Lack of Collagen XVIII/Endostatin Exacerbates Immune-Mediated Glomerulonephritis

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ABSTRACT
Collagen XVIII is a component of the highly specialized extracellular matrix associated with basement membranes of epithelia and endothelia. In the normal kidney, collagen XVIII is distributed throughout glomerular and tubular basement membranes, mesangial matrix, and Bowman’s capsule. Proteolytic cleavage within its C-terminal domain releases the fragment endostatin, which has antiangiogenic properties. Because damage to the glomerular basement membrane (GBM) accompanies immune-mediated renal injury, we investigated the role of collagen XVIII/endostatin in this disorder. We induced anti-GBM glomerulonephritis in collagen XVIII−/−null and wild-type mice and compared the resulting matrix accumulation, inflammation, and capillary rarefaction. Anti-GBM disease upregulated collagen XVIII/endostatin expression within the GBM and Bowman’s capsule of wild-type mice. Collagen XVIII/endostatin-deficient mice developed more severe glomerular and tubulointerstitial injury than wild-type mice. Collagen XVIII/endostatin deficiency altered matrix remodeling, enhanced the inflammatory response, and promoted capillary rarefaction and vascular endothelial cell damage, but did not affect endothelial proliferation. Supplementing collagen XVIII-deficient mice with exogenous endostatin did not affect the progression of anti-GBM disease. Taken together, these results suggest that collagen XVIII/endostatin preserves the integrity of the extracellular matrix and capillaries in the kidney, protecting against progressive glomerulonephritis.


The major constituents of all basement membranes (BMs) are predominantly laminins, nidogen/entactin, collagen IV, and heparan sulfate proteoglycans (HSPGs).1,2 HSPGs are a class of biomolecules that consist of a core protein with covalently attached heparan sulfate sugar side chains. HSPGs are involved in biologic processes such as glomerular filtration, cell adhesion, migration, proliferation, and differentiation,3–5 which are mediated by the binding of chemokines, cytokines, enzymes, growth factors, or other bioactive molecules.6 Collagen XVIII (Col 18) is a HSPG associated with BMs of almost all epithelia and endothelia. This collagen contains an N-terminal noncollagenous domain (NC-11), 10 collagenous domains alternating with 9 noncollagenous repeats, and a C-terminal noncollagenous domain (NC-1).7 In the normal kidney, Col 18 is distributed throughout glomerular and tubular BMs, mesangial matrix, and Bowman’s capsule in both humans and mice.8,9

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Inactivating mutations in the human gene for Col 18, COL18A1, have been identified in patients with Knobloch syndrome, which is an autosomal recessive disorder characterized by the occurrence of vitreoretinal degeneration with retinal detachment, high myopia, macular degeneration, occipital encephalocoele, and minor renal abnormalities.\textsuperscript{10,11} The kidney of Col 18/endostatin-null mice exhibits no abnormalities on light microscopy, where expansion of the mesangial matrix and thickened proximal tubular BM are observed on electron microscopy.\textsuperscript{7,8} The study of Utriainen et al.\textsuperscript{8} suggested that Col 18/endostatin may have a role in maintaining the structural integrity of the extracellular matrix in the normal kidney, whereas its role in susceptibility and progression of inflammatory glomerular diseases remains to be clarified.

Proteolytic cleavage within NC-1 of Col 18 releases a fragment termed endostatin, which has been shown to have anti-angiogenesis activity in vitro and in vivo.\textsuperscript{1,12–16} Endostatin is an endogenous angiogenesis inhibitor and is detected in the circulation at a physiologic level of 20 to 50 ng/ml in serum.\textsuperscript{1,7,17–19} Col 18/endostatin-null mice display enhanced tumor growth when implanted with tumor cells that are unable to produce Col 18.\textsuperscript{18} In contrast, overexpression of circulating endostatin in the transgenic mice leads to reduced tumor growth and vascularization.\textsuperscript{18} Nonetheless, it is still unclear whether endostatin plays a role in renal disease as an endogenous angiogenesis inhibitor.

As a major component of the ultrafiltration apparatus in the kidney, the glomerular basement membrane (GBM) is constantly exposed to serum flow and pressure and thus needs to be functionally sound and to stringently maintain its structural integrity. Mouse anti-GBM glomerulonephritis (GN) is characterized by damage of GBM followed by invasion of inflammatory cells, accumulation of mesangial matrix, and destruction of the glomerular capillary network, finally resulting in the development of glomerular sclerosis.\textsuperscript{20–22}

In this study, we induced anti-GBM disease in Col 18/endostatin-null mice to test the hypothesis that Col 18/endostatin is critical for maintaining the integrity of the GBM and capillaries in the glomerulus. We demonstrate that Col 18/endostatin-deficient mice with anti-GBM disease have altered distribution of extracellular matrix, accelerated inflammatory responses, and more severe endothelial cell (EC) damage compared with wild-type (WT) mice with this disease. These results suggest that Col 18/endostatin may play an important role in preserving the integrity of extracellular matrixes and capillary vessels in the kidneys of patients with immune complex glomerulonephritis.

RESULTS

Col 18/Endostatin Expression Was Upregulated in the GBM and Bowman’s Capsule in WT Mice with Anti-GBM Disease

In control Col 18/endostatin-null mice, no renal histologic abnormalities were seen by light microscopy compared with WT mice (Figure 3, A and B). The renal cortex of the control WT mice showed faint Col 18/endostatin staining within glomerular and tubular BMs and Bowman’s capsule (Figure 1). Upon induction of anti-GBM GN in WT mice, Col 18/endostatin protein expression was clearly upregulated within the GBM and Bowman’s capsule and Col 18 mRNA expression was significantly elevated in the renal cortex on day 6 (Figure 1, A arrows and B).

Col 18/Endostatin-Null Mice Demonstrated Enhanced Renal Injury upon Induction of Anti-GBM Disease

Five days after induction of anti-GBM GN, several Col 18/endostatin-null mice began to die and other mice became very feeble with signs of severe edema and ascites and died within 7 days, whereas all WT mice survived up to day 12. Among the mice with anti-GBM GN, urine protein excretion on day 6 was significantly higher in Col 18/endostatin-null mice than in WT mice (Figure 2A). Renal function deteriorated significantly in
nephritic Col 18/endostatin-null mice compared with that in nephritic WT mice as assessed by the blood urea nitrogen (BUN) and serum creatinine (Scr) levels on day 6 (Figure 2B; Supplemental Figure 1). In WT mice, cell proliferation in the glomerulus, glomerular thrombosis, and crescent formation were seen on day 6 after induction of anti-GBM GN (Figure 3, A and C; Supplemental Figure 2A). In nephritic Col 18/endostatin-null mice, the glomeruli were more enlarged (Figure 3A) and the total score of the glomerulus, score of glomerular thrombosis, and crescent formation were significantly higher than those in nephritic WT mice (Figure 3C; Supplemental Figure 2A). However, there were no significant differences in the total score of the interstitium, score of infiltration of mononuclear cells, score of tubular damage, and score of interstitial fibrosis (Figure 3B; Supplemental Figure 2B).

Heterologous and Autologous Antibody Responses in Col 18/Endostatin-Null and WT Mice
In nephritic Col 18/endostatin-null and WT mice, similar amounts of heterologous and autologous antibodies were deposited on the GBM on day 6, as semiquantitatively assessed by the binding of rabbit anti-GBM antibody and mouse IgG, respectively (Figure 4). In addition, there was no obvious difference in the amount of complement deposited along the GBM on day 6, as semiquantitatively assessed by glomerular C3 staining, between Col 18/endostatin-null and WT mice (Figure 4). Deposition of rabbit and mouse IgG and C3 was detected in the mesangial area of glomeruli of nephritic Col 18/endostatin-null mice, but not in nephritic WT mice (Figure 4, arrows). No deposition of rabbit and mouse IgG and C3 was observed in the GBM and mesangium of glomeruli of control Col 18/endostatin-null and WT mice (data not shown).

Glomerular Inflammation Was Augmented in Col 18/Endostatin-Null Mice with Anti-GBM Disease
In the renal cortex of WT mice, the influx of polymorphonuclear leukocytes (PMN), macrophages/monocytes (M/Mo), and CD8\(^+\) cells significantly increased on day 6 after induction of anti-GBM GN (Figure 5, A through C; Supplemental Figure 3, A through C). The influx of PMN into glomeruli and interstitium and M/Mo into glomeruli were significantly higher in nephritic Col 18/endostatin-null mice than in nephritic WT mice (Figure 5, A and B; Supplemental Figure 3, A and B), whereas similar levels of glomerular and interstitial infiltration of CD8\(^+\) cells were observed in the two groups of nephritic mice (Figure 5C; Supplemental Figure 3C). Urine monocyte chemoattractant protein-1 (MCP-1) excretion significantly increased in nephritic Col
ALtered Matrix Deposition Was Observed in Col 18/Endostatin-Null Mice

Because Col 18/endostatin has also been detected in the mesangial matrix of the normal kidney,8,9 we studied control and nephritic kidneys from Col 18/endostatin-null and WT mice by electron microscopy. Among controls, the mesangial matrix expanded in some of the glomeruli of Col 18/endostatin-null mice compared with that in WT mice, as shown previously (Figure 6A).8 After induction of anti-GBM disease, the mesangial matrix became further expanded and massive fibrinoid thrombi were seen in the capillary loops on day 6 in the glomeruli of Col 18/endostatin-null mice compared with WT mice (Figure 6B). Immunohistochemical analyses showed that deposition of collagen IV and perlecan on the mesangial matrix increased in the glomeruli of nephritic Col 18/endostatin-null mice compared with that in nephritic WT mice (Figure 6C and D arrows).

Col 18/Endostatin Deficiency Led to Severe Glomerular and Interstitial Capillary Loss

Because endostatin is known to be a potent endogenous angiogenesis inhibitor,7,18,19 we compared the density of glomerular and interstitial capillaries in Col 18/endostatin-null and WT mice. In WT mice, the capillary area as assessed by CD31+ endothelial cells in the glomeruli and interstitium dramatically decreased on day 6 of anti-GBM GN (Figure 7A). Unexpectedly, Col 18/endostatin-null mice with anti-GBM disease showed further progressive loss of glomerular capillaries: severe vascular injury was observed in the glomeruli of Col 18/endostatin-null mice compared with that in WT mice (Figure [NTS]). In contrast, the density of interstitial capillaries was equal in Col 18/endostatin-null and WT mice on day 6 after the onset of anti-GBM GN (Figure 7A). Whereas vascular endothelial growth factor (VEGF) expression in the glomeruli and cortex of WT mice increased after induction of anti-GBM GN, VEGF expression significantly decreased along with the density of glomerular capillaries in the glomeruli and cortex of Col 18/endostatin-null mice (Figure 7, A and B).

Capillary rarefaction was extensively induced at the late stage in anti-GBM disease models,23,24 and accelerated capillary repair due to the lack of the endogenous angiogenesis inhibitor may be overlooked in the kidney of Col 18/endostatin-null mice. Therefore, we decided to use new rabbit anti-mouse GBM serum (nephrotoxic serum 18/endostatin-null mice compared with that in nephritic WT mice (Figure 5D). In nephritic Col 18/endostatin-null mice, the elevated influx of M/Mo into the glomeruli was consistent with the increase in renal synthesis of MCP-1.

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suggesting that the progression of anti-GBM disease was dependent on the dosage of COL18A1 gene (Supplemental Figure 4, A through D). Nephritic Col 18/endostatin-null mice, compared with nephritic WT mice, displayed damage of the central area of the liver related to abnormal hepatic circulation, proliferation of lymphocytes within the splenic red pulp, and influx of PMN into alveolar septa, whereas there were no differences in the heart, testis, and degree of elevation of serum liver enzymes between nephritic Col 18/endostatin-null and WT mice (Supplemental Figure 8; and data not shown).

In addition, nephritic Col 18/endostatin-null mice on day 14 exhibited severe EC damage, detachment and disappearance of ECs, and thickening of capillary walls with prominent double outlines in the glomeruli, and also necrotizing lesions, thrombosis, cellular crescent formation, and rupture of the GBM, some of which were also detected in the glomeruli of WT mice (Figures 9, A through I). These results were confirmed by electron microscopy. Narrowing and loss of capillary lumens were evident with EC swelling and loss of fenestra, and the subendothelial space was expanded with thickening of the GBM in nephritic Col 18/endostatin-null mice (Figure 9J).

We also administered mouse endostatin protein or peptide (mP1) to nephritic Col 18/endostatin-null mice to study the role
of endogenous circulating endostatin on the progression of anti-GBM GN. The antiangiogenic effects of the endostatin protein and mP1 were demonstrated in the growth factor–supplemented Matrigel plug assay (Supplemental Figure 9). Supplementation with the protein and peptide did not affect the Scr level nor the total scores, although the serum endostatin concentration in treated Col 18/endostatin-null mice was similar to that in non-treated nephritic WT mice (Figure 10, A through C; Supplemental Figures 5 and 10). No positive staining of Col 18/endostatin was detected within the renal section of treated Col 18/endostatin-null mice by immunohistochemical staining (data not shown). These results suggest that circulating soluble endostatin has little effect on the progression of anti-GBM disease.

**DISCUSSION**

In this study, we focused on Col 18/endostatin because its antiangiogenic properties have been extensively character-
The first major finding of this study was that Col 18/endostatin expression in the GBM and Bowman’s capsule is upregulated during anti-GBM GN and that its absence in Col 18/endostatin-null mice enhanced inflammation, matrix accumulation, and capillary rarefaction in the injured kidney. These results suggest that increased deposition of Col 18/endostatin in the GBM and Bowman’s capsule may be associated with protective effects against renal disease.

Col 18/endostatin deficiency led to increased glomerular influx of PMN and M/Mo, but not CD8⁺ cells, demonstrating that Col 18/endostatin deficiency could, by itself, accelerate the progression of anti-GBM disease at the early stage.⁵,2⁰⁻³³ In nephritic Col 18/endostatin-null mice, deposition of heterologous and antologous antibodies, C3, collagen IV, and perlecain was observed in the mesangium, where additional immunological responses including influx of PMN and M/Mo into the glomeruli may enhance early progression of anti-GBM disease. Col 18/endostatin may have an anchoring function that prevents loosening of the GBM; that is, it may serve as a scaffold on which other BM proteins interact. Previous studies demonstrated that endostatin bound to other BM components such as perlecain and laminin.⁷⁻⁹,3⁴ Lack of or altered interaction between endostatin and laminin has also been suggested to contribute to the other phenotypes of Col 18/endostatin-null mice and patients with Knobloch syndrome.⁷,3⁵ Lack of interaction between endostatin and perlecain may contribute to the augmentation of renal damage in nephritic Col 18/endostatin-null mice because mutations in human perlecain were related to renal abnormalities including thickening of glomerular and tubular BMs and expansion of mesangium,³⁶ which were shown in Col 18/endostatin-null mice. However, the precise role of Col 18/endostatin in the human kidney remains unclear.

MCP-1 has been demonstrated to be the dominant chemokine involved in M/Mo recruitment in anti-GBM disease and the binding properties of MCP-1 to HSPGs have been well defined in several reports.²¹,2⁴,³¹,3⁷ In particular, Col 18 plays a role in enhancing renal inflammation because Col 18 bound to locally produced MCP-1 and presented it to infiltrated monocytes, leading to their adhesion to vascular cell adhesion molecule-1.³⁷⁻³⁹ How-

Figure 9. Nephritic Col 18/endostatin-null mice on day 14 exhibit severe EC damage as well as necrotizing lesions, cellular crescent formation, and rupture of the GBM in the glomeruli. (A through I) Glomerulus and tubulointerstitium from female nephritic Col 18/endostatin-null [KO(+), n = 3] and WT [WT(+), n = 7] mice on day 14 were stained with periodic acid–Schiff and periodic acid–methenamine silver reagents. Necrotizing lesions (A), thrombosis, cellular crescent formation (B), detachment of ECs (D), thickening of capillary walls (E), rupture of the GBM (F), and mesangial cell proliferation (G) were extensively observed in the glomeruli from KO(+), compared with those in WT(+) (C). (H and I) Degeneration of the tubular epithelium with cast formation was extensively observed in KO(+). Representative pictures are shown. Scale bar: 50 μm. (J) In KO(+) glomeruli, narrowing and loss of capillary lumens were evident with swelling of ECs (S) and loss of fenestra (arrow), and the subendothelial space was expanded with thickening of the GBM (E). Mesangial matrix also accumulated (M) and diffuse effacement of foot process in podocytes was noted (F). In contrast, in WT(+) glomeruli the glomerular capillaries were well preserved and ECs were present along the GBM, whereas mild swelling of ECs was noted with well-preserved fenestra of ECs. Focal effacement of foot process in podocytes was also noted (F). Scale bar: 2 μm.
The second major finding of our study was that Col 18/endostatin-null mice exhibited enhanced inflammation, and capillary rarefaction. Furthermore, endostatin deficiency did not prevent glomerular and interstitial capillary loss that occurred in this renal disease model. These results indicate that Col 18/endostatin plays an important role in preserving the integrity of the extracellular matrix and capillary vessels in the kidneys and inhibiting the progression of anti-GBM disease.

### Figure 10.
Intravenous administration of mouse endostatin protein has no effect on the progression of anti-GBM disease. Mouse endostatin protein or saline as a control was intravenously injected into male Col 18/endostatin-null mice during anti-GBM GN (Exp II) for 8 days. (A) Analysis of circulating endostatin levels in the sera of nephritic WT [WT(+), n = 6], Col 18/endostatin-null [KO(+), n = 4], and endostatin-supplemented KO(+) [KO(+) + Endo, n = 5] mice and control WT [WT(−), n = 6] and KO [KO(−), n = 4] mice on day 6. Endostatin was not detected in the sera of KO(+) mice, whereas KO(+) + Endo mice had a similar circulating endostatin level to that in WT (+) mice. Each bar represents the mean ± SEM. **P < 0.01, compared with WT(+) group. ††P < 0.01, compared with KO(+) + Endo group. (B and C) Scr, the percentage of crescent formation, and the influx of M/Mo in the glomeruli were evaluated. There were no differences in Scr, the percentage of crescent formation, nor the influx of M/Mo between KO(+) and KO(+) + Endo mice. Each bar represents the mean ± SEM. **P < 0.01, compared with WT(+) group. *P < 0.05, compared with WT(+) group.

In conclusion, the lack of Col 18/endostatin augmented several phenotypic responses in the renal injury of anti-GBM nephritis. The augmentation of renal injury may potentially be mediated by altered distribution of matrix accumulation, enhanced inflammation, and capillary rarefaction. Furthermore, endostatin deficiency did not prevent glomerular and interstitial capillary loss that occurred in this renal disease model. These results indicate that Col 18/endostatin plays an important role in preserving the integrity of the extracellular matrix and capillary vessels in the kidneys and inhibiting the progression of anti-GBM disease.
Hematologic Evaluation and Determination of Albuminuria

The levels of Scr and BUN were determined by standard methods using the following kits: CORTase-POD (Kainos, Tokyo, Japan) for Scr and Urease-GLDH (Kyowa, Tokyo, Japan) for BUN. Spot urine was collected when mice were sacrificed. Albumin (Sigma, St. Louis, MO) and creatinine (Cayman Chemical, Ann Arbor, MI) concentrations in the urine were estimated using a colorimetric assay according to the manufacturer’s recommendations. Urine albumin excretion was estimated as the quotient of urine albumin and urine creatinine.43

Histologic Analysis

The kidney, liver, heart, spleen, lung, and testis were fixed in 10% buffered formalin and embedded in paraffin. The paraffin sections were stained with periodic acid–Schiff, periodic acid–methenamine silver, Masson’s trichrome, or hematoxylin and eosin and evaluated by light microscopy. Morphologic changes in the glomeruli or tubulointerstitial area were scored on a scale of 0 to 5: 0, none of the glomeruli or no tubulointerstitial area was affected by morphologic changes; 1, <20%; 2, <40%; 3, >60%; 4, >80%; 5, full. In the analysis of glomeruli, scores were assigned for intraglomerular cell proliferation, glomerular thrombosis, and extracapillary cellular proliferation (crescent formation), and the total score was the sum of the three scores. Crescent formation was quantified by calculation of the percentage of affected glomeruli. In the analysis of interstitium, scores were assigned for infiltration of mononuclear cells, damage of tubules, and interstitial fibrosis, and the total score was the sum of the three scores. Fifty glomeruli per mouse were assessed for glomerular damage and at least 20 separate 200× fields per mouse were assessed for tubulointerstitial damage. The scoring was performed by two independent observers on blinded sections.

Immunohistochemistry

Immunohistochemical staining was performed as described previously.44 Briefly, frozen kidney sections were cut at a thickness of 4 μm and fixed in cold (−20°C) 100% acetone for 3 minutes. For the immunofluorescence study, cryostat sections were stained with Alexa Fluor 568–conjugated goat anti-rabbit IgG (1:200), Alexa Fluor 488–conjugated rat anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA) or rat anti-mouse C3 antibodies (1:50; MP Biomedicals, Solon, OH) for heterologous antibodies, autologous antibodies, and complement, respectively. In addition, the sections were stained with the following primary antibodies: Alexa Fluor 488– and Alexa Fluor 568–conjugated secondary antibodies. For primary antibodies, we used goat anti-mouse collagen type IV (1:50; Millipore, Billerica, MA), goat anti-mouse Col 18 (1:25; R&D Systems, Minneapolis, MN) for the C-terminal NC-1 domain, rat anti-mouse CD31 (1:50; BD PharMingen, San Diego, CA) for vascular endothelial cells, goat anti-mouse VEGF antibodies (1:25; R&D Systems, Minneapolis, MN), and rat anti-mouse perlecann (1:50; Fitzgerald Industries, Concord, MA). Vectashield (Vector Laboratories, USA) antigenic mounting medium was applied and imaged by immunofluorescence microscopy (BX51; Olympus, Japan). As for the immunohistochemical study, the cryostat sections were quenched with intrinsic peroxidase by H₂O₂ and incubated with rat anti–mouse neutrophil (clone 7/4) (1:20; Immunotech, Prague, Czech Republic) for PMN, rat anti-mouse CD11b (1:50; BD Pharmingen, San Diego, CA) for vascular endothelial cells, goat anti-mouse VEGF antibodies (1:25; R&D Systems, Minneapolis, MN), and rat anti-mouse perfecan (1:50; Fitzgerald Industries, Concord, MA). Vectashield (Vector Laboratories, App Imaging, Burlingame, CA) antigenic mounting medium was used as the substrate. Fifty glomeruli per mouse were assessed for glomerular damage and at least 15 separate 200× fields per mouse were assessed for tubulointerstitial changes. In each group, the stained cells were counted and the stained area was quantified using NIH Image J, software for image processing and analysis developed by the National Institutes of Health (http://www.nih.gov), and averaged.

Electron Microscopy

Kidneys from Col18/endostatin-null and WT mice were minced into 1-mm³ pieces and were fixed in 2.5% glutaraldehyde in 0.05 M sodium phosphate (pH 7.4) overnight followed by postfixation in 1%
osmium tetroxide in 0.05 M sodium phosphate (pH 7.4) for 1.5 hours. Subsequently, the tissue were dehydrated in a graded series of ethanol and propylene oxide and embedded in an epoxy resin. Tissue sections were cut at 80-nm thickness and stained with uranyl acetate and lead citrate. Specimens were imaged using an electron microscope (JEM-1010; JEOL, Japan).

Measurement of Urine MCP-1 and Serum Endostatin Concentrations

MCP-1 concentrations in the spot urine were estimated using ELISA according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). Urine MCP-1 excretion as a marker of glomerular MCP-1 production was estimated as the quotient of urine MCP-1 and urine creatinine. Serum endostatin concentrations were measured as described previously.18

RNA Isolation and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from frozen renal cortex using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Reverse transcription of 300 ng RNA was performed with PrimeScript RT Reagent Kit (Takara, Shiga, Japan) and cDNA was used as template in RT-PCR using SYBR Premix Ex Taq II (Takara, Shiga, Japan) with gene-specific primers [Col 18; (forward) GTG GCC ATC GTC AAC CTG AA (reverse) AGT TGA CCC TGG GAG CCA GA, VEGF; (forward) CTG GAT ATG TTT GAC TGC TGT GGA (reverse) GYT TCT GGA AGT GAG CCA ATG TG, glyceraldehyde-3-phosphate dehydrogenase (GAPDH); (forward) ATG GGG TGA GCC CGG TGC TG (reverse) GYT GAT GTC ATC ATA CTT GG] on the Mini Opticon RT-PCR system (Bio-Rad, Hercules, CA). Expression of the housekeeping gene GAPDH was measured to quantify gene expression using the relative-expression method with the standards graph.

Matrigel Plug Assay

In vivo Matrigel plug assay was performed as described previously.45 Briefly, Matrigel (BD Biosciences, San Jose, CA) was mixed with 20 units/ml of heparin, 50 ng/ml of VEGF (R&D Systems, Minneapolis, MN), and 0.5 μM mouse endostatin or 10 μM mP1 or PBS. The Matrigel mixture was injected subcutaneously into the back of WT mice. Six days after the injection, all mice were sacrificed. The Matrigel plugs were removed and fixed with 4% paraformaldehyde and 10% formalin. They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were examined using 30 different fields by light microscopy, and the number of blood vessels at 400× magnification were counted and averaged. All sections were coded and observed by an investigator who was blinded for study protocols.

Statistical Analysis

The number of mice used for each experiment is provided in the figure legends. The significance of differences between two groups was assessed by t test or Mann-Whitney test. Statistical analyses among more than two groups were performed using ANOVA. As needed, additional analysis was carried out using the t test with Bonferroni correction to identify significant differences. Kaplan-Meier curves were used for survival analysis, and the log-rank (Mantel-Cox) test was used to determine statistical significance. P < 0.05 was considered to be statistically significant.

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DISCLOSURES

None.

REFERENCES


