Calpains Contribute to Vascular Repair in Rapidly Progressive Form of Glomerulonephritis: Potential Role of Their Externalization


Objective—Calpains, calcium-activated proteases, mediate the angiogenic signals of vascular endothelial growth factor. However, their involvement in vascular repair has not been investigated and the underlying mechanisms remain to be fully elucidated.

Methods and Results—A rapidly progressive form of glomerulonephritis in wild type and transgenic mice expressing high levels of calpastatin, a calpain-specific inhibitor, was studied. Calpastatin transgene expression prevented the repair of peritubular capillaries and the recovery of renal function, limiting mouse survival. In vitro analysis detected a significant reduction of both intracellular and extracellular calpain activities in transgene expressing cells, whereas Western blotting revealed that proangiogenic factors vascular endothelial growth factor and norepinephrine increased calpain exteriorization. In vitro, extracellular calpains increased endothelial cell proliferation, migration and capillary tube formation. In vivo, delivery of nonpermeable extracellular calpastatin was sufficient to blunt angiogenesis and vascular repair. Endothelial cell response to extracellular calpains was associated with fibronectin cleavage, generating fibronectin fragments with proangiogenic capacity. In vivo, fibronectin cleavage was limited in the kidney of calpastatin transgenic mice with nephritis.

Conclusion—This study demonstrates that externalized calpains participate in angiogenesis and vascular repair, partly by promoting fibronectin cleavage and thereby amplifying vascular endothelial growth factor efficiency. Thus, manipulation of calpain externalization may have therapeutic implications to control angiogenesis. (Arterioscler Thromb Vasc Biol. 2012;32:335-342.)

Key Words: angiogenesis ■ kidney ■ pathology ■ vascular biology

Calpains are calcium-activated neutral cysteine proteases.1,2 Two major isoforms, calpain μ and m which require micromolar and millimolar Ca2+ concentrations for activity, respectively, are ubiquitously expressed, whereas the other isoforms are tissue-specific forms. Their activity is limited by calpastatin, a specific endogenous inhibitor that contains 4 equivalent inhibitory domains.1,2 Calpains play an important role in inflammatory process. First, they are involved in the activation of NF-κB, and thereby in the NF-κB–dependent expression of proinflammatory cytokines and adhesion molecules.3 Second, calpains are critical for inflammatory cell adhesion and chemotaxis and inflammatory mediator processing.4,5 Third, calpains are implicated in the cleavage of the heat shock protein 90, which is required for both binding and anti-inflammatory efficacy of glucocorticoids.6 Finally, calpains are externalized during inflammatory process and play a role in the microenvironment of inflammatory cells.7 We have recently demonstrated that calpains participate in the development of inflammatory lesions in an acute model of antiglomerular basement membrane nephritis.8

In endothelial cells, calpain activity contributes to the hydrolysis of cytoskeletal proteins,9 thereby facilitating shear stress-induced endothelial cell alignment,10 and lymphocyte or leukocyte transendothelial migration.11,12 Importantly, vascular endothelial growth factor (VEGF) increases m-calpain expression and activity in those cells.13–15 In turn, calpain activity participates in angiogenesis process, possibly by promoting cytoskeleton reorganization,13 rear-cell detachment,14 and/or nitric oxide generation.15 However, whether activation of calpains plays any role in the vascular repair process besides the formation of new blood vessels is still unknown.

In severe and progressive kidney diseases, there is a rarefaction of peritubular capillaries which precedes the
development of tubulo-interstitial fibrosis and the decline of renal function.\textsuperscript{16–20} This process is the hallmark of experimental and human kidney diseases regardless of their cause, such as antiglomerular basement membrane models of glomerulonephritis, anti-Thy-1.1 model of acute glomerulonephritis, remnant kidney model, aging associated renal disease, chronic cyclosporine A nephropathy, and kidney transplantation.\textsuperscript{16,17} Progressive loss of peritubular capillaries is explained by a decrease in postglomerular flow from diseased glomeruli and an inappropriate activation of endothelial cells by factors such as angiotensin II, which allows rolling and sticking of leukocytes, thus limiting oxygen delivery.\textsuperscript{18,19} Hypoxia and inflammatory conditions lead to endothelial cell death. Nevertheless, endothelium has a strong potential to regenerate via the proliferation of resident progenitors and/or the recruitment of circulating endothelial progenitor cells.\textsuperscript{17}

This angiogenesis process results from the local balance between pro- and antiangiogenic factors.\textsuperscript{16} A loss of the angiogenic VEGF expression and a marked expression of the antiangiogenic factor thrombospondin-1 limit the repair of peritubular capillaries and promote the development of interstitial fibrosis.\textsuperscript{21,22}

Herein we took advantage of transgenic mice constitutively expressing high levels of calpastatin\textsuperscript{8} to study the impact of calpains/calpastatin balance on regeneration of peritubular capillaries in a severe model of antiglomerular basement membrane nephritis. Our results show that calpain activity is involved in this repair process. Surprisingly, extracellular calpains are particularly effective in capillary formation, suggesting that manipulation of calpain externalization rather than intracellular expression may have therapeutic implications to favor or inhibit angiogenesis.

Methods

Calpastatin transgenic (CalpTG) mice were created and characterized in the laboratory.\textsuperscript{8} Glomerulonephritis was induced in male C57BL/6 wild type (WT) or CalpTG mice as described previously.\textsuperscript{23} Renal injury was evaluated on days 7 and 14. Specimens of kidney were cut for histochemistry and immunohistochemistry studies. Calpain activity and expression were determined with a fluorescence assay described previously\textsuperscript{8} and a Western assay, respectively. Human umbilical vein endothelial cells (HUVECs) were obtained to assess endothelial cell proliferation, monolayer repair, transmigration, and capillary formation.

For an expanded Materials and Methods, see the Supplemental Data, available online at http://atvb.ahajournals.org.

Results

CalpTG Mice Show a Defect in Both Formation of New Blood Vessels and Vascular Repair

To explore the importance of calpain/calpastatin balance in renal vascular repair, we compared WT and CalpTG mice. We first localized the transgene product in the kidney by immunohistochemistry (Figure 1A). Calpastatin expression was detectable in only a few tubules and in peritubular capillary cells. The intensity of this staining increased markedly in CalpTG as compared to WT mice. Then, to verify the role of calpain/calpastatin balance in the formation of new blood vessels in our mouse model, we used a Matrigel plug assay.\textsuperscript{13,24} Hemoglobin content in Matrigel plugs 10 days after subcutaneous injection, which measured blood vessel formation in the presence of VEGF-A, was significantly reduced in CalpTG as compared with WT recipients (Figure 1B). Finally, to assess the role of calpain/calpastatin balance in renal vascular repair, a rapidly progressive form of glomerulonephritis was induced in mice. Antiglomerular basement membrane serum (a total of 1.5 mg total protein/g body weight) was injected intravenously over 3 consecutive days, as previously described.\textsuperscript{23} Survival curves show that the number of surviving mice decreased much more rapidly for CalpTG than WT mice (Supplemental Figure 1A). Blood urea nitrogen levels rose rapidly during the acute phase of immunologic injury until day 7 and then over the course of 14 days improved toward baseline values in WT mice while being even higher in CalpTG mice (Supplemental Figure 1B).
Peritubular capillary density was quantified in 2 ways after mouse CD31 (PECAM) or panendothelial cell antigen (MECA-32) immunostaining of endothelial cells. First, the mean number of capillary cross-sections per high power field (hpf) at 200× magnification was measured in tubulointerstitial areas (Supplemental Figure IC–ID and Supplemental Figure II). As expected, capillary density significantly decreased in all mice at day 7 after glomerulonephritis induction. One week later, this defect was reversed in WT mice while persisting in CalpTG mice. Second, peritubular capillary density was quantified by computer image analysis of MECA-32-stained area. At day 14, this area increased, consistent with capillary dilatation (Supplemental Figure II). Again, such a staining pattern was significantly altered in CalpTG as compared to WT mice.

Altogether, these results underscore the involvement of calpains in vascular repair in addition to their established role in the formation of new blood vessels.

**Extracellular Activity of Calpains Is Limited by Calpastatin Transgene Expression and Amplified by Proangiogenic Factors**

Calpains are intracellular calcium-activated proteases that are partly externalized. To assess the respective consequences of calpastatin transgene expression on intracellular and extracellular calpain activities, we decided to analyze both polyoma middle T-transformed mouse brain capillary endothelial cells (bEND.3 cells) transfected with the cDNA clone of endothelial cells, they are available without in vitro culture. Indeed, in contrast to microvascular control cDNA, and T cells isolated from the spleen of CalpTG and WT mice. Again, such a staining pattern was significantly altered in CalpTG as compared to WT mice.

Thus, the lack of new blood vessel formation and vascular repair in CalpTG mice is potentially due to a decrease in intracellular and/or extracellular calpain activity.

**Hypoxia and inflammatory conditions are responsible for the expression of a great diversity of proangiogenic factors including peptidic (e.g., VEGF) and nonpeptidic (e.g., norepinephrine) mediators.** To further identify the respective roles of intracellular and extracellular activities of calpains in angiogenesis process, we measured the effect of VEGF and norepinephrine on calpain activity and expression in endothelial cells. Human umbilical vein endothelial cells were exposed to the indicated concentrations of VEGF and norepinephrine for 24 hours and calpain activity was determined by measuring the calpain-specific cleavage of fluorescent 7-amino-4-methylcoumarin (AMC) (n=5). *P<0.05, significant difference from untreated cells. Extracellular Activity of Calpains Is Limited by Calpastatin Transgene Expression and Amplified by Proangiogenic Factors

Figure 2. Effect of proangiogenic factors vascular endothelial growth factor (VEGF) and norepinephrine on calpain activity and expression in endothelial cells. A, Effect of proangiogenic factors on intracellular and extracellular activities of calpain. Human umbilical vein endothelial cells were exposed to 10 nmol/L norepinephrine (N) or 10 ng/mL VEGF (V) for 4 hours before Western blot analysis. Blots are representative of 3 independent experiments. Histograms show means±SEM (n=3). *P<0.05, significant difference from untreated cells.

that did not achieve statistical significance and a statistically significant rise of m-calpain, as demonstrated by Western blot analysis (Figure 2B). Calpain secretion is thought to be in an unconventional way due to the lack of N-terminal classic secretion signal peptide. Underlying mechanisms are not known but might involve microparticle formation and shedding. To determine whether microparticles are carrying calpains, microparticles were isolated from the conditioned medium of norepinephrine-treated endothelial cells. They were identified by flow cytometry as events measuring 0.1 to 1.0 μm that stained positive for annexin V bound to extracellular calpain activity into the extracellular milieu was not attributable to increased apoptosis or necrosis of the treated cells as evident by the similar percentage of mortality (propidium iodide-positive cells) of the norepinephrine-treated and control untreated cells (5.6±0.9 and 6.5±1.0, respectively). Externalization of calpain activity was associated with a rise in extracellular μ-calpain, a trend that did not achieve statistical significance and a statistically significant rise of m-calpain, as demonstrated by Western blot analysis (Figure 2B). Calpain secretion is thought to be in an unconventional way due to the lack of N-terminal classic secretion signal peptide. Underlying mechanisms are not known but might involve microparticle formation and shedding. To determine whether microparticles are carrying calpains, microparticles were isolated from the conditioned medium of norepinephrine-treated endothelial cells. They were identified by flow cytometry as events measuring 0.1 to 1.0 μm that stained positive for annexin V bound to extracellular calpain activity into the extracellular milieu was not attributable to increased apoptosis or necrosis of the treated cells as evident by the similar percentage of mortality (propidium iodide-positive cells) of the norepinephrine-treated and control untreated cells (5.6±0.9 and 6.5±1.0, respectively). Externalization of calpain activity was associated with a rise in extracellular μ-calpain, a trend that did not achieve statistical significance and a statistically significant rise of m-calpain, as demonstrated by Western blot analysis (Figure 2B). Calpain secretion is thought to be in an unconventional way due to the lack of N-terminal classic secretion signal peptide. Underlying mechanisms are not known but might involve microparticle formation and shedding. To determine whether microparticles are carrying calpains, microparticles were isolated from the conditioned medium of norepinephrine-treated endothelial cells. They were identified by flow cytometry as events measuring 0.1 to 1.0 μm that stained positive for annexin V bound to extracellular calpain activity into the extracellular milieu was not attributable to increased apoptosis or necrosis of the treated cells as evident by the similar percentage of mortality (propidium iodide-positive cells) of the norepinephrine-treated and control untreated cells (5.6±0.9 and 6.5±1.0, respectively). Externalization of calpain activity was associated with a rise in extracellular μ-calpain, a trend...
sured the effect of calpain on DNA synthesis in HUVECs and found a dose-dependent increase with a maximal effective concentration of 1 μg/ml (Figure 3A). Such a proliferative response, nearly comparable to that obtained with 2% serum, was dependent on the activity of the protease, because calpastatin addition blocked calpain effects (Supplemental Figure V). Because both μ- and m-calpain accumulated in the culture medium of endothelial cells (Figure 2B), we addressed the respective contribution of either externalized calpain to the process of HUVEC proliferation. To this aim, HUVECs were cultured with endothelial cell conditioned media first depleted of μ- and/or m-calpain by absorption on

Figure 3. Effect of extracellular calpains on endothelial cell proliferation, migration, capillary formation, and microvessel outgrowth from mouse aortic explants. A, Human umbilical vein endothelial cells (HUVECs) (50×10³ cells/well) were cultured in basal medium with or without 2.0% fetal calf serum (FCS) or the indicated concentrations of μ-calpain (top; n=5) or with endothelial cell conditioned media (eCM) first depleted of μ- and/or m-calpain by absorption on wells coated with specific antibodies or nonspecific immunoglobulin G (IgG) (bottom; n=4). Additions were performed at 24 and 48 hours. BrdU was added to the wells at the later time. After further incubation (24 hours), DNA synthesis was measured using 5-bromo-2-deoxyuridine assay kit. *P<0.05 and **P<0.005, significant difference from untreated cells (top) or cells treated with non depleted eCM (bottom). B, After a cell-free zone (0.5 mm) was created in the monolayer, HUVECs, were incubated for the indicated periods of time with (gray circles) or without (white circles) μ-calpain (2 μg/mL). Thereafter, monolayer repair was measured and expressed as percentage of wound surface area (n=4). **P<0.01, significant difference from untreated cells. C, HUVECs in suspension were seeded on Matrigel and incubated for 20 hours with or without μ-calpain in the presence of 0.5% FCS and with or without calpeptin or calpastatin in the presence of 2.0% FCS. The density of formed tubes was analyzed by measuring total capillary length by high power field. (n=4–6). *P<0.02 and ****P<0.0002, significant difference from untreated cells. D, Aortic explants from wild type (WT) or Calpastatin transgenic (CalpTG) mice adherent to Matrigel were treated as indicated for 7 days. Quantification of total microvessel outgrowth demonstrates a significant increase in aortic rings exposed to vascular endothelial growth factor (VEGF), norepinephrine (NE), or extracellular μ-calpain and, conversely, a significant decrease in aortic rings treated by extracellular calpastatin. (n=3–5). *P<0.05, significant difference from untreated aortic explants.
wells coated with specific antibodies. Both μ- and m-calpain depletion significantly limited DNA synthesis in HUVEC under these experimental conditions, and the effect of μ-calpain depletion was additive to that of m-calpain (Figure 3A). Thus, the 2 exteriorized calpains participate in endothelial cell proliferation.

Second, we analyzed the ability of HUVEC to migrate into a denuded area using a 2D scratch assay. Wound closure measured after 3, 6, and 9 hours was significantly faster after addition of calpain in the culture medium (Figure 3B).

Third, we examined the role the extracellular calpains play in the ability of endothelial cells to form vessels in vitro. HUVECs were incubated in Matrigel for 24 hours and endothelial tube formation was analyzed by measuring the total length of capillaries formed per high power field at 400× magnification. Addition of calpain enhanced significantly endothelial cell tube formation, whereas, conversely, there was a reduction of serum-induced tube formation in the presence of factors inhibiting extracellular (non permeant calpastatin) and/or intracellular (calpeptin) calpain activity (Figure 3C and Supplemental Figure VI).

Finally, we measured the formation of vascular structures in an ex vivo sprouting assay. Addition of calpain to the culture medium resulted in increased sprouting from mouse aortic rings similar to that of VEGF and norepinephrine (Figure 3D). Conversely, endothelial sprout formation was limited by the presence of calpastatin or calpeptin.

Extracellular Calpains Induce Both Formation of New Blood Vessels and Vascular Repair In Vivo

As secreted calpains contribute to angiogenesis process in vitro, we needed to verify their specific role in new blood vessel formation in vivo. To this aim, we used again the Matrigel plug assay. Corresponding to Matrigel red colors, hemoglobin content and number of capillary cross-sections per high power field were significantly reduced after addition of calpastatin to the plugs (Figure 4A). Similarly, during wound healing process, local delivery of calpastatin significantly limited the formation of both blood and lymphatic vessels (Figure 4B).

Proangiogenic Function of Extracellular Calpains Involves Fibronectin Cleavage

Appearance of calpains in extracellular milieu leads potentially to proteolytic processing of a number of proteins at the surface of endothelial cells (eg, integrin subunits involved in endothelial cell attachment) and/or in extracellular matrix (eg, fibronectin (FN), laminin, and collagens). Our previous studies indicated that extracellular calpains play a role in tubular epithelium regeneration after renal ischemia/reperfusion via the detachment of FN fragments from the extracellular matrix, consistent with findings of other groups. Thus, to identify molecular mechanisms whereby extracellular calpains speed up endothelium regeneration, we evaluated the ability of calpains to hydrolyze proteins of extracellular matrix in 1D SDS-PAGE experiments (Supplemental Figure VII). After exposure to human μ-calpain or porcine m-calpain, collagen I, collagen IV, and laminin remained intact. Intact human FN appeared with an apparent molecular weight (MWapp.) >250 kDa. But additional protein bands with lower MW were also observed after incubation with calpain at MW=210 kDa and MW=140 kDa, respectively named (FN+C1)α and (FN+C1)β. Fragmentation of FN increased as a function of calpain concentration, eventually leading to the release of a 30 to 40 and 60 kDa breakdown products. The main bands were excised from the gel and after in-gel tryptic digestion, the resulting peptide digests were analyzed by MALDI-TOF/TOF. The obtained MS and MS/MS data were used for a database search, leading to the identification of the human FN (NCBI, gi | 119590951) for the 3 protein bands, namely FN, (FN+C1)α, and (FN+C1)β, with highly significant scores of 172, 341, and 255, respectively. Because of the lower MW of the (FN+C1)α and (FN+C1)β proteins, one can assume that incubation of FN with calpain results in the hydrolysis/degredation of the intact FN leading to truncated forms. MS results obtained from the analysis of the truncated proteins (FN+C1)α and (FN+C1)β show that this proteolysis occurs in the N-terminal part of the intact FN (Supplemental Table I). The peptides mass fingerprint from (FN+C1)α and (FN+C1)β contained peptides only present in the C-terminal part of the FN compared to the peptides observed for the intact FN, which were located all...
whereby externalized calpains induce endothelial cell mobilization. These data suggest an extracellular mechanism not. Moreover, calpains dramatically increased HUVEC mi-

Figure 5. Effect of calpain-induced fibronectin fragments on endothelial cell mobilization. A, Human umbilical vein endothelial cells (HUVEC) (10 000 cells/well) were incubated for 48 hours with or without native or μ- or m-calpain-treated fibronectin (5 μg/mL) and with or without vascular endothelial growth factor (VEGF) (10 ng/mL). Cell growth was determined by using a BrdU assay. (n=7). #P<0.05, significant difference from control with VEGF. B, HUVEC (20 000 cells/well) were plated onto 24-well transmigration inserts (membrane pore size, 8 μm). The lower chamber contained medium with or without native or calpain-treated fibronectin (5 μg/mL) and VEGF (10 ng/mL). After 24 hours, transmigration was quantified by hematoxylin staining and cell counting. (n=6). **P<0.0005 and *P<0.01, significant difference from control; #P<0.05, significant difference from native fibronectin.

along the sequence. Moreover, the MS/MS sequencing of peptides located in the C-terminal part of the intact FN, in position 1354 to 1382 and 1836 to 1859 in both proteins (FN-C1)α and (FN-C1)β confirmed that these truncated forms contained both the cell binding domain IIIα and the VEGF binding domain IIIβ.

Importantly, it is, to our knowledge, the first to uncover the role of exteriorized calpains in angiogenesis process both in vitro and in vivo. Additionally, we show that calpain-dependent fragmentation of FN in extracellular matrix contributes to the induction of endothelial cell proliferation and migration in vitro. As a consequence, we propose that modification of calpain externalization is a potential target for either limiting angiogenesis (eg, in tumor development) or amplifying vascular repair (eg, in ischemia/inflammation).

To explore the role of calpains in vascular repair besides its known involvement in the formation of new blood vessels, we studied a model of loss of kidney capillaries. In progressive autologous/accelerated form of anti-glomerular basement membrane glomerulonephritis in mouse, rarefaction of peritubular capillaries causes peritubular ischemia that is responsible for tubulointerstitial damage, even more than proteinuria. Here, anti-glomerular basement membrane antiserum was administered over 3 consecutive days, inducing also a severe accelerated form of glomerulonephritis. This model thus differs from that described in our previous study of calpain regulation where a single injection of anti-glomerular basement membrane antiserum caused a transient heterologous form of glomerulonephritis. Our present results demonstrate that limiting calpain activity by expressing calpastatin transgene does not affect the destruction but prevents completely the repair of peritubular capillaries, as assessed by 2 different techniques. Importantly, this is the first evidence to demonstrate the role of calpains in vascular repair besides neoangiogenesis. Further studies are clearly needed to understand the different downstream mechanisms involved in this process.

Up to now, studies performed mainly in vitro have implicated a role for intracellular calpains in angiogenesis. They mediate VEGF effects on cytoskeleton reorganization in endothelial cells, possibly through the activation of the Rho signaling pathway. Ezrin, a protein involved in cytoskeletal remodeling, would contribute to calpain localization to plasma membrane and, hence, to VEGF-dependent activation of endothelial nitric oxide synthase. As a consequence, endothelial NO production enhances endothelial cell migration, proliferation, and response to angiogenic factors. However, VEGF-induced calpain activity would also destabilize the microtubule skeleton, thereby preventing the integration of neovascular networks and causing the formation of vascular blind ends.

At variance with these previous studies, our own studies implicate calpain externalization as an important intermediate in angiogenesis process. Although calpains are considered as
intracellular proteases, a few studies show that they are partly externalized. Obviously, calpains may leak out from injured and dying cells, but the release of intracellular calpains from lymphocytes or parathyroid cells among other cells is not due to cell death. The results of our study demonstrate for the first time the exteriorization of μ-calpain and m-calpain from endothelial cells in response to proangiogenic factors, VEGF, and norepinephrine. Calpastatin is not secreted under these conditions, consistent with our findings of an increase in extracellular activity of calpains. Surprisingly, VEGF increased and norepinephrine decreased calpain activity in endothelial cells. One possible interpretation of our data here is that VEGF activates intracellular calpain activity, possibly through an effect on membrane-specific localization rather than cell expression of calpains, whereas norepinephrine limits intracellular calpain activity by increasing calpastatin expression, as shown in Western blot experiments. Actually, the fact that 2 main proangiogenic factors increase constant calpain exteriorization and not intracellular expression suggests that extracellular calpains participate in angiogenesis process, even if this role is not exclusive to them.

Our present in vitro studies strongly demonstrate an important contribution of exteriorized calpains as proangiogenic signals and as potential downstream effectors of different proangiogenic factors. Extracellular μ- and m-calpains promoted endothelial cell proliferation, migration, and capillary formation. Conversely, specific inhibition of extracellular calpains by calpastatin blunted serum-induced capillary formation from either endothelial cell suspensions or vessel explants. Next, experiments were performed to investigate signaling pathways that were associated with angiogenesis in response to extracellular calpains. Previous studies have suggested a role for protease-induced cleavage products of extracellular matrix proteins in vascular development and repair. Because calpains are known to cleave FN, we focused our studies on this protein. Exposure of native human FN to calpains generated various fragments encompassing the C-terminal heparin-II domains 9 to 10 and 13 to 14 which bind the α5β1 integrin and VEGF, respectively. Such cleavage products increased capillary formation in vitro significantly more than native FN did, confirming previous studies. In addition, cleavage of FN by calpains would be a limiting factor of endothelial cell binding to extracellular matrix. Interestingly, a weakening of the linkage between extracellular matrix and the cytoskeleton of endothelial cells is sufficient to trigger an intracellular signaling cascade leading to capillary formation. In the absence of subendothelial FN matrix, endothelial cells are thought to undergo a switch from αvβ3 to αvβ1 integrin-based adhesion, associated with a disruption of adherens junctions. In turn, αvβ3 engagement promotes angiogenesis, in part via interaction with VEGF receptor 2. Thus, FN cleavage by extracellular calpains would promote angiogenesis by both generating proangiogenic fragments and limiting FN adhesive functions.

Besides FN in the extracellular matrix, calpains are known to cleave latency-associated peptide and, hence, to activate transforming growth factor-β at the surface of endothelial cells. As a consequence, mature transforming growth factor-β could promote angiogenesis through direct effects on endothelial cells and indirectly in stimulating VEGF synthesis by nonendothelial cells. This intriguing possibility remains to be investigated.

The contribution of other potential mechanisms and their likely interaction with various signaling pathways in angiogenesis is difficult to evaluate in vitro. Thus, we tested in vivo whether inactivation of extracellular calpains by nonpermeant calpastatin would translate into deficiencies in angiogenesis and vascular repair. Once again, we showed that inhibition of extracellular calpains alone resulted in defect of capillary formation and vascular repair in Matrigel plug and skin wound healing models, respectively.

In summary, the present studies provide evidence that calpain exteriorization from endothelial cells is induced by proangiogenic factors and is crucial for the angiogenesis process. They shed new light on the fundamental aspects of angiogenesis, adding a novel member to the list of extracellular proteases involved in angiogenesis, and point to new directions in the exploration of therapeutic strategies.

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Disclosures

None.

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Detailed Methods

Mice and induction of nephritis. All procedures involving these animals were conducted in accordance with national guidelines and institutional policies. Calpastatin transgenic (CalpTG) mice were created and characterized in the laboratory (1). The presence and the expression of the cDNA clone of rabbit calpastatin were identified in founder transgenic mice by PCR and RT-PCR analysis, respectively. All animals used in these studies were homozygous for the transgene and backcrossed into the C57BL/6 background > nine generations.

Glomerulonephritis was induced in male C57BL/6 wild type (WT) or CalpTG mice by intravenous administration of a total of 1.5 mg protein of sheep anti-mouse GBM serum / g body weight, administered over three consecutive days (days 0, 1, and 2), as described previously (2).

Analyses of renal function. Blood urea nitrogen (BUN) was measured by a colorimetric method.

Immunohistochemical analyses. Renal fragments embedded in paraffin were cut into 3 µm sections. Calpastatin expression was assessed using a polyclonal primary antibody (Affinity Bioreagents; 1:200). Endothelial cells were immunostained with purified rat anti-mouse panendothelial cell antigen (MECA-32; BD Biosciences) or with purified rat anti-mouse CD31 antibody (MEC 13.3; BD Biosciences). Samples were revealed with Single Stain
Mouse MAX PO (rat) Histofine (Nichirei Biosciences). Density of peritubular capillaries was
determined on pictures at 200 x magnification by using Image J software.

**Matrigel plug assay.** Angiogenesis was determined in vivo using a Matrigel plug assay, as
previously described (3). Briefly, a volume of 0.6 mL Matrigel (BD Biosciences) containing
50 ng VEGF, 10 U heparin, and calpastatin human recombinant domain I or calpastatin
peptide negative control, was injected subcutaneously near the abdominal midline under
isoflurane anesthesia. After 10 days, mice were euthanatized and Matrigel was withdrawn
carefully. To determine blood vessel formation, haemoglobin content in the Matrigel plug was
measured using the Drabkin’s reagent (Sigma-Aldrich) and calculated using a standard with a
known concentration of haemoglobin.

**Skin wound healing assay.** 8 weeks-old C57BL/6 female mice (Jackson laboratories) were
anesthetized by inhalation of 4.9% isofluorane (Aerane®, Baxter, Deerfield, IL) at a 300
ml/min ambient air flow. After depilation, 5 mm surgical wounds were generated using a
punch biopsy device. Wound edges were injected immediately after surgery and on days 3
and 5, either with 120 µl of recombinant calpastatin (40 µg/mL) or with control peptide for
calpastatin (40 µg/ml). n=6 mice per group. Wounds were left uncovered until they were
harvested. Mice were euthanized in a CO₂ chamber. Wound tissues were embedded in O.C.T
compound and frozen at -80°C. Blood and lymphatic vessel count and surface were measured
on 3 different fields on double-labeled cryosection for CD31 (BD Pharmingen) and Lyve-1
(Abcam), respectively. Measurements were done using a fluorescence microscope (Leica),
with an imaging digital camera and a motorized measurement platform (Exploranova®).
**Cell cultures.** After written informed consent was obtained from donors, human umbilical vein endothelial cells (HUVECs) were isolated with collagenase perfusion of term umbilical cord vein. They were cultured in Endothelial Cell Basal Medium 2 (ECBM2; PromoCell) supplemented as indicated by the provider. HUVECs between passage 2 and 3 were used for experiments. The bEND.3, a polyoma middle T–transformed mouse brain capillary endothelial cell line, was obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. These cells were transfected transiently with 3 µg of PCI expression vector including or not the cDNA clone of rabbit calpastatin (PM 194) using a Nanofectin Kit (PAA Laboratories).

Spleen cells from WT and CalpTG mice were isolated by passing the tissue through a nylon membrane. They were depleted of erythrocytes by 90 sec exposure to ACK lysing buffer (BioWhittaker), washed, and resuspended in RPMI-1640 medium supplemented with 10% FCS, 1% glutamine, 10 mM HEPES, 0.05 mM β-mercaptoethanol, and penicillin / streptomycin. T cells were isolated from this preparation using the mouse CD3⁺ T cell enrichment kit (Stem Cell Technologies) according to the manufacturer’s instructions.

**Endothelial cell proliferation.** To perform BrdU incorporation assays, HUVECs (50×10³ cells/well) were cultured in medium (ECBM2 supplemented with 0.5% fetal bovine serum) with or without

- additional 2.0% fetal bovine serum,
- μ-calpain purified from human erythrocytes (Calbiochem),
- 50% conditioned media harvested at 4 h from VEGF-treated endothelial cells, and first depleted or not of μ- and/or m-calpain by absorption on wells coated with specific antibodies or isotype control antibody for 1 h prior to adding into culture,
- native human fibronectin (Sigma)
fibronectin fragments obtained by incubation of native human fibronectin in KRH medium supplemented with 2mM calcium and 2 µg/mL human µ-calpain or porcine m-calpain. After 24h at 37°C, calpain activity was no more detectable.

Additions were performed at 24 and 48 h. BrdU was added to the wells at the later time. After further incubation (24 hours), cells were fixed, denatured, and immunostained with the anti-BrdU antibody (Cell Proliferation ELISA, BrdU, Roche, manufacturer's instructions).

**Flow cytometric detection of microparticles.** The bEND.3 endothelial cells were cultured with 100 nM norepinephrine for 24h. At the end of incubation period, the culture medium was removed and centrifuged at 800 g for 5 min, and at 4,500 g for 5 min to pellet contaminating cells. Supernatants were eventually centrifuged at 18,000 g for 45 min. The resulting microparticle-containing pellet was washed in PBS and dispersed in 100 µl Annexin V binding buffer and Annexin V-FITC (Annexin V-FITC Apoptosis detection kit, Sigma-Aldrich), according manufacturer’s instructions. For calpain detection, microparticles were permeabilized using the BD cytofix/cytoperm kit (BD Biosciences) according manufacturer’s instructions. Primary antibody (rabbit anti-mouse µ-calpain, Santa Cruz Biotechnology) or isotype-matched control (rabbit IgG, Santa Cruz Biotechnology) was added and samples were incubated on ice for 20 min. After washing, microparticles were incubated with secondary antibody (AlexaFluor® 647 goat anti-rabbit, Invitrogen) on ice for 20 min and then washed 3 times. Resulting samples were run on a Gallios flow cytometer (Beckman Coulter) and microparticles were identified by gating of size (less than or equal to 1 µm) and by binding of Annexin V to phosphatidylserine. Standard beads of different diameters (0.5-3 µm, Megamix) were used for size calibration. Calpain-positive microparticles were identified by fluorescence levels greater than two standard deviations above the isotypic controls.
**Endothelial cell migration.** Two different assays were performed. For endothelial monolayer repair assay, 7x10⁴ HUVEC were cultured to confluence in each of the two wells of a Culture-Insert (Ibidi). After 24 h, the Culture Insert was removed and the cell monolayer including a central cell-free gap of 0.5 mm was covered with fresh medium with or without µ-calpain (Calbiochem) or porcine m-calpain (Calbiochem). Gap surface area was analyzed at 3, 6, and 9h by phase contrast microscopy.

For transmigration assay, 2x10⁴ HUVECs in culture medium devoid of VEGF were added onto the upper chamber of 24-well transmigration inserts (Costar Transwell; membrane pore size, 8µm), the lower chamber being filled with medium containing native human plasma fibronectin (Sigma-Aldrich) or calpain-treated fibronectin fragments (5 µg/mL) and VEGF (10 ng/mL). After 24 h, transmigration was quantified by haematoxylin staining and cell counting.

**In vitro angiogenesis assay.** 6x10⁴ HUVECs in suspension were seeded on Matrigel in 24-wells and incubated for 20h with or without human µ-calpain in the presence of 0.5% FCS and with or without calpeptin or calpastatin in the presence of 2.0% FCS. The density of formed tubes was analyzed by measuring total capillary length or total segment number by hpf.

**Aortic ring outgrowth assay.** The thoracic aorta from WT or CalpTG mice was cut into 1mm segments that were placed on Matrigel and covered with culture medium devoid of VEGF and supplemented with recombinant human VEGF (BioVendor Laboratory Medicine), norepinephrine (Aguettant), human µ-calpain (Calbiochem), purified human calpastatin domain I and peptide negative control (Calbiochem), or calpeptin (Biomol). After 7 days,
mean total vessel outgrowth was measured for each ring at 200x magnification, by using Analysis software.

**Sequence analysis of fibronectin and fibronectin fragments.** Samples of native human fibronectin (Sigma) were incubated at 37°C for 1 h in KRH medium supplemented with 2mM calcium. Human µ-calpain 2 μg/mL was added twice at 30 min intervals. After calpain heat-inactivation, proteins were separated on 1D-SDS PAGE gel (12%). The bands stained with colloidal blue were excised, destained, washed twice with deionized water/ACN (1/1), and dried in ACN. Proteins in the bands were reduced with 10 mM dithiotreitol in 100 mM ammonium bicarbonate at 56°C for 45 min, and alkylated with 55 mM iodoacetamide at room temperature for 30 min in the dark. Then, gel pieces were washed twice with deionized water/ACN (1/1) and dried in ACN. Finally, spots were soaked in a 50 mM ammonium bicarbonate containing 8 ng/µL of trypsin Gold mass spectrometry grade (Promega) for 45 min on ice. After removing residual trypsin, the gel pieces were incubated in 50 mM ammonium bicarbonate at 37°C overnight. Digested peptides were collected in the supernatant and acidified in 0.1 % TFA.

Desalting and concentration of the samples – Microcolumns packed with Poros oligo 20R2 resin (Applied biosystems) were prepared as described previously (4). For each sample, two consecutive micropurifications were performed. The flowthrough from the first microcolumn containing possibly unbound peptides was systematically recovered via a second microcolumn. Finally, the bound peptides were directly eluted from both columns onto the MALDI target using 0.6 µL of the α-cyano-4 hydroxy cinnamic acid (CHCA) matrix solution. This matrix was prepared as follows: it was dissolved in 50% ACN, 50% H2O
containing 0.1% TFA at a concentration of 5 mg/mL. The dried droplet method for target preparation was chosen (5).

Mass Spectrometry – Positive ions MALDI-TOF as well as MALDI-TOF-TOF mass spectra were recorded on the Applied Biosystems 4700 Proteomics Analyzer instrument. MALDI-TOF MS was performed in reflector mode (focus mass at 2100 u) near the threshold of laser fluence. Calibration was performed using external standards (Proteomix 4 LaserBio Labs). MALDI-TOF-TOF experiments were carried out in CID mode with gas (N2, ~ 2x10-7 Torr) with collision energy of 1 keV. Typically the precursor ion (M+H+) was selected in a window (-5u, 5u) centred on the first isotope. Data Explorer version 4.6 software was used to analyze the spectra.

Protein identification – The peptide mass fingerprints (PMF) and MS/MS for the trypsin digests were submitted to a MASCOT sequence query search (www.matrixscience.com) after advanced based line correction, noise removal (standard deviation to remove = 2), and peak deisotoping (MASCOT search parameters were: NCBI nr 20090528 as the database; MSDB 20060831 and Swissprot 57.3 were also systematically searched in parallel, all entries or homo sapiens for the taxonomy, oxidized M and carbamidomethyl C as variable modifications, 2 missed cleavages allowed for trypsin, 40ppm for the peptide mass tolerance and 0.1 Da for the MS/MS tolerance). To be qualified as a positive identification, a protein’s score had to equal or exceed the minimum significant score threshold at P>0.05.

**Statistical analysis.** Data are expressed as mean ± SEM. The results were analyzed by t test (for comparison of 2 groups) or 2-way ANOVA for multiple comparisons of time courses. Results with P < 0.05 were considered statistically significant.
Supplemental References


Supplemental Figures and Figure Legends

Supplemental Figure I: CalpTG mice develop more severe renal injury than WT mice.

WT (gray) and CalpTG (black) mice were injected with anti-GBM antiserum over three consecutive days starting at day 0. (A) Survival curves show that the percentage of surviving mice decreased more rapidly for CalpTG than WT mice ($P<0.05$). (B) Blood urea was measured using an autoanalyzer. *$P<0.01$ and ***$P<0.005$, significant difference from WT control.
Supplemental Figure II: CalpTG mice exhibit defective vascular repair. Kidneys from WT (gray bars) and CalpTG mice (black bars) were immunostained with anti-mouse panendothelial cell antigen antibody (MECA-32).

Upper panel. Peritubular capillary densities were quantified by measuring the number of capillary cross-sections per high power field. *P<0.05, significant difference from corresponding control at day 0; #P<0.05, significant difference from WT at day 14.

Lower panel. Peritubular capillary density, as quantified by computer image analysis of MECA-32 immunostaining (area in µm²) in each high power field at day 14. **P=0.005, significant difference from WT.
Supplemental figure III: Effect of calpastatin transgene expression on intracellular and extracellular calpain activities. (A) Spleen CD3\(^+\) T cells (3.10\(^6\) in 500 µL) isolated from WT and CalpTG mice were stimulated for 24 h with 1 µg/mL α CD3 mAb. (B) Polyoma middle T–transformed mouse brain capillary endothelial cells (bEND.3 cells) transfected with the PCI expression vector including the cDNA clone of rabbit calpastatin (CalpTG) or not (WT) were incubated for 4 h. Intracellular and extracellular calpain activities were determined by measuring the calpain-specific cleavage of fluorescent AMC. N=6; *P<0.05, **P<0.01, ***P<0.001, significant difference from WT.
Supplemental Figure IV: Flow cytometric detection of μ-calpain in microparticles from endothelial cells. Microparticles were isolated from the medium of bEND.3 endothelial cells treated with norepinephrine for 24h, using differential centrifugations. Shown are controls, including samples of microparticles stained with FITC (A) or nonspecific IgG-Alexa 647 (B). Microparticles stained positive for annexin V-FITC (C) of which 8.52% displayed μ-calpain-Alexa 647 (D).
Supplemental Figure V: The effect of extracellular calpains on endothelial cell proliferation requires the activity of the protease. HUVECs (50×10³ cells/well) were cultured in basal medium including 0.5% FCS with or without 2 µg/ml μ- or m-calpain together with or without 20 µg/ml calpastatin. Additions were performed at 24 and 48 h. BrdU was added to the wells at the later time. After further incubation (24 hours), DNA synthesis was measured using BrdU assay kit (n = 4). * P <0.01 and # P <0.005, significant difference from untreated cells and calpastatin-treated cells, respectively.
Supplemental Figure VI: Effect of calpain activity on endothelial cell capillary formation. HUVECs in suspension were seeded on Matrigel and incubated for 20h with or without μ-calpain in the presence of 0.5% FCS and with or without calpeptin or calpastatin in the presence of 2.0% FCS. Representative photomicrographs of fields corresponding to the experiments shown in Fig. 3C. Bars, 50 µm.
Supplemental Figure VII: Effect of calpains on extracellular matrix proteins. Samples of native human fibronectin, collagen IV, collagen I or laminin (Sigma) were incubated at 37°C for 1 h in KRH medium supplemented with 2mM calcium. Either μ-calpain (μ-) or m-calpain (m-) was added twice at 30 min intervals. After calpain heat-inactivation, proteins were separated on 1D-SDS PAGE gel (12%) and the bands were stained with colloidal blue.
Supplementary Table: Identification by mass spectrometry of characteristic peptide units in human fibronectin fragments generated upon cleavage by human α-calpain.

<table>
<thead>
<tr>
<th></th>
<th>FN</th>
<th>(FN+C1)\textsubscript{A}</th>
<th>(FN+C1)\textsubscript{B}</th>
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<tbody>
<tr>
<td>\textbf{MW\textsubscript{theo}}</td>
<td>262 kD (259 kD without signal peptide)</td>
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<tr>
<td>\textbf{MW\textsubscript{app}}</td>
<td>&gt; 250 kD</td>
<td>210 kD</td>
<td>140 kD</td>
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<tr>
<td>Number of peptides observed in the PMF (MS)</td>
<td>40</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>Sequence of the intact FN covered by the observed peptides</td>
<td>From 273 to 2180</td>
<td>From 831 to 2180</td>
<td>From 831 to 2180</td>
</tr>
<tr>
<td>Position of the peptides fragmented by MS/MS in the intact FN</td>
<td>959-976 (101)</td>
<td>1130-1157 (63)</td>
<td>959-976 (101)</td>
</tr>
<tr>
<td></td>
<td>1354-1382 (82)*</td>
<td>1354-1382 (82)*</td>
<td></td>
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<tr>
<td></td>
<td>1836-1859 (24)**</td>
<td>1836-1859 (21)**</td>
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</tr>
<tr>
<td></td>
<td>2045-2060 (30)</td>
<td>2045-2060 (30)</td>
<td></td>
</tr>
</tbody>
</table>

Peptides labelled with * or ** belong to the C-terminal heparin-II domains of fibronectin (type III repeats), III\textsubscript{9} cell binding domain (1356 to 1449) and III\textsubscript{14} VEGF-binding domain (1813 to 1901) of the intact human fibronectin, respectively.