Low flow enhances platelet activation after acute experimental arterial injury

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Purpose: **Vascular smooth muscle cell (VSMC) proliferation and migration to the subintima or intimal hyperplasia (IH) occur after arterial injury and are thought to be induced by mitogenic factors released from activated platelets. Because low flow (LF) and shear have been attributed to the localization and progression of IH, we postulated that hemodynamic factors may regulate the degree of platelet activation, as measured by plasma thromboxane B_2 (TXB_2) and platelet-derived growth factor-AB (PDGF-AB) release at regions of experimental arterial injury.**

Methods: **The right common carotid artery (CCA) was subjected to balloon injury in 18 New Zealand White male rabbits. Flow in the injured CCA was reduced by out-flow ligation (LF group, n = 6) or increased by ligation of the left CCA (high flow [HF] group, n = 6). In six other animals, flow was preserved (normal flow [NF] group). Mean blood flow and pressure in the right CCA were measured thereafter at 10 and 30 minutes. Plasma TXB_2 and PDGF-AB levels were determined with the enzyme-linked immunosorbent assay method in each animal with blood samples taken systematically before injury (baseline) and in the distal CCA at similar time points.**

Results: **At 10 minutes, mean blood flow was reduced from 20 ± 2 ml/min in the NF group to 7 ± 1 ml/min in the LF group animals ($p < 0.01$) and increased to 32 ± 2 ml/min in the HF group animals ($p < 0.05$). Mean arterial blood pressure did not differ among the groups. Hemodynamic parameters were similar at 10 and 30 minutes. TXB_2 levels were more than fourfold greater in the LF group than in the HF and NF groups at both time points ($p < 0.05$). In addition, there was a twofold increase in plasma PDGF-AB level at 10 minutes in the LF group compared with baseline levels ($p < 0.05$).**

Conclusion: **Platelet activation at regions of acute vascular injury was determined to be flow dependent. Upregulated platelet activity in low flow conditions may be due to increased platelet exposure time to subendothelial collagen and is greatly attenuated if normal or increased flow is present. (J Vasc Surg 1998;27:910-8.)**

Platelet activation and aggregation play a prominent role in the initial response of the vessel wall to mechanical injury. It has been suggested that platelet activation and aggregation at regions of intima or medial injury mediate intimal hyperplasia (IH) and eventual lumen stenosis after vascular reconstruction or angioplasty.[1-3] A major feature of the response to this injury is an increase in the number of vascular smooth muscle cells (VSMCs) in the intima.[4] Platelets adhere to sites of injury, undergo morphological transformation, release granular contents such as adenosine diphosphate (ADP) and thromboxane (TX) A_2, and express a receptor, glycoprotein IIb/IIIa, that is essential for fibrinogen cross-linking, platelet aggregation, and thrombus formation.[5] These processes also result in the release of chemotactic and mitogenic growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β, and basic fibroblast growth factor, which induce VSMC proliferation and migration after vascular injury.[6-8] Recent studies have indicated that antibodies to fibrinogen glycoprotein IIb/IIIa receptor may offer complete inhibition of platelet function and help abrogate the initial "response to injury" process.[9-11]

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Hemodynamic factors (i.e., flow and shear stress) may modulate platelet activity and thrombosis formation either directly[12,13] or indirectly by regulating the mechanism controlling endothelial coagulation. For example, shear stress induces significant increases in levels of prostacyclin, tissue plasminogen activator, and thrombomodulin receptor messenger RNA.[14-18] Evidence also has accrued that low flow (LF) and shear stress are associated with the localization and progression of IH.[19-21] We previously studied how hemodynamic factors modulate key molecular events that influence matrix degradation and VSMC cell migration to the subintima and IH.[22] In the current study, we examined the role of blood flow in regulating the degree of platelet activation after experimental arterial injury.

We measured acute plasma levels of TXB_2, a stable metabolite of TXA_2 that is released from activated platelets, and of PDGF-AB, a mitogenic factor that is released after platelet aggregation in relation to different flow conditions subsequent to balloon injury of the rabbit carotid artery.

MATERIALS AND METHODS

Animal model. Adult New Zealand White male rabbits, weighing 3.0 to 3.5 kg and fed normal rabbit chow (n = 18), were used for the study. Anesthesia was induced with intramuscular ketamine hydrochloride (40 mg/kg) and xylazine (5 mg/kg) and maintained with 1% halothane via endotracheal intubation. A 2F balloon catheter was introduced twice via the facial branch of the right common carotid artery (CCA) for 10 cm to induce mural endothelial denudation and the usually associated subjacent medial injury of the entire length of the right CCA. The balloon was consistently inflated with 0.2 ml of normal saline. Housing and handling of animal were in compliance with "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 80-23, revised 1985).

Flow modulation and hemodynamic measurements. Blood flow in the right CCA was reduced through ligation of three of the four terminal CCA branches (LF group, n = 6) or increased through ligation of the contralateral left CCA (high flow [HF] group, n = 6) as described by Langille et al.[23] In six other animals, no hemodynamic manipulation was undertaken, and flow was preserved (normal flow [NF] group). Mean blood flow was measured with the use of transit time ultrasonography (Transonics, Ithaca, N.Y.) in the right CCA before and after flow modulation. Mean arterial pressure was monitored and recorded with an indwelling transduced 23-gauge catheter in the facial artery. Flow and blood pressure measurements were obtained after denudation injury (baseline), at 10 and 30 minutes after blood flow manipulation.

Measurement of plasma TXB_2. Blood was drawn from the central artery of the ear before operation (baseline) and from the right facial artery distal to the injured CCA via a catheter at 10 and 30 minutes after blood flow modulation. Blood was centrifuged immediately at 800 *g* for 20 minutes. Platelet-rich plasma was collected and stored at –20° C until the assay was performed.

Plasma TXB_2 was measured with an enzyme immunoassay kit[24] (Cayman Chemical, Ann Arbor, Mich.). Before the plasma was added to the assay well, it (0.25 ml) was purified through a C-18 reverse phase Sep-Pak cartridge (Millipore, Milford, Mass.) after the removal of precipitated proteins. The cartridge was rinsed with 5 ml of Ultra-Pure water, followed by hexane, and then eluted with 5 ml of ethyl acetate containing 1% methanol. After evaporation under a stream of dry nitrogen, the dry samples were dissolved in 0.5 ml of assay buffer (100 mmol/L K_2HPO_4, pH 7.4, 1.5 mmol/L NaN_3, 0.4 mol/L NaCl, 1 mol/L EDTA, and 0.1% bovine serum albumin). We added 50 µl of samples or standard to the wells of the plate, which had been coated with mouse monoclonal antibody, followed by 50 µl of acetylcholinesterase linked to TXB_2 and 50 µl of TXB_2-specific rabbit antiserum. The plate was then incubated for 18 hours at room temperature. After washing, the plate was developed with 200 µl of Ellman's reagent, a substrate to acetylcholinesterase that consists of acetylthiocholine and 5,5´-dithio-bis(2-nitrobenzoic acid). Absorbance was determined with an enzyme-linked immunosorbent assay (ELISA) reader (Spectra Max 340, Molecular Devices, Menlo Park, Calif.) at 412 nm. The concentration of TXB_2 in each sample was calculated by identifying the percentage of bound sample divided by the percentage of maximum bound sample on the standard curve.

Determination of plasma PDGF-AB levels. Plasma was obtained as described above. PDGF-AB levels were detected with a solid-phase ELISA technique[25] (Quantikine immunoassay kit, R&D Systems, Minneapolis, Minn.). Of each sample or standard, 100 µ l was loaded onto the wells of the plate, which had been coated with a murine monoclonal antibody specific for human PDGF-AA.

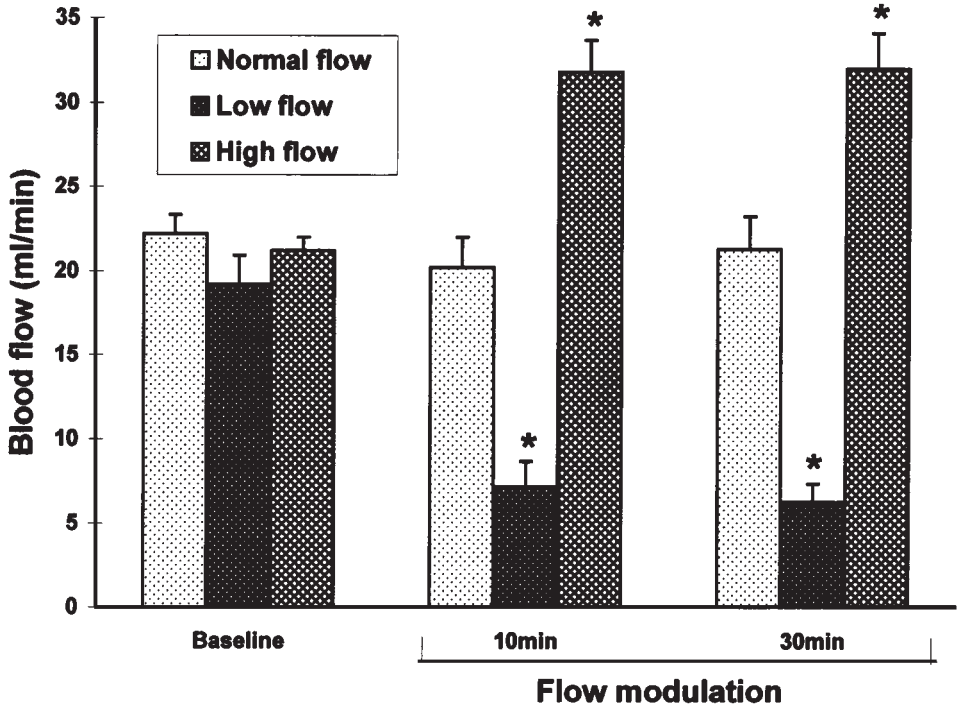

Fig. 1. Blood flow measurement in the three experimental groups. Blood flow was markedly reduced in the LF group and significantly increased in the HF group compared with the NF group at 10 and 30 minutes after flow modulation. There were no differences among flow rates in the different groups before flow modulation (baseline).
Data are presented as mean ± SEM.
*$p < 0.05$ compared with the NF group.

After incubation for 2 hours at room temperature, the plate was washed and incubated with a horseradish peroxidase-linked polyclonal antibody specific for human PDGF-BB for an additional 2 hours at room temperature. After washing, 100 µl of substrate solution (tetramethylbenzidine) was added to each well. The absorbance of the immunoreaction was read on a Kinetic microplate reader (Molecular Devices) at 450 nm. The concentration of PDGF-AB in each sample was calculated based on the standard curve, which was plotted on log/log paper.

Data analysis. Analysis of variance (ANOVA) was used to compare the mean responses in the three groups defined by animals assigned to the targeted LF, NF, and HF rates (these were performed on both the original and log-transformed scales; the latter scale was used to stabilize the variance across groups). The relationship between mean flow and TXB_2 values was calculated with Spearman's rank-order correlation coefficients. Results were expressed as mean value ± SEM. A value of $p < 0.05$ was considered to be significant.

RESULTS

Hemodynamic parameters. Mean baseline blood flow did not differ among groups: 22 ± 1 ml/min, 19 ± 2 ml/min, and 21 ± 1 ml/min in the NF, LF, and HF groups respectively. At 10 minutes, out-flow ligation was successful in reducing flow by threefold to 7 ± 1 ml/min in the LF group ($p < 0.01$), whereas ligation of the contralateral left CCA increased baseline flow by 1.5-fold to 32 ± 2 ml/min in the HF group ($p < 0.05$) compared with the NF group (20 ± 2 ml/min). At 30 minutes, flow rates in the different groups were similar to those measured at 10 minutes (Fig. 1). Mean arterial pressure (MAP) in the right CCA did not differ among the LF, HF, and NF groups at all time points and ranged from 49 mm Hg to 56 mm Hg.

Plasma TXB_2 levels under different flow conditions. Systemic TXB_2 levels before injury (baseline) ranged from 0.5 to 0.7 ng/ml. After blood flow modulation, there was a greater than fourfold increase in TXB_2 levels in the LF group at 10 and 30 minutes (3.6 ± 0.9 ng/ml and 3.8 ± 1.4 ng/ml, respectively) compared with the NF (0.8 ± 0.3

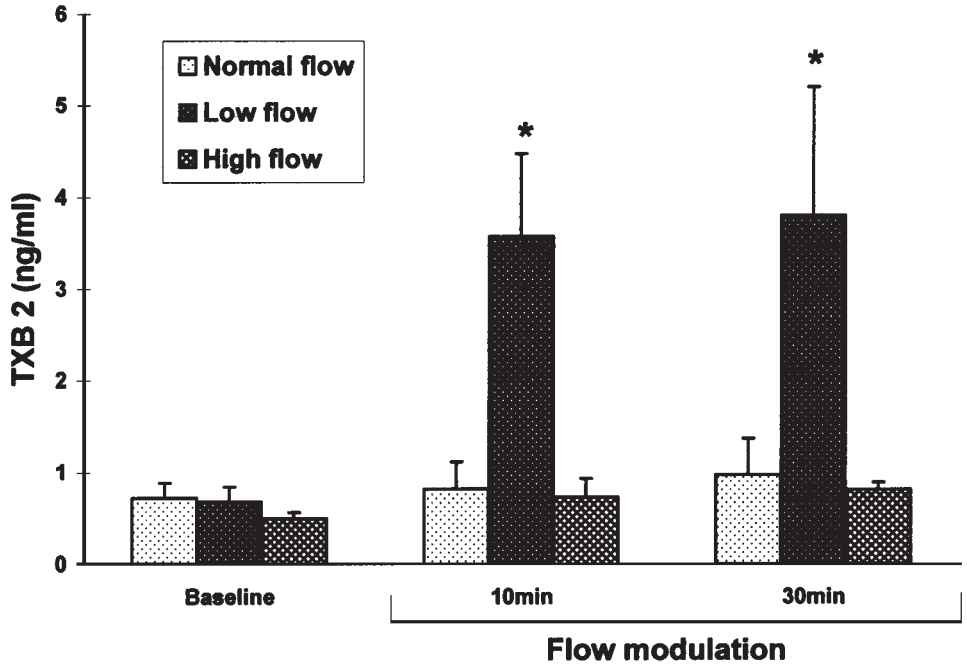

Fig. 2. Plasma TXB_2 levels in the three experimental groups. There was a greater than fourfold increase in TXB_2 levels in the LF group at 10 and 30 minutes after blood flow modulation compared with the NF and HF groups. TXB_2 levels were minimally increased in the NF and HF groups compared with baseline levels. Data are presented as mean ± SEM.
*$p < 0.05$ compared with the NF and HF groups.

ng/ml and 1 ± 0.4 ng/ml) and HF (0.7 ± 0.2 ng/ml and 0.8 ± 0.1 ng/ml) groups ($p < 0.05$) at similar time points. TXB_2 levels in the NF and HF groups were not significantly different from baseline values (Fig. 2). Spearman's rank-order correlation coefficients are significant between TXB_2 levels and mean flow at 10 minutes ($r = 0.51$, $p = 0.029$) and 30 minutes ($r = 0.58$, $p = 0.012$). There is clustering of the low and high values (Fig. 3). The plots consistently show an increase in TXB_2 levels when LF conditions prevail.

This is further substantiated by ANOVA of the log-transformed scales and standard deviation values of the LF, NF, and HF groups. At 10 minutes, the null hypothesis that all three groups have the same mean can be rejected ($F = 6.16$, $p = 0.011$). The pairwise comparisons indicate that the differences between the LF and NF groups and the LF and HF groups are significant ($p < 0.05$) but that the difference between the NF and HF groups is not (p = NS). At 30 minutes, although the F test value from the ANOVA is significant ($F = 3.83$, $p = 0.045$), the pairwise group differences are not significant after Tukey's allowance for multiple comparisons.

Plasma PDGF-AB levels under different flow conditions. Systemic plasma PDGF-AB (baseline) ranged from 31 to 36 pg/ml. At 10 minutes, there was close to a twofold increase in PDGF-AB levels in LF group compared with control values (57.6 ± 6.3 pg/ml vs 34 ± 4.6 pg/ml, $p < 0.05$). At 30 minutes, PDGF-AB levels declined to values close to baseline (39.5 ± 6.2 vs 34 ± 4.6 pg/ml, p = NS). A similar trend was found in the NF group, but this was not significantly different from control values (44 ± 4.4 vs 36 ± 2.3 ng/ml, p = NS). PDGF-AB levels did not differ in the HF group from control values at either the 10 or 30 minute time point (35 ± 1.8 and 24.8 ± 4.9 ng/ml vs 31 ± 2.2 ng/ml, respectively, p = NS) (Fig. 4).

DISCUSSION

Deendothelialization and medial injury results in the immediate adhesion of platelets to interstitial collagen in the injured vessel wall. The attached platelet releases ADP and activates the arachidonic acid synthesis pathway to produce TXA_2, a potent chemoattractant and smooth muscle cell mitogen, leading to additional platelet recruitment and

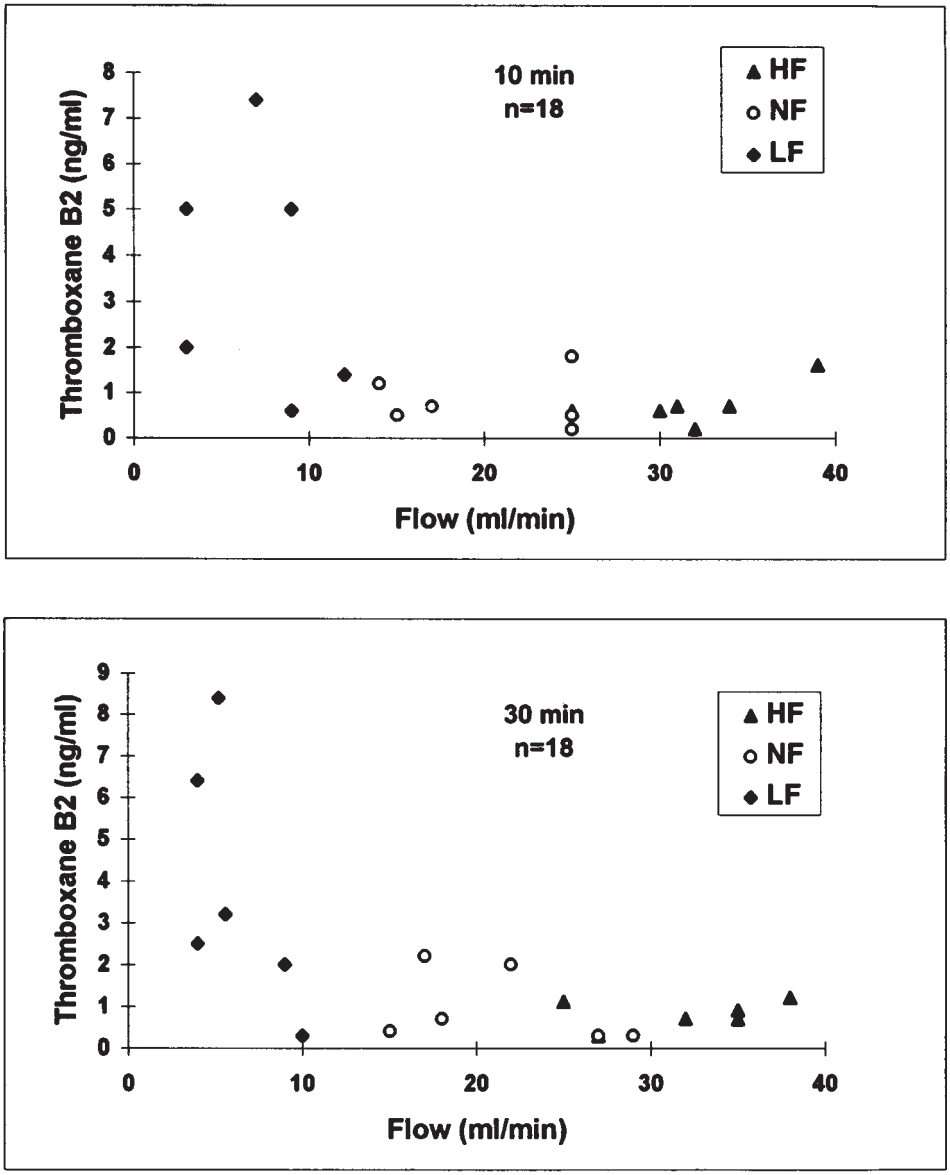

Fig. 3. The individual flow levels and TXB_2 levels were plotted. The plots consistently show an increase in TXB_2 levels when LF conditions prevail.

platelet aggregation.[5] Because TXA_2 released from activated platelet is unstable and is rapidly metabolized, its stable metabolite TXB_2 has been used as a measure of platelet activation.[26] The findings of this study indicate that platelet activation at regions of experimental arterial injury is flow dependant. In addition, upregulated platelet activity under LF conditions was accompanied by acute increases in plasma PDGF-AB levels.

Hemodynamic shear forces have been shown to exert both direct and indirect effects on platelet activity. In vitro, normal platelets fail to aggregate when placed in an aggregometer (low shear environment) unless an agonist such as collagen or ADP is added. However, platelets exposed to shear stress levels of 60 to 80 dynes/cm in a cone-and-plate viscometer (high shear environment) aggregate without the addition of an exogenous agonist.[27] A number of mechanisms by which shear forces induce platelet aggregation have been elucidated. High shear stress induces a conformational change in the platelet membrane and thereby increases its affinity

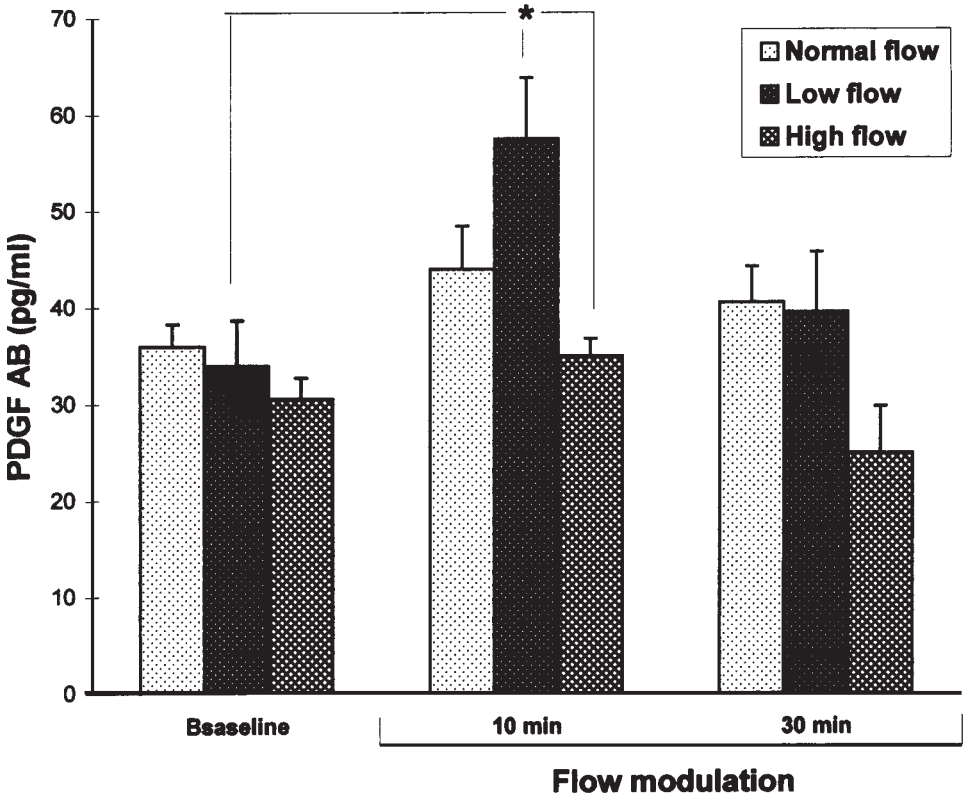

Fig. 4. Plasma PDGF-AB levels in the three experimental groups. PDGF-AB levels were increased close to twofold in the LF group compared with baseline levels at 10 minutes and then returned to baseline levels by 30 minutes. A similar trend was observed in the NF group, but this trend was not statistically significant. In the HF group, PDGF-AB levels were not significantly changed from baseline values.
Data are presented as mean ± SEM.
*$p < 0.05$ compared with baseline levels and the HF group at 10 minutes.

to von Willebrand's factor on glycoprotein IIb/IIIa and increases adhesion to the subendothelium.[28] Shear forces have also been reported to increase cytosolic free Ca^{2+} concentration, and the dense granule release by which shear forces activate platelets is unknown but may be related to a shear-sensitive Ca^{2+} channel.[29]

Mazeaud et al.[30] investigated platelet aggregation in relation to in vivo shear forces in hypertensive patients and found that ex vivo platelet reactivity to ADP or collagen was inversely related to the in vivo brachial artery blood flow velocity, wall shear rate, and stress. It has been shown that shear stress may indirectly regulate platelet activity by stimulating the production of endothelium and smooth muscle-derived antiaggregatory agents, such as prostacyclin,[15,31] endothelium-derived relaxing factor,[32] and nitric oxide.[33] Shear also increases the synthesis of tissue plasminogen activator[17] and plasmin,which cleaves the platelet glycoprotein lb receptor and thus attenuates platelet activation and its aggregation to the injured vessel wall.[34] Thus, platelet function and aggregation in response to hemodynamic shear are governed by a dynamic equilibrium between its direct effects on platelet activation and its indirect role in enhancing the production of antiaggregatory agents from the vessel wall.

The role of flow in regulating platelet activity in vivo after arterial injury has been previously studied in stenoses. Merino et al[35] demonstrated that under critical local high shear rate conditions, platelet deposition was enhanced, although the hemodynamic parameters differed from those of the current study. In their study, exceedingly high shear was induced by placing an external ring to the damaged area, which produced a significant stenosis. In the current study, flow was modulated within a lower range of shear rate values. Furthermore, the stenosis model is complicated by the development of turbulence and regions of flow separation, whereas the

flow profile in the rabbit CCA is close to being laminar because of its uniform diameter and lack of side branches. Kohler and Jawien[36] used electron microscopy to investigate the degree of platelet adhesion in relation to flow at regions of arterial injury in rats. No difference was found between the LF and HF groups at 1 hour and 7 days after balloon injury. These findings may be accounted for by the narrow range of flow modulation (±30%), which may minimally influence platelet adhesion, or by the delayed time points selected for the study. Previous investigation have demonstrated that platelet adhesion to the balloon-injured rabbit aorta predominantly occurs during the first 30 minutes after injury,[37] the time course used in the current study.

We previously demonstrated that there are two types of IH found in experimental anastomotic regions: suture line IH, which represents vascular healing, and arterial floor IH, which develops in regions of flow oscillation and relatively low shear.[19] Increasing flow by creating a distal arteriovenous fistula markedly reduced anastomotic IH in experimental end-to-side polytetrafluoroethylene anastomoses.[21] Low shear stress also was found to promote IH and cellular proliferation in vein and endothelialized prosthetic grafts.[38,39] Kraiss et al.[39] demonstrated that blood flow reduction is associated with enhanced VSMC proliferation and PDGF-A expression. Because a putative shear stress-responsive element (GAGACC) has been identified in the 5′-flanking region of the PDGF-B gene,[40] LF- and shear-induced IH may therefore be related indirectly to shear-mediated PDGF secretion from activated platelets and VSMCs.

To determine whether cytokine release from activated platelets at regions of arterial injury is modulated by flow, we assessed plasma PDGF-AB levels of blood samples from the distal CCA. Plasma PDGF-AB levels were significantly increased at 10 minutes after injury and normalized by 30 minutes in the LF group. Early release of PDGF-AB by LF may favor intimal VSMC migration and proliferation. A recent study by Caplice et al.[41] demonstrated that PDGF-AB released into coronary circulation after vascular injury peaked at 5 minutes and returned to preangioplasty levels by 30 minutes. Autologous serum obtained from a patient's coronary sinus after percutaneous transluminal coronary angioplasty promoted proliferation of the patient's VSMCs in culture. This result implicated that early release of biologically active growth factors, even in small quantities, may act locally in a synergistic fashion to produce a significant mitogenic signal. Because platelets are thought to be a major source of PDGF,[42] the observed peak in plasma PDGF-AB levels in the current study is more than likely of platelet origin, although some contribution from the underlying injured VSMCs is possible. VSMCs are known to secrete a variety of biologically active growth factors (e.g., PDGF, basic fibroblast growth factor, epidermal growth factor) immediately after mechanical injury.[43] Further studies, including that of platelet function and activity using specific monoclonal antibodies for glycoprotein IIb/IIIa, a receptor blockade, will help elucidate the role of hemodynamic factors in cytokine release from the injured medial smooth muscle cells.

It is important to recognize the limitations inherent to the experimental design of the current study. A longer time course assessment and a more direct quantitative evaluation of platelet adhesion using radiolabeling techniques and scanning electron microscopy ideally would help further our understanding of the role of hemodynamics in platelet activation, aggregation, and adhesion in this in vivo model.

CONCLUSIONS

We present experimental evidence that suggests LF is conductive to platelet activation and the release of mitogenic factors involved in the pathogenesis of IH. Of interest is the positive effect of NF or increased flow on this phenomenon. These findings also support the concept that objective assessment of flow parameters after vascular intervention is critical in selecting patients in need for antithrombotic therapy and predicting interventions at risk for failure for which there is a need for close hemodynamic surveillance.

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DISCUSSION

Dr. Thomas W. Wakefield (Ann Arbor, Mich.). In this investigation, Dr. Bassiouny and colleagues have shown the importance of flow rate on platelet activation with an inverse correlation between flow rate and thromboxane B_2 levels. In addition, a two-fold increase in platelet-derived growth factor-AB early in the low-flow group was not noted in the normal-flow or high-flow groups. Intimal hyperplasia was only present in the low-flow animals. The authors hypothesize that up regulation and platelet activation in areas of low flow may be related to increased platelet-exposure time to subendothelial collagen. This study is an important elegant study that adds much to our knowledge about arterial injury and the response to that injury.

I have the following questions:

First, is there any information in your model on the activation of other blood elements, such as leukocytes, in the areas of low flow with prolonged resins times?

Second, as you suggested at the end of your presentation, some authors have made the recommendation for use of oral anticoagulants in situations of low-flow velocities after distal bypass graft procedures, especially those involving prosthetic grafts. Your data also would suggest that antiplatelet agents should be incorporated into these treatment regimens. Can you please comment on this concept?

Third, do changes in intimal hyperplasia occur at more than or less than a threshold flow velocity, either in your model or in patients?

Additionally, could you tell us if the most important factor here is flow rate or shear rate?

And finally, on the basis of your own experience and review, is low shear or high shear most contributing to the development of intimal hyperplasia?

Like all good investigative work, this study raises as many new areas for further investigation as it answers. I congratulate the authors on the excellent presentation and thank them for sending me the manuscript. I also thank the Midwestern Vascular Surgical Society for the opportunity to discuss this paper.

Dr. Hisham S. Bassiouny. Thank you for these interesting questions. I will try to answer them in order.

We did not find any evidence of lymphocytic or leukocytic infiltration in the histopathologic specimens that were examined at 1 week. However, an early inflammatory reaction may have been present but not detected because we did not use some specific labeling techniques for lymphocytes or leukocytes.

With regards to the question of anticoagulants in patients who undergo infrainguinal prosthetic bypass surgery, I think that the key findings of the study suggest that measurement of the intraoperative and postoperative hemodynamic parameters may help select those patients who in fact may benefit from antithrombotic therapy. At this time we are looking perspectively into the value of impedance measurements, flow rates, and pressure gradients in vein grafts to assess the value of these parameters in relation to immediate and long-term patency of infrainguinal vein grafts. To correlate these hemodynamic variables with the presence of intimal hyperplasia in vivo is difficult, however, we are hopeful that future imaging methods may help us understand and characterize this common cause of restenosis more clearly.

I do not know the threshold flow velocity below which intimal hyperplasia does occur. There is experimental evidence that would suggest that if the shear values dropped to less than 5 dyne/cm^2, intimal thickening might in fact be enhanced. As we know, normal shear stress values in the human arterial vessels range anywhere from 10 to 20 dyne/cm^2.

And finally, do I think that flow rate or shear rate is the most important factor? This question is a complex question. This is because of the direct effect of shear rate on platelet activation. This effect is seen in high shear stress values of 60 to 80 dyne/cm^2. The direct effect of hemodynamic shear on platelet activation is to be contrasted with the beneficial effect of or shear stress on production of platelet antiaggregatory agents from the vessel wall. High-flow conditions have been shown to enhance tissue plasminogen activator from the endothelial cells and smooth muscle cells. So, the net effect is related to a dynamic equilibrium between the direct effect of the shear forces on the platelet and on modulation of the secretion of antiaggregatory agents from the intima and media.