Published in final edited form as:

Microvasc Res. 2011 January ; 81(1): 108–116. doi:10.1016/j.mvr.2010.09.010.

Acute Venous Occlusion Enhances Matrix Metalloprotease Activity. Implications on Endothelial Dysfunction

Tom Alsaigh¹, Elizabeth S. Pocock^{1,2}, John J. Bergan², and Geert W. Schmid-Schönbein¹

¹ Department of Bioengineering, The Institute for Engineering in Medicine, University of California San Diego, La Jolla, California 92093-0412

² Department of Surgery University of California San Diego La Jolla, California 92093-0412

Abstract

Venous hypertension is associated with microvascular inflammation, restructuring, and apoptosis, but the cellular and molecular mechanisms underlying these events remain uncertain. In the present study we tested the hypothesis that elevated venous pressure and reduction of shear stress induces elevated enzymatic activity. This activity in turn may affect endothelial surface receptors and promote their dysfunction. Using a rodent model for venous hypertension using acute venular occlusion, microzymographic techniques for enzyme detection, and immunohistochemistry for receptor labeling, we found increased activity of the matrix metalloproteases (MMPs) -1, -8 and -9 and tissue inhibitors of metalloproteases (TIMPs) -1,-2 in both high and low-pressure regions. In this short time frame we also observed that elevated venule pressure led to two different fates for the vascular endothelial growth factor receptor-2 (VEGFR2); in higher-pressure upstream regions some animals exhibited higher VEGFR2 expression, while others displayed lower levels upstream compared to their downstream counterparts with lower pressure. VEGFR2 expression was, on average, more pronounced upon application of MMP inhibitor, suggesting possible cleavage of the receptor by activated enzymes in this model. We conclude that venous pressure elevation increases enzymatic activity which may contribute to inflammation and endothelial dysfunction associated with this disease by influencing critical surface receptors.

Keywords

matrix metalloproteases; inflammation; vascular endothelial growth factor-2; occlusion; mesentery; venule; endothelial cell

Introduction

Chronic venous insufficiency (CVI) is a common condition that is characterized by severe destruction of venous valves leading to reflux of blood and culminating in venous hypertension. (Kaplan, R.M et al., 2003; Langer, R.D et al., 2005). Venous disease is preceded by an inflammatory cascade that involves leukocyte attachment to venous endothelium, oxygen free radical production, endothelial and parenchymal apoptosis, and

Correspondence: Dr. Geert W. Schmid-Schönbein, Department of Bioengineering, The Institute for Engineering in Medicine, University of California San Diego, La Jolla, California 92093-0412, Tel: 858 534 3852, Fax: 858 534 5722, gwss@bioeng.ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

gradual distension and restructuring of the venous wall (Bergan JJ et al., 2006). The underlying mechanisms leading to inflammation, restructuring and breakdown of the venous wall and surrounding structures are, however, not well understood.

In recent years, matrix metalloproteases (MMPs) have emerged as possible contributors to the pathologies associated with venous disease. MMPs are proteolytic enzymes responsible for the synthesis and degradation of the extracellular matrix (ECM). In addition, MMPs have also been shown to cleave ECM receptor proteins (e.g. integrins), growth factor receptors (e.g. FGF receptor-1), the insulin receptor, and cell adhesion molecules, thereby derailing cellular signaling and other important cellular functions (Kajita, M et al., 2001; Levi, E et al., 1996; Noe, V et al., 2001; Streuli, C et al., 1999; DeLano et al., 2008). MMPs are also involved in the regulation of apoptosis (Egeblad, M et al., 2002). A number of studies have suggested that MMPs, especially MMP-1, -2, -3, -9 and -12 and their endogenous inhibitors (TIMPs), may play a role in the pathogenesis of venous hypertension and structuring of the venous wall (Buján J et al., 2000; Sansilvestri-Morel P et al., 2007; Sansilvestri-Morel P et al., 2005; Pascarella L et al., 2005; Woodside KJ et al., 2003; Zamboni P et al., 2005) (Herouy Y et al., 1998; Tarlton JF et al., 1999). Yet, there are no studies that have examined MMP activity over different time periods of venous pressure elevation to identify early versus later stage MMP expression patterns. We have previously found elevated MMP activity in venules and other microvessels in the spontaneously hypertensive rat model (SHR). This activity was associated with cell damage and cleavage of the extracellular domain of membrane receptors (DeLano, F.A et al., 2008; Tran, E.D., 2010). Among these receptors is the vascular endothelial growth factor receptor 2 (VEGFR2), a receptor partially responsible for promoting cell survival and inhibiting apoptosis.

Hence, this study was designed to test the hypothesis that acute venous hypertension would result in high activity of MMPs in the venule wall. We have also tested whether this enhanced activity can promote cleavage of key receptors such as VEGFR2.

Materials and Methods

Animals

The animal protocol was reviewed and approved by the Animal Subjects Committee of the University of California, San Diego and conforms to the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health (NIH Publication No. 85-23, 1996). Eight-week-old male Wistar rats (300–350 g, $n=48$) were purchased from Charles River Breeding Laboratories (Wilmington, Mass). The left femoral vein was cannulated under general anesthesia (pentobarbital sodium, Abbott Laboratories, North Chicago, IL, 50 mg/kg, I.M.). Cannulation ensured sustained anesthesia for the duration of the experimental procedure. The animals breathed spontaneously without tracheotomy.

Mesentery

The rat mesentery was exposed through a midline incision and gently draped over a transparent pedestal on a heated animal stage (at 37°C). The mesentery tissue was continuously superfused (2.0 ml/min) with Krebs-Henseleit bicarbonate-buffered solution (37°C) containing a 95% N₂-5% CO₂ gas mixture. Care was taken to avoid any reduction of a fluid suffusion over the tissue since even short periods of drying cause instantaneous apoptosis.

Intravital Microscopy and Microhemodynamics

Images of the rat mesenteric microcirculation were recorded with an intravital microscope (Leica) by use of a color charge-coupled device camera (DEI-470, Optronics Engineering;

Goleta, CA; frame rate 1/125 s for bright field and 1/2 s for fluorescence light). All images were recorded on videotape (Model AG-a270P, Panasonic; Tokyo, Japan) and digitally stored for analysis.

The microcirculation was viewed with a water immersion objective lens ($\times 25$, numerical aperture = 0.60, Leitz; Wetzlar, Germany). To elicit fluorescent images, the preparation was illuminated with a 200-W mercury lamp. The light was passed through a quartz collector, heat filter (KG-2, Zeiss; Oberkochen, Germany), and fluorescent filter set (L3 filter cube, Ploempak, Leitz). Single microscopic fields ($\sim 300 \mu\text{m} \times 350 \mu\text{m}$) containing arterioles and venules were examined. Four mesenteries were investigated for each enzyme substrate, with one obstruction of a venule in each tissue (Takase S et al., 2002).

Venule Occlusion

A closed end micropipette on a micromanipulator was used to gently compress a local region along the venule and thereby obstruct blood flow for selected short periods (15 minutes occlusion). To minimize damage to the vessel, the micropipette was blunted by exposing the open tip to a flame for a brief time period. The tip of the micropipette ($\sim 50 \mu\text{m}$) was carefully lowered onto the venule without impeding blood flow in adjacent vessels (e.g. the parallel arteriole) during occlusion (Figure 1). Therefore the use of a local micropipette for these occlusions does not obstruct blood flow in vessels other than the venule under investigation.

Occlusion, if maintained for an extended period on mesenteric venules, results in a lack of reperfusion. Therefore the occlusion protocol was designed such that each 15 minute occlusion period was followed by 15 minutes of reperfusion, and this cycle was repeated three times for a total of 90 minutes. This approach ensures a sustained elevation of venous pressure with significantly reduced likelihood of venous stasis caused by prolonged occlusion (Takase S et al., 2000).

Microzymography and Measurements

To investigate enzymatic activity in the mesentery in vivo during and after venous occlusion, the following fluorescent enzyme substrates were used: for MMP-8 (*N*-(2,4-Dinitrophenyl)-Pro-Leu-Gly-Cys(Me)-His-Ala-D-Arg amide), and for MMP1/9 (*N*-(2,4-Dinitrophenyl)-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg amide) ($1.5 \mu\text{mol/L}$, Sigma Aldrich Inc., St. Louis, MO). In addition as a part of a pilot study to determine the activity of the major enzymes that become activated during the acute occlusion period the following fluorescent substrates were investigated: MMP-2 (7-Methoxycoumarin-4-acetyl-Pro-Leu-Ala-Nva-L-(2,4-dinitrophenyl)diaminopropionyl-Ala-Arg-amide), MMP-3 (7-Methoxycoumarin-4-acetyl-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-(2,4-dinitrophenyl)Lys amide), lipase (4-Methylumbelliferyl oleate) ($1.5 \mu\text{mol/L}$, Sigma Aldrich Inc., St. Louis, MO), and elastase activity was tested with the fluorogenic elastase substrate (MeOSuc-AAPV-AMC) ($1.5 \mu\text{mol/L}$, Calbiochem, catalogue number 324740-25MG). Measurements of enzymatic activity were obtained by superfusion of the mesentery with each individual fluorogenic substrate ($1.5 \mu\text{mol/L}$, diluted with Krebs-Henseleit bicarbonate-buffered solution) in the suffusate. Substrate is added to the mesentery suffusion 10 minutes prior to image collection and continuously throughout the duration of the experiment. Substrate cleavage products produce fluorescence with an intensity that is proportional to the enzyme activity in the tissue subtracted from auto-fluorescence values of the tissue. Reproducibility of measurements was within 3%. A broad based MMP inhibitor (GM6001, 50nmol/L , Millipore) was applied directly into the suffusate in subsequent experiments in order to attenuate MMP activity.

Enzyme activity along the venular endothelium was determined by measurement of fluorescent intensity (Image J, v1.34, Public Domain Software, NIH). Measurements were taken at the start of the first occlusion, followed by repeated occlusion and reperfusion (each for 15 minutes) over a total period of 90 minutes.

Two different analytical measurements were carried out. For MMP-8 measurements, the fractions of fluorescent leukocytes were determined by superposition onto bright field images with the co-localized leukocytes (#leukocytes/100µm venule endothelium). The fluorescent intensity values for MMP-1/9 activity were determined by tracing 100µm of the venule at approximately 50µm upstream and downstream of the occlusion site. Measurements of the same vessel were made five times and averaged to minimize random experimental error. The mean fluorescence intensity values were used to represent the MMP activity in endothelial cells.

Immunohistochemistry and Histology

After the rat was sacrificed, mesentery tissue sectors were excised from the intestine and quickly placed on a microscope slide to dry. Adipose tissue was removed. Fixation was performed with cold acetone for 10 minutes, then stored overnight in 1X Phosphate Buffered Saline (PBS). The next day slides were incubated with 5% normal goat serum (NGS) blocking solution. Additionally, tissue sectors were blocked with Avidin and Biotin (Vector Laboratories Inc.) solutions for 15 minutes each. After repeated PBS washing, the tissue sections were stained for either extracellular VEGFR2 antibody (1:200 dilution in 5% NGS, Genetex Inc.) or TIMP-1,-2 antibody (1:200 in 5% NGS, Calbiochem) overnight at 4 degrees. Slides were subsequently incubated with secondary antibody (goat anti-chicken or goat anti-mouse IgG biotinylated, Bethyl Laboratories Inc.) diluted 1:1000 for 90 minutes at room temperature, stained with DAB solution (Vector Laboratories, Inc.) and dehydrated with alcohol and cleared with xylene.

The density of extracellular VEGFR2 labeling was measured on individual microvessels in the form of light absorption, A , such that

$$A = -\ln\left(\frac{I}{I_0}\right)$$

I is the light intensity over the tissue and I_0 the light intensity over the background. The measurements on the tissue specimens were made in a blinded fashion to the operator. Repetition of the light absorption measurement over the *same* tissue gives virtually identical values (error less than 1%) and has minimal operator error.

Wright's stain was used to distinguish between leukocyte types and to identify presence of neutrophils in occluded regions. Briefly, mesentery sectors were excised, fixed in cold acetone (10 minutes) and subsequently stained with Wright's stain (1 minute). Slides were washed, dehydrated and cover-slipped for imaging.

Statistics

Measurements are presented as mean $\pm$ standard deviation. Differences between the upstream, downstream, and control tissue segments were first analyzed using one-way analysis of variance. A Scheffe's comparison test was subsequently performed to test for differences among means of each group. $P < 0.05$ was considered significant.

Results

MMP-1/9 Activity

MMP-1/9 activity as detected by the microzymography was present on venular endothelial cells (Fig. 2). During the repeated occlusion/reperfusion cycles there was an overall upregulation of enzymatic activity compared with control measurements (without occlusions), with segments upstream of the occlusion having higher activity compared to those measured downstream (Fig. 2A–C). Furthermore, after superfusion of the mesentery with a broad-acting MMP inhibitor (GM6001), there was a significantly lower fluorescence level associated with MMP activity both upstream and downstream from the occlusion point (Fig. 2A lower panel & 2C). Despite the presence of migrating and adherent leukocytes on and around the endothelium, there was no evidence of MMP-1/-9 release by these white blood cells in any rats tested (n=4). This evidence suggests that the endothelial cells serve as the main source of these proteases in these experiments.

MMP-8 Activity

Increased leukocyte derived MMP-8 activity was elevated after 90 minutes (Fig 3A). The number of leukocytes interacting with the venular endothelium per unit length increased after each successive occlusion and reperfusion period both upstream and downstream of the occlusion point (Fig. 3B). There was not only a significant influx of rolling and adherent leukocytes on the endothelium upstream and downstream of the occlusion point, but many of these leukocytes exhibited an upregulation of MMP-8 activity. This may be part of the early injury response following the occlusion. In the control group, there was only a small increase in fluorescence associated with migrating and adherent leukocytes. After application of an MMP inhibitor (GM6001), the fluorescent intensity due to cleavage of MMP-8 substrate was significantly attenuated (Fig. 3C). However, bright field images showed an influx of rolling leukocytes around the exposed mesentery sectors, thus indicating that the release of MMP-8 by the leukocytes was mediated by the acute injury mechanism due to the occlusions. Many of the adherent leukocytes were neutrophils with segmented nucleus (Fig. 3D).

MMP-2,-3

In contrast, no change in MMP-2,-3 activity was measured by microzymography after 90 minutes of occlusion and reperfusion (Figure 4).

TIMP-1,-2 Labelling

The presence of TIMP-1 and TIMP-2 was detected after the occlusion, with upstream portions of venules exhibiting higher levels of anti-proteolytic activity than downstream and control segments (Figure 5).

Extracellular VEGFR2 Labelling

At the end of the experiment, mesentery tissue sectors were instantly excised and labelled with an antibody against the extracellular domain of VEGFR2. The venules in four out of six rats exhibited on average a 4.6% lower receptor density upstream compared to downstream from the occlusion site (Fig. 6A&B). This trend could be inhibited by GM6001 (Fig. 6C&D). However, this pattern was not uniform among all mesenteries we studied. The venules in two out of six rats expressed a 3.7% increase in receptor density upstream of the occlusion site compared to downstream, while three sectors treated with the MMP inhibitor expressed less receptor than the untreated ones. Altogether, our results, although not statistically different, indicate that the levels of the extracellular domain of VEGFR2 tend to

be lower upstream of the occlusion site and not different downstream (Fig 6E). Inhibition of MMPs tends to preserve the expression of the receptor.

Mesentery venules in sham animals exhibited a heterogeneous label density for the extracellular domain of VEGFR2 from one endothelial cell to the next but were on average uniform along the length of the venules (Figure 7).

Discussion

Venous insufficiency needs to be studied in its earliest stages in man at a time when preventative measures could be applied to ameliorate or even abort the degenerative process. Both primary and chronic venous insufficiency are characterized by venular degeneration and an inflammatory reaction that involves leukocyte attachment to venous endothelium, oxygen free radical production, apoptosis, gradual distension and restructuring of the venous wall (Bergan JJ et al., 2006).

In the present study we explored the role of venular occlusion on MMP activity. We found that a shift in hemodynamic stresses during venous occlusion serve as a stimulatory factor in the upregulation of degrading enzyme activity. The current results in the rat mesentery provide the first indication that acute venous occlusion is accompanied within minutes by MMP and TIMP upregulation in-vivo. Venous occlusion is accompanied by a change in blood pressure, which depends on location upstream (about 30 mmHg pressure elevation in the mesentery during the occlusion, Takase et al., 2002) or downstream (with lower pressure that does not significantly change during the occlusion) of the occlusion site, and at the same time reduction of shear stress to a level that is the same in the up- and downstream venular segment. The protease activation occurs within minutes and involves MMPs that are blocked by a broad range MMP inhibitor (GM6001).

The events described herein precede the longer-term inflammatory events in venous disease, which eventually result in vein valve damage and weakening of the venous wall (Bergan JJ et al., 2006; Bergan JJ et al., 2008; Pascarella, L et al., 2005; Pascarella, L et al., 2005; Takase S et al., 2004). In the current acute microvascular model, an increase in venous pressure causes vein wall expansion (Takase S et al., 2000), and the corresponding increase in vein wall mechanical stress augments the expression and activity of MMPs (Pascarella, L et al., 2008) and TIMP-1,-2 levels. The imbalance between MMP and TIMP levels leading to increased levels of extracellular matrix proteins has been documented in venous disease (Badier-Commander C, et al., 2000). The presence of MMPs and TIMPs in this acute venous occlusion model suggests that there is an important early interplay between protease and inhibitor during events that precede the development of venous disease.

Among a multitude of degrading enzymes that we tested in the current acute model of venous occlusion only MMP-8 and MMP-1/-9 activity were found to be elevated while MMP-2, -3, elastase and lipase activities were unchanged. MMP-1, -2, -3, -9 and -12 activity has been detected in chronic human models of venous hypertension (Buján J et al., 2000; Sansilvestri-Morel P et al., 2007; Sansilvestri-Morel P et al., 2005; Pascarella L et al., 2005; Woodside KJ et al., 2003; Zamboni P et al., 2005). In the current model there is insufficient time for de-novo synthesis of new MMPs, and likely both MMP-8 and MMP-1/-9 are already constitutively expressed. MMP-8 accumulates via the leukocytes and MMP-1/-9 are likely already expressed in endothelium and are merely activated during the venous occlusion. The MMPs expressed during chronic pressure elevation also includes enzymes derived from macrophages (MMP-12) that are not present in significant numbers in acute situations. The time course that leads from an initial MMP profile to one expressed in chronic venous disease remains to be determined and may be influenced by cofactors (e.g.

hormones, obesity and intestinal proteases) (Gilliver SC et al., 2007) that are likely present in chronic venous hypertension but less so in acute forms.

In the current model the MMP-8 accumulates and exhibits activity in the lumen of the venules that is co-localized with adhering leukocytes, while MMP-1/-9 is upregulated by endothelial cells. Leukocytes (especially neutrophils), as major carriers of MMP-8, accumulate during the acute occlusion period in increasing numbers predominantly on the high-pressure, upstream side of the occlusion (Takase S et al., 2002). Thus it is possible that the major contribution of the MMP-8 activity in the current model is derived from the attachment of leukocytes to the venular endothelium.

A primary event during occlusion is the reduction of the fluid shear stress on the endothelium as well as on circulating cells in the occlusion segment. Laminar shear stress has been shown to have a variety of effects on endothelial cells, including suppression of specific stages of the normal cell cycle (Akimoto S et al., 2000; Lin K et al., 2000), prevention of apoptosis (Dimmeler S et al., 1999; Dimmeler S et al., 1996), activation of Thr/Ser and Tyr kinases (Chen KD et al., 1999), and activation of integrin mediated mechanotransduction (Shyy, J.Y et al., 2002). In light of the evidence that fluid shear stress may have a strong physiological effect on a broad range of cell activities (Busse R et al., 2006; Davies PF et al., 2005; Makino A et al., 2007), a reduction of the fluid shear stress may have a direct impact on normal anti-inflammatory activities that would otherwise prevent the accumulation of degradative enzyme activity.

Overall the results show that the upstream segment of a venule, which is under elevated pressure during the occlusion, tends to be subject to higher levels of proteolytic activity compared with venular segments not subject to occlusion. Elevated pressure is associated with increased stretch of the venule and its endothelium. Venous occlusion with elevated pressures causes more oxygen free radical formation, higher numbers of leukocytes attached to venular endothelium, and higher levels of apoptosis compared with the venules experiencing the same reduction in fluid shear stress, but not the elevated blood pressure (Takase S et al., 2002). This is consistent with the in-vitro evidence that stretch of cells applied acutely over short period of time leads to activation of MMP (Asanuma K et al., 2003).

Among a variety of receptors that may be influenced by MMPs, we selected VEGFR2 as a major signalling molecule for endothelial cells. In light of the importance of VEGFR2 for survival of the endothelium, a possible enzymatic destruction of this receptor may be a potential signal for reduced viability of endothelial cells, possible apoptosis, along with the loss of capillaries (rarefaction) (Tran, E.D et al., 2010). Our results in the acute venous occlusion model indicate that MMPs may have a dual role in venular endothelial cells. They may be involved with upregulation of receptors and with possible receptor destruction. It is possible that cleavage of VEGFR2 enhances a compensatory response where endothelial cells release intracellular stores of the receptor onto the cell surface, thereby compensating for the loss of receptor due to MMP-mediated destruction. We are limited in our efforts to determine if the receptor has been destructed because of the inability to measure intracellular levels of VEGFR2 in this in-vivo model. No suitable antibody currently exists for this purpose. The possibility of receptor internalization by stressed endothelial cells in this model remains a possibility. The presence of MMP activation may also affect other receptors and be the start for an actual breakdown of the structure of the venous wall with venous enlargement and eventually failure of the venous valves.

The issue of MMP involvement is important for understanding the etiology of chronic venous disease and requires longer studies in which hormonal regulation and hemodynamic

factors are controlled individually and MMP activity is monitored. Amelioration of the inflammatory process that causes damage to venous structures is a potential target for pharmacological intervention and therapy. The possible dual mechanism for MMPs in affecting expression patterns of VEGFR2 remains still to be further explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Rafi Mazor for his critical reading of this manuscript.

Grants: This work was supported by a Grant from the La Jolla Vein Institute Foundation and in part by NIH Grant HL 10881.

References

- Akimoto S, Mitsumata M, Sasaguri T, Yoshida Y. Laminar shear stress inhibits vascular endothelial cell proliferation by inducing cyclindependent kinase inhibitor p21. *Circ Res* 2000;86:185–190. [PubMed: 10666414]
- Allegra C, Bergan J. Update on fundamental causes and management of chronic venous insufficiency. Executive summary. *Angiology* 2003;54(Suppl 1):S1–3. [PubMed: 12934751]
- Asanuma K, Magid R, Johnson C, Nerem RM, Galis ZS. Uniaxial strain upregulates matrix-degrading enzymes produced by human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2003;284:H1778–1784. [PubMed: 12543633]
- Badier-Commander C, Verbeuren T, Lebard C, Michel J-B, Jacob M-P. Increased TIMP/MMP ratio in varicose veins: a possible explanation for extracellular matrix accumulation. *J Pathol* 2000;192:105–112. [PubMed: 10951407]
- Bergan JJ, Schmid-Schönbein GW, Smith PD, Nicolaides AN, Boisseau MR, Eklof B. Chronic venous disease. *N Engl J Med* 2006;355:488–498. [PubMed: 16885552]
- Bergan JJ, Pascarella L, Schmid-Schonbein GW. Pathogenesis of primary chronic venous disease: Insights from animal models of venous hypertension. *J Vasc Surg* 2008;47(1):183–192. [PubMed: 18178472]
- Buján J, Jurado F, Gimeno MJ, Garcia-Honduvilla N, Pascual G, Jiménez J, Bellón JM. Changes in metalloproteinase (mmp-1, mmp-2) expression in the proximal region of the varicose saphenous vein in young subjects. *Phlebology* 2000;15:64–70.
- Busse R, Fleming I. Vascular endothelium and blood flow. *Handb Exp Pharmacol* 2006:43–78. [PubMed: 16999224]
- Chen KD, Li YS, Kim M, Li S, Chien S, Shyy J. Roles of receptor tyrosine kinases, integrins, and Shc in the mechanotransduction in response to fluid shear stress. *J Biol Chem* 1999;274:18393–18400. [PubMed: 10373445]
- Cummins PM, von Offenber Sweeney N, Killeen MT, Birney YA, Redmond EM, Cahill PA. Cyclic strain-mediated matrix metalloproteinase regulation within the vascular endothelium: A force to be reckoned with. *Am J Physiol Heart Circ Physiol* 2007;292:H28–42. [PubMed: 16951049]
- Davies PF, Spaan JA, Krams R. Shear stress biology of the endothelium. *Ann Biomed Eng* 2005;33:1714–1718. [PubMed: 16389518]
- DeLano FA, Schmid-Schönbein GW. Proteinase activity and receptor cleavage: Mechanism for insulin resistance in spontaneously hypertensive rat. *Hypertension* 2008;52:415–423. [PubMed: 18606910]
- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–605. [PubMed: 10376603]
- Dimmeler S, Haendeler J, Rippmann V, Nehls M, Zeiher AM. Shear stress inhibits apoptosis of human endothelial cells. *FEBS Lett* 1996;399:71–74. [PubMed: 8980122]

- Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161–174. [PubMed: 11990853]
- Gilliver SC, Ruckshanthi JP, Atkinson SJ, Ashcroft GS. Androgens influence expression of matrix proteins and proteolytic factors during cutaneous wound healing. *Lab Invest* 2007;87:871–881. [PubMed: 17607299]
- Herouy Y, May AE, Pornschlegel G, Stetter C, Grenz H, Preissner KT, Schopf E, Norgauer J, Vanscheidt W. Lipodermatosclerosis is characterized by elevated expression and activation of matrix metalloproteinases: Implications for venous ulcer formation. *J Invest Dermatol* 1998;111:822–827. [PubMed: 9804345]
- Kajita M, et al. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J Cell Biol* 2001;153:893–904. [PubMed: 11381077]
- Kaplan RM, Criqui MH, Denenberg JO, Bergan J, Fronek A. Quality of life in patients with chronic venous disease. San Diego population study. *J Vasc Surg* 2003;37(5):1047–1053. [PubMed: 12756353]
- Kolbach DN, Hamulyak K, Prins MH, Neumann HA, Cleutjens JP. Severity of venous insufficiency is related to the density of microvascular deposition of PAI-1, uPA and von Willebrand factor. *Vasa* 2004;33(1):19–24. [PubMed: 15061043]
- Langer RD, et al. Relationships between symptoms and venous disease: the San Diego population study. *Arch Intern Med* 2005;165(12):1420–1424. [PubMed: 15983292]
- Levi E, et al. Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. *Proc Natl Acad Sci USA* 1996;93:7069–7074. [PubMed: 8692946]
- Lin K, Hsu PP, Chen BP, Yuan S, Usami S, Shyy J, Li YS, Chien S. Molecular mechanism of endothelial growth arrest by laminar shear stress. *Proc Natl Acad Sci U S A* 2000;97:9385–9389. [PubMed: 10920209]
- Makino A, Shin HY, Komai Y, Fukuda S, Coughlin M, Sugihara-Seki M, Schmid-Schönbein GW. Mechanotransduction in leukocyte activation: A review. *Biorheology* 2007;44:221–249. [PubMed: 18094448]
- Noe V, et al. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001;114:111–118. [PubMed: 11112695]
- Pascarella L, et al. Mechanisms in experimental venous valve failure and their modification by Daflon 500 mg. *Eur J Vasc Endovasc Surg* 2008;35(1):102–110. [PubMed: 17890112]
- Pascarella L, Penn A, Schmid-Schönbein GW. Venous hypertension and the inflammatory cascade: Major manifestations and trigger mechanisms. *Angiology* 2005;56 (Suppl 1):S3–10. [PubMed: 16193224]
- Pascarella L, Schönbein GW, Bergan JJ. Microcirculation and venous ulcers: a review. *Ann Vasc Surg* 2005;19(6):921–927. [PubMed: 16247708]
- Pascarella L, Schmid-Schönbein GW, Bergan J. An animal model of venous hypertension: the role of inflammation in venous valve failure. *J Vasc Surg* 2005;41(2):303–311. [PubMed: 15768014]
- Raffetto JD, Khalil RA. Mechanisms of varicose vein formation: valve dysfunction and wall dilation. *Phlebology* 2008;23(2):85–98. [PubMed: 18453484]
- Sansilvestri-Morel P, Fioretti F, Rupin A, Senni K, Fabiani JN, Godeau G, Verbeuren TJ. Comparison of extracellular matrix in skin and saphenous veins from patients with varicose veins: Does the skin reflect venous matrix changes? *Clin Sci (Lond)* 2007;112:229–239. [PubMed: 17020541]
- Sansilvestri-Morel P, Rupin A, Jullien ND, Lembrez N, Mestries-Dubois P, Fabiani JN, Verbeuren TJ. Decreased production of collagen type iii in cultured smooth muscle cells from varicose vein patients is due to a degradation by mmps: Possible implication of mmp-3. *J Vasc Res* 2005;42:388–398. [PubMed: 16088212]
- Shyy JY, Chien S. Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* 2002;91:769–775. [PubMed: 12411390]
- Streuli C. Extracellular matrix remodelling and cellular differentiation. *Curr Opin Cell Biol* 1999;11:634–640. [PubMed: 10508658]
- Takase S, Lerond L, Bergan JJ, Schmid-Schönbein GW. Enhancement of reperfusion injury by elevation of microvascular pressures. *Am J Physiol Heart Circ Physiol* 2002;282:H1387–1394. [PubMed: 11893575]

- Takase S, Lerond L, Bergan JJ, Schmid-Schönbein GW. The inflammatory reaction during venous hypertension in the rat. *Microcirculation* 2000;7:41–52. [PubMed: 10708336]
- Takase S, Pascarella L, Bergan JJ, Schmid-Schönbein GW. Hypertension-induced venous valve remodeling. *J Vasc Surg* 2004;39:1329–1334. [PubMed: 15192576]
- Tarlton JF, Bailey AJ, Crawford E, Jones D, Moore K, Harding KD. Prognostic value of markers of collagen remodeling in venous ulcers. *Wound Repair Regen* 1999;7:347–355. [PubMed: 10564563]
- Tran ED, DeLano FA, Schmid-Schönbein GW. Enhanced matrix metalloproteinase activity in the spontaneously hypertensive rat: VEGFR-2 cleavage, endothelial apoptosis, and capillary rarefaction. *J Vasc Res* 2010;47:423–431. [PubMed: 20145415]
- Woodside KJ, Hu M, Burke A, Murakami M, Pounds LL, Killewich LA, Daller JA, Hunter GC. Morphologic characteristics of varicose veins: Possible role of metalloproteinases. *J Vasc Surg* 2003;38:162–169. [PubMed: 12844106]
- Zamboni P, Scapoli G, Lanzara V, Izzo M, Fortini P, Legnaro R, Palazzo A, Tognazzo S, Gemmati D. Serum iron and matrix metalloproteinase-9 variations in limbs affected by chronic venous disease and venous leg ulcers. *Dermatol Surg* 2005;31:644–649. discussion 649. [PubMed: 15996413]

Figure 1.

Micrograph of a typical postcapillary venule (V) and occlusion strategy. A micropipette with a rounded tip was gently lowered onto the vessel until blood flow in the venule came to a halt. The direction of the flow in the venules before occlusion is shown by the arrow. Unbranched venules were chosen, and all measurements were taken 50µm upstream (US) or downstream (DS) from the occlusion site, with the occlusion site being avoided for measurements due to injury to the vessel. Such an occlusion leads to about 30 mmHg increase in micropressure upstream from the occlusion site in this model (Takase S et al, 2002).

C.

Figure 2.

Selected microvascular images of (A) MMP-1/-9 upregulation by endothelial cells upstream and downstream of the occlusion site in the rat mesentery. MMP-1/-9 activity control sequence and MMP-1/-9 activity during occlusion and superfusion with GM6001 (MMP inhibitor) shown. Comparison of fluorescent intensity values (B) without MMP inhibitor and (C) with inhibitor, $n=4$ for all groups. V=Venule. A=Arteriole. Arrows demonstrate increased enzyme release by venule endothelial cells. Bars on graphs represent periods of venular occlusion. * $p<0.05$ for all time points in comparison of the means of the three groups by ANOVA. †Scheffe's comparison test of US vs Control at 95% Confidence interval ($p<0.05$), DS vs Control ($p<0.05$).

Figure 3.

(A) Upregulation of MMP-8 activity via leukocyte release at T=0 and T=90 upstream from the occlusion point. Arrows indicate absence/appearance of leukocytes. Comparison of percentage of fluorescing leukocytes (B) without MMP inhibitor and (C) with inhibitor, n=4 for all groups. Arrows indicate leukocyte-fluorescence co-localization. (D) Leukocyte morphology shows the typical segmented nucleus of neutrophils (N) within the occluded venule. Length bar = 10µm. Graph bars represent periods of venular occlusion. *p<0.05 for all time points in comparison of the means of the three groups by ANOVA. †Scheffe's comparison test of US vs Control at 95% Confidence interval (p<0.05), DS vs Control (p<0.05), and US vs DS (p<0.05).

Figure 4.

Selected microvascular images of MMP-2,-3 activity by endothelial cells upstream of the occlusion site in the rat mesentery at 0 and 90min. Graphs detail the activity level of these MMPs throughout the in vivo experiment. Note the unchanged levels of these proteases throughout the occlusion experiment.

Figure 5.

After 90 minute occlusion and reperfusion of postcapillary venule, mesentery sectors were excised and labeled for TIMP-1,-2 presence. Panels represent venule lengths (bar) 50 μ m upstream and downstream of the occlusion site, an adjacent control vessel, and a venule labeled with a normal mouse IgG as an antibody control. Note the increased presence of TIMPs 1 and 2 on vessels affected by postcapillary occlusion experiment.

Figure 6.

After 90 minute occlusion and reperfusion of postcapillary venule, mesentery sectors were excised and labeled for extracellular VEGFR2 using immunohistochemical staining techniques. Selected microvascular images shown represent venule lengths (bar) 50µm upstream and downstream of the occlusion site. Panels A and B and represent the mesentery sector of one animal without superfusion of MMP inhibitor during acute occlusion. Panels C and D represent a mesentery sector superfused with MMP inhibitor during acute occlusion. Arrows indicate endothelial cell VEGFR2 label density. Note the variable label density among different animals. A total of four animals per group (with and without inhibitor) were examined for extracellular VEGFR2 destruction (E). No statistically significant difference was seen between the groups shown.

Figure 7.

Figures A and B represent postcapillary venules located adjacent to occluded venules from two different mesentery preparations in the acute occlusion experiment. These venules were not obstructed, nor were they manipulated in any way during the occlusion experiment. Note the heterogeneous extracellular VEGFR2 label density along the length of the venules. Panel C represents a normal chicken IgG control stain for the extracellular VEGFR2 antibody used.