceded a 3-h shear exposure, etc. All control cases are again included in one data set because the same overall experimental conditions applied for each group (10- to 11-h total experiment time at 37°C, 5% CO₂-95% air with no SS applied). Here, 52.68 ± 8.99 cells/field migrated under control conditions (n = 23), 41.91 ± 14.19 cells/field in response to 1 h of 20 dyn/cm² SS (n = 7), 27.53 ± 8.06 cells/field in response to 2 h of 20 dyn/cm² SS (n = 5), 24.33 ± 13.83 cells/field in response to 3 h of 20 dyn/cm² SS (n = 5), and 9.69 ± 6.22 cells/field in response to 4 h of 20 dyn/cm² SS (n = 6). Although there appears to be a consistent decreasing trend in cells migrated per field as SS duration increases, only the 4-h data set is significantly different from control (P < 0.001).
SS inhibited SMC migration both in the center of the insert, where there is relatively little shear, and the perimeter of the insert, where shear is maximum. Examining the central area of inserts, 135.06 ± 14.29 cells/central field migrated in control (no shear) conditions. In control inserts, the same volume of medium was removed as in SNAP experiments, but it was replaced with an equal volume of fresh medium not containing SNAP. Conversely, experiments were conducted in which 100 μM 1-NAME was added to inserts before exposure to 4 h of 20 dyn/cm² SS. In control inserts, the same volume of medium was removed as in 1-NAME inserts, but it was replaced with an equal volume of fresh medium with no 1-NAME. During shear exposure, control inserts were treated as described in Effect of SS on SMC migration. Addition of 1-NAME abolished the shear-mediated inhibition of SMC migration seen earlier, as 135.06 ± 16.61 cells/field migrated under control conditions (n = 4) and 135.97 ± 23.49 cells/field migrated when exposed to 20 dyn/cm² SS and 100 μM 1-NAME (n = 4). The difference between the two groups is statistically significant (P < 0.05).
difference between the two groups was not statistically significant (Fig. 4).

Experiments were also conducted to determine whether L-NAME had an effect on SMC migration in the absence of SS. L-NAME (100 μM) was added to selected inserts as described above, but in place of SS, both control and L-NAME inserts were then placed in a 37°C, 5% CO₂-95% air incubator for the remainder of experimentation (9–10 h). SMC migratory activity was then quantified as described above. L-NAME in the absence of SS had no significant effect on SMC migration, as 83.5 ± 4.58 cells/field migrated under control conditions (no L-NAME; n = 5) and 85.73 ± 3.16 cells/field migrated in the presence of L-NAME (n = 5).

**Effect of SS on MMP-2 production and activity.** The cumulative results of ELISA conducted on conditioned medium to determine the effects of 4 h of 10 or 20 dyn/cm² SS on MMP-2 production and activation are shown in Fig. 5. Medium from cells exposed to 4 h of 10 dyn/cm² SS had a total MMP-2 concentration of 13.55 ± 2.93 ng/ml (n = 4; Fig. 5A), whereas cells from companion control (no shear) experiments had a total MMP-2 concentration of 14.28 ± 2.84 ng/ml (n = 4). Medium collected from cells subjected to 4 h of 20 dyn/cm² SS contained 11.50 ± 1.95 ng/ml total MMP-2 (n = 6), and medium from companion control experiments contained 13.76 ± 0.97 ng/ml total MMP-2 (n = 6). These data sets

![Fig. 3. Effect of NO donor (S-nitroso-N-acetyl-penicillamine; SNAP) on SMC migration. Cells were seeded onto Matrigel-coated cell culture inserts and first incubated for 6–7 h. SNAP (500 μM) was then added to selected inserts. Under control conditions, an equal volume of medium was removed as in experimental inserts but replaced with fresh medium containing no SNAP. All inserts were then incubated for 4 more hours, after which migration levels were quantified. n, No. of times each experimental condition was completed. Data are presented as means ± SE. Statistically different from control (no SNAP): *P < 0.05.](http://ajpheart.physiology.org/)

![Fig. 4. Effect of L-NAME on shear-induced inhibition of SMC migration. SMCs were seeded on Matrigel-coated cell culture inserts and incubated for 6–7 h before exposure to 4 h of 20 dyn/cm² SS. L-NAME (100 μM) was added to inserts to be subjected to SS 1 h after initial cell seeding. The shear rod was in place but remained stationary throughout the duration of the experiment under control conditions. n, No. of times each experimental condition was completed. Data are presented as means ± SE. When cells had been incubated with L-NAME, exposure to SS had no significant effect on their migratory activity.](http://ajpheart.physiology.org/)

![Fig. 5. Effect of SS on matrix metalloproteinase (MMP)-2 production and activation. SMCs were exposed to SS, and conditioned medium samples from both control and sheared cells were used in ELISA to determine the total (proenzyme + active) and active MMP-2 concentrations. A: total MMP-2 concentrations in conditioned medium obtained from cells exposed to 4 h of either 10 or 20 dyn/cm² SS or control conditions. B: active MMP-2 concentrations in conditioned medium obtained from cells exposed to 4 h of either 10 or 20 dyn/cm² SS or control conditions. Control conditions were those in which the shear rod was in place but remained stationary throughout the completion of the experiment. n, No. of times each experimental condition was completed. Data are presented as means ± SE. Statistically different from companion control: *P < 0.05, **P < 0.01.](http://ajpheart.physiology.org/)
showed no statistically significant effects of SS on total MMP-2 production.

The effect of 4 h of 10 or 20 dyn/cm² SS on active MMP-2 levels is shown in Fig. 5B. Four hours of 10 dyn/cm² SS significantly (P < 0.05) reduced the active MMP-2 concentration (4.04 ± 0.19 ng/ml active MMP-2 in conditioned medium; n = 4) compared with control (4.88 ± 0.26 ng/ml active MMP-2; n = 4). Four hours of 20 dyn/cm² SS also significantly (P < 0.01) inhibited active MMP-2 concentration (2.56 ± 0.34 ng/ml active MMP-2 in conditioned medium; n = 9) compared with control (3.74 ± 0.26 ng/ml; n = 9).

Effect of NO levels on MMP-2 production and activity. After determining the role of NO levels on SMC migration (Figs. 3 and 4), we next quantified the effect of changes in NO concentration on MMP-2 production and activity levels through the use of ELISA. In conditioned medium collected from cells exposed to SNAP, the total MMP-2 concentration was 6.32 ± 0.31 ng/ml (n = 5), whereas the total MMP-2 concentration in control conditioned medium was 11.51 ± 0.77 ng/ml (n = 5). The difference was statistically significant (P < 0.001; Fig. 6). Conditioned medium from control cells had an active MMP-2 concentration of 2.07 ± 0.09 ng/ml (n = 5), and medium collected from SNAP-exposed cells had an active MMP-2 concentration of 1.44 ± 0.16 ng/ml (n = 5). The difference between the two groups was also statistically significant (P < 0.01; Fig. 6).

Conditioned medium collected from inserts subjected to 100 μM L-NAME before exposure to 20 dyn/cm² SS had a total (proenzyme + active) MMP-2 concentration of 28.82 ± 1.72 ng/ml (n = 4), and conditioned medium from companion control inserts had a total MMP-2 concentration of 27.77 ± 1.50 ng/ml (n = 4). The difference between the two groups was not statistically significant (Fig. 7). Conditioned medium from inserts subjected to L-NAME before 20 dyn/cm² shear had an active MMP-2 concentration of 7.40 ± 0.71 ng/ml (n = 4), whereas control conditioned medium contained 7.43 ± 0.34 ng/ml active MMP-2 (n = 4). The difference was again not statistically significant (Fig. 7).

Effect of MMP-2 inhibition on SMC migration. To further ensure that SMCs were responding to changes in MMP-2 activity, experiments were conducted in which 10 μM MMP-2 inhibitor (soluble in DMSO) was added to inserts in the same manner as described in the SNAP experiments above. The addition of MMP-2 inhibitor significantly decreased SMC migration levels as shown in Fig. 8: 76.95 ± 7.49 (n = 7) cells/field migrated in control inserts, and 31.38 ± 11.86 (n = 6) cells/field migrated in experiments in which MMP-2 inhibitor was added (P < 0.05). We also conducted experiments to determine whether DMSO alone had a significant effect on SMC migration: 59.85 ± 19.32 (n = 6) cells/field migrated when DMSO alone was added to inserts, which was not significantly different from control (no DMSO or MMP-2 inhibitor).

Effect of SS on SMC PDGF-AA production. Finally, we determined whether SMCs increased their production of PDGF-AA after exposure to SS. Cells were exposed to 4 h of 20 dyn/cm² SS or to control conditions as described above. However, after completion of SS exposure, SMCs were lysed and samples were used in Western blotting procedures. A representative blot is shown in Fig. 9A. There was no significant effect of 4 h of 20 dyn/cm² SS on the SMC production of PDGF-AA, as the PDGF-AA content in sheared cells was 95% of that in control cells (n = 3; Fig. 9B). To ensure that the presence of Matrigel had no effect on Western blots, SMCs were also grown on glass slides without Matrigel and exposed to 4 h of 20 dyn/cm² SS, and lysates from both control and experimental conditions were run in Western blots. Again, there was no effect of SS on SMC PDGF-AA production, as sheared cells contained 97% of the PDGF-AA content of control cells (n = 3; Fig. 9B).
DISCUSSION

The primary findings of this study were that 1) fluid SS applied directly to SMCs grown on Matrigel-coated cell culture inserts inhibits their migration toward a known chemoattractant (PDGF-BB) in a dose-dependent manner (Fig. 1); 2) this inhibition is due to the NO-mediated reduction in MMP-2 levels (Figs. 2–8); and 3) 4 h of 20 dyn/cm² SS does not affect SMC PDGF-AA production (Fig. 9). One other study examined the effect of SS on SMC migration indirectly by exposing cells to shear in culture and then transferring them to a migration assay (29), and another study applied pulsatile shear to SMCs and examined their spreading activity, which takes into account both migration and proliferation (35). The present study is the first to apply SS directly to SMCs while they were migrating in a quantifiable migration assay.

Abnormally high SMC proliferation in the media and subsequent migration to the intima are recognized as hallmark processes in IH. It has been well documented that IH is attenuated in areas of increased blood flow (SS; Refs. 5, 19, 20, 24), and the data presented here are consistent with those observations (Fig. 1). Increased flow has been shown to inhibit IH both in common carotid arteries (CCAs) denuded of their endothelium by balloon catheter injury (SMCs exposed to direct blood flow; Ref. 19) and endothelialized vascular grafts (5, 20, 24). A role for interstitial flow SS in the control of IH was suggested by experiments in which a silicone collar was placed around rabbit CCAs (9). Marano et al. (27) used a silicone collar to induce intimal thickening in rabbit CCAs. Increased flow conditions diminished the IH caused by the collar, and this reduction was attributed to increased SMC NO production in response to elevated flow (and subsequent interstitial SS). It is also plausible that, in studies in which IH was suppressed under elevated flow conditions in endothelialized vascular grafts (5, 20, 24), the increased flow elevated EC production of NO, which in turn diffused to the underlying SMCs and suppressed their migratory activity. Although it is possible that these elevated NO levels acted to suppress SMC proliferation, it is also plausible that NO inhibited SMC migration, leading to the reduction in IH.

![Graph](image)

Fig. 8. Effect of MMP-2 inhibition on SMC migration. SMCs were seeded onto Matrigel-coated cell culture inserts and incubated for 6–7 h. MMP-2 inhibitor (10 μM) was then added to selected experimental inserts. Under control conditions, an equal volume of medium was removed as in experimental inserts, but it was replaced with fresh medium containing no MMP-2 inhibitor. All inserts were then incubated for 4 more hours, after which migration levels were quantified. *P < 0.05.

Fig. 9. Effect of SS on SMC PDGF-AA production. SMCs were seeded onto Matrigel-coated inserts or glass slides and subjected to 4 h of 20 dyn/cm² SS. Cells were then lysed, and samples were used in Western blotting procedures. A: representative Western blot for PDGF-AA (mol mass ~30 kDa) is shown. C, control (no shear); S, SS. B: relative PDGF-AA content in control and SS samples. *Statistically different from control (no MMP-2 inhibitor): *P < 0.05.
The effect of increased NO on MMP activity is controversial. In one study (40), MMP-2 levels were examined 3 days after placing elevated flow through left CCAs of rabbits. Active MMP-2 levels were significantly increased on exposure to elevated flow (and elevated NO produced by EC), and when 1-NAME was used to inhibit NO synthesis, MMP levels fell. In another study, the endothelial NOS (eNOS) gene was transferred to SMCs and its effects on SMC migration and MMP-2 levels were examined (15). eNOS gene transfer inhibited SMC migration but, in contrast to the work discussed above, it also inhibited MMP-2 activity in conditioned medium. Addition of a NO donor (DETA NONOate) to medium also reduced MMP-2 activity. Thus it seems that NO has multiple effects on SMC MMP-2 production and/or activation, the balance of which is likely determined by the physiological and/or pathological state of the vessel.

Because MMP-2 is secreted in a proenzyme form and activated on the cell surface, we examined the effect of both SS and NO levels on the SMC production (secretion) and activation of MMP-2. It should be noted that we decided to look specifically at MMP-2 because it has been shown in other work to be necessary for rat aortic SMC migration (33) and its expression is elevated under altered flow conditions (4, 17).

Palumbo et al. (29) also reported that 15 h of 12 dyn/cm² SS downregulated MMP-2 but did not affect MMP-9 in bovine aortic SMCs. Addition of SNAP to the upper well of inserts for 4 h served to inhibit significantly both total (proenzyme + active) and active MMP-2 levels in conditioned medium (Fig. 5). It is of note that significant inhibition of both pro- and active MMP-2 levels in SMC conditioned medium was reported in response to 15 h of 12 dyn/cm² SS (29), and therefore it seems that inhibition of pro-MMP-2 by SS (>4 h) occurs on a longer timescale than inhibition of active-MMP-2 (<4 h).

Reductions in mRNA and protein for MT-MMP, which activates pro-MMP-2 on the cell surface, were also reported after 15 h of SS but not after 3 h (29). It seems plausible that this inhibition of MT-MMP happens on a timescale of roughly 4 h (the time point measured in our work) and that it is responsible for the reduction in active MMP-2 reported here. Considering these data together, it appears that decreases in active MMP-2 levels are involved in the SS/NO-mediated inhibition of SMC migration. The reduction in total MMP-2 concentration after SNAP administration appears to have no effect on SMC migration by itself and is likely due to the larger dose used in the present work (500 μM) compared with other studies of SM C migration (10 μM; Ref. 13). We also report here decreased SMC migration levels in the presence of 10 μM MMP-2 inhibitor I (Fig. 8), providing further evidence for a role of MMP-2 in SMC migration.

Finally, we conducted experiments to determine the effect of SS on the production of PDGF-AA by SMCs. PDGF-AA has been shown to have virtually no chemotactic activity on its own (7) and to actually inhibit SMC migration induced by PDGF-BB when used in a modification of the Boyden chamber assay (21). SMC PDGF-AA mRNA expression has also been shown to be increased under both high (41)- and low (23)-flow conditions. We thus hypothesized that SMCs might produce more PDGF-AA when exposed to elevated SS levels, and this would suppress their migration in an autocrine fashion. Our results, however, showed no influence of 4 h of 20 dyn/cm² SS on SMC PDGF-AA protein expression (Fig. 9). This is likely due to the fact that SS was applied within a shorter time frame in our experiments (4 h) than in other studies (41) that reported SMC PDGF-AA expression in response to increased flow (a minimum of 24-h exposure to modified flow conditions). Thus it is possible that SMCs do produce PDGF-AA when exposed to elevated SS, which effectively inhibits their migration, but this mechanism may operate within a longer time frame than was tested in our experiments.

In conclusion, this study provides the first direct evidence that fluid SS acts to inhibit SMC migration. The mechanism of SS action involves the NO-mediated downregulation of MMP-2 levels. Although this study broadened our fundamental understanding of the role of SS in SMC migration, the use of a more physiological three-dimensional interstitial flow model is required for improved understanding.

GRANTS

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