Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Substance P-Mediated Expression of the Pro-Angiogenic Factor CCN1 Modulates the Course of Colitis

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Substance P (SP) regulates important intestinal functions, such as mucosal permeability, motility, chloride secretion, and inflammation via the neurokinin-1 receptor (NK-1R). Previous reports showed that vascularization and expression of angiogenic factors are evident in the colonic mucosa of rats with colitis and patients with inflammatory bowel disease. Here we determined whether SP is associated with angiogenesis. Human NCM460 colonocytes stably transfected with the human NK-1R (NCM460-NK-1R cells) and mice with dextran sodium sulfate-induced colitis were used. We found that expression of the angiogenic factor CCN1 was increased in the colons of patients with Crohn's disease and ulcerative colitis. Mucosal extracts from inflammatory bowel disease patients induced human intestinal microvascular endothelial cell migration that was inhibited by blockade of CCN1 and its receptor integrin $\alpha\beta 3$. Both the degree of angiogenesis and CCN1 expression were elevated in the colons of mice with dextran sodium sulfate-induced colitis, which was reduced by treatment with the NK-1R antagonist CJ-12255. SP also increased CCN1 expression in NCM460-NK-1R colonocytes. SP exposure to human intestinal microvascular endothelial cells co-cultured with NCM460-NK-1R cells induced angiogenic activity that was inhibited by CCN1 silencing. In addition, intracolonic overexpression of CCN1 induced angiogenesis in mouse colon. Thus, SP mediates angiogenesis via

CCN1 during colitis. (Am J Pathol 2008, 173:400–410; DOI: 10.2353/ajpath.2008.080222)

Substance P (SP), an 11-amino acid neuropeptide member of the tachykinin family isolated by Leeman and Chang,¹ is localized in the central nervous system,² sensory neurons,³ enteric nerves⁴ and immune cells.⁵ SP binding to its high-affinity neurokinin-1 receptor (NK-1R) modulates important intestinal functions, such as mucosal permeability,⁶ motility,⁷ chloride secretion,⁸ and inflammation, via activation of COX-2, PGE₂, and nuclear factor- κ B-regulated cytokine genes.^{9,10} SP-NK-1R interaction also induces proliferation of colonic epithelial cells that involves activation of metalloproteinases and transactivation of the epithelial growth factor receptor.¹¹ SP also exerts an anti-apoptotic effect in colonic epithelial cells via Akt phosphorylation *in vitro* and *in vivo*.¹²

Angiogenesis is a critical component of colonic inflammation. The occurrence of angiogenesis and expression of angiogenic factor have been established in inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC).¹³ In both CD and UC mucosa, the local microvasculature undergoes intensive inflammation-dependent angiogenesis. Evidence from a sponge angiogenesis model¹⁴ and synovial inflammation in rats indicates that SP may mediate angiogenesis in nonintestinal tissues.^{18–21} However, whether SP or any other neuropeptide mediates vascular angiogenesis during colonic inflammation is unknown and the mecha-

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nisms governing these responses have never been examined.

Members of the CCN family, including CCN1 (cysteine-rich 61, Cyr61), CCN2 (connective tissue growth factor, CTGF), CCN3 (nephroblastoma, NOV), CCN4/5/6 (Wnt-inducible secreted protein, WISP-1, -2, and -3), are 30- to 40-kDa cysteine rich proteins¹⁵ that stimulate mitosis, adhesion, apoptosis, extracellular matrix, angiogenesis, and tumor growth.¹⁶ Recent data indicate that CCN1, -2, and -3 bind to cell surface integrins and thereby activate intracellular signaling, including kinase phosphorylation and gene transcription.¹⁵ CCN1 plays a pivotal role in vasculogenesis during embryogenesis because mice lacking CCN1 cannot develop *in utero* because of vascular defects in the placenta, confirming the importance of this protein in endothelial cell proliferation and angiogenesis.^{17,18} However, the expression and role of CCN1 in the intestinal tract has never been investigated.

In this study, we found increased CCN1 levels in the colons of UC and CD patients and in the colonic mucosa of mice with experimental colitis. We also demonstrate that intracolonic overexpression of CCN1 induced colonic vascularization *in vivo* and that SP increased expression of this angiogenic factor in human colonic epithelial cells.

Materials and Methods

Human Colonic Tissues

Colon samples from UC and CD patients were obtained as previously described.¹⁹ Total RNA was isolated from eight pairs of inflamed colon and matching adjacent normal regions and then converted into cDNA. All patients gave informed consent and the Beth Israel Deaconess Medical Center Institutional Review Board approved the protocol.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Analyses

Total RNA was isolated from colon tissues by Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA using the Superscript III reverse transcription kit (Invitrogen). The PCR reactions were run in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). The levels of CCN1, TACR1 (NK-1R), TAC1 (SP), and VEGFA, VEGFB, VEGFC, and VEGFD mRNA were determined using their respective real-time primer sets obtained from Applied Biosystems according to the manufacturer's instructions. Levels were normalized to equal levels of GAPDH mRNA and results were expressed as fold induction compared to their respective controls.

Cell Cultures

NCM460 cells overexpressing NK-1R (NCM460-NK-1R), previously generated by us,⁹⁻¹² were cultured in M3D

medium (INCELL, San Antonio, TX) containing 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Human intestinal microvascular endothelial cells (HIMECs) were generously provided by Dr. Claudio Fiocchi (Department of Pathobiology, The Cleveland Clinic Foundation, Cleveland, OH)¹³ and cultured for five to six generations in MCDB131 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Cambrex, East Rutherford, NJ) on fibronectin-coated culture plates (Becton-Dickinson, Franklin Lakes, NJ). Cells were treated with SP or human CCN1 protein (Peprotech Co., Rocky Hill, NJ) as indicated.

Mouse Colon Tissue Preparation

Male, 8- to 10-week old C57BL6 mice ($n = 6$ per group) (Charles River Laboratories, Wilmington, MA) were maintained at the animal facility under standard conditions. Animal studies were approved by the institutional animal care and use committee of Beth Israel Deaconess Medical Center. Mice received standard pelleted chow and tap water *ad libitum*, except the colitis group, which received water containing dextran sodium sulfate (DSS) 5% (w/v), as previously described.^{9,11,12} Groups of mice were also injected intraperitoneally with 200 μ l of phosphate-buffered saline (PBS) containing the NK-1R antagonist CJ-12255 (2.5 mg/kg/twice per day) or PBS. After 5 days, mice were sacrificed by carbon dioxide euthanasia. Colon tissues were dissected and homogenized in immunoprecipitation buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and equal amounts of protein (40 μ g/lane) were subjected to Western blotting. Some colon tissues were also fixed in formalin and paraffin-embedded for immunohistochemistry.

Western Blot Analyses

Cells were lysed in 1 $\times$ lysis buffer (62.5 mmol/L Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, and 1% 2-ME). Equal amounts of cell extracts were fractioned by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membranes (400 mA for 2 hours at 4°C; Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat milk in TBST (50 mmol/L Tris, pH 7.5, 0.15 mol/L NaCl, 0.05% Tween 20), and then incubated with antibodies against phospho-integrin β 3, CCN1²⁰ (Santa Cruz Biotechnology) and β -actin (Sigma). Horseradish peroxidase-labeled antibody was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and was exposed to X-ray film (Fujifilm; Fisher, Pittsburgh, PA). In some experiments, Western blot bands were quantified by densitometry using Scion Image Analysis software (Frederick, MD, USA).

Promoter Activity of CCN1 Gene

The DNA fragments of the CCN1 promoter (1.1 kb) upstream of the translational start site were cloned by

PCR from human colonocyte genomic DNA and after confirming the sequence identity, subcloned into a pGL3 vector (pGL3-CCN1). NCM460-NK-1R cells were seeded in 12-well plates (2×10^6 cells/plate) overnight and transiently transfected with the pGL3-CCN1 construct together with an internal control pRL-TK (Promega, Madison, WI) as previously described.⁹ Transfected cells were serum-starved for 24 hours and then treated with SP for up to 24 hours. The relative promoter activity of CCN1 in equal amounts of cell extracts was measured using a dual luciferase reporter assay system (Promega).

CCN1 Knockdown by siRNA Transfection

NCM460-NK-1R colonocytes were seeded in 12-well plates (2×10^5 cells/well) overnight and transiently transfected with equal amounts of CCN1 or control siRNA (Dharmacon, Chicago, IL) using the X-tremeGene transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.^{9,10,12} Reduced endogenous CCN1 expression was confirmed by Western blot analyses.

Immunohistochemistry of von Willebrand Factor (vWF) for Endothelial Cell Staining

Colon tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After incubation with blocking buffer, sections were incubated with a rabbit polyclonal anti-vWF antibody (AB7356; Millipore, Billerica, MA) overnight at 4°C. After washing, sections were incubated with donkey anti-rabbit IgG and slides were stained with an ABC kit for color development (sc-2018, Santa Cruz Biotechnology). Sections were photographed under the microscope and computerized vWF-stained image analysis was performed using the Scion Image Software as previously described.^{13,21,22}

In Vivo Transfection of CCN1

Human CCN1 cDNA clone no. TC310465 was obtained from Origene (Rockville, MD). The CCN1-overexpressing construct was mixed with polyethyleneimine-based transfection reagent (*in vivo* Jet, 201-50G; Polyplus, San Marcos, CA) in a N:P ratio = 5 (polyethyleneimine nitrogen to DNA phosphate ratio). Before induction of colitis, C57/BL6 mice were starved overnight and then anesthetized by pentobarbital sodium (60 mg/kg i.p.), followed by intracolonic injection of CCN1 cDNA/*in vivo* Jet mixture (100 μ g/200 μ l/mice) (day 0). Mice were returned to consciousness and provided with 5% DSS in their drinking water or water alone for 5 days *ad libitum*. A separate group of mice were injected with the same amount of enhanced green fluorescent protein (EGFP)-expressing plasmid. CCN1 transfection was repeated on day 3 to increase transfection efficiency.

Immunofluorescent Staining of CCN1 and EGFP

Colon tissues were embedded in OCT solution. Frozen sections were made, fixed in 10% formalin, and permeabilized using 0.5% Triton X-100. After incubation with blocking buffer, slides were incubated with a rabbit polyclonal anti-CCN1 antibody, anti-green fluorescent protein (GFP) antibody, or 4,6-diamidino-2-phenylindole stain (blue emission signal to stain nuclei) overnight at 4°C. Samples were then washed with PBS and stained by rhodamine (red) or fluorescein isothiocyanate (green)-conjugated secondary antibodies for 1 hour. Slides were then rinsed and mounted with 4,6-diamidino-2-phenylindole mounting solution. Images were analyzed with an Axioskop-2 microscope (Carl Zeiss, Thornwood, NY).

In Vitro Angiogenesis

HIMECs (5×10^3 cells/well) were seeded in 96-well plates coated with ECMatrix (*in vitro* angiogenesis kit, no. ECM625; Millipore). In co-culture experiments, NCM460-NK-1R colonocytes were pretransfected with control or CCN1 siRNA as described above and then 1×10^3 cells were added to HIMEC cultures. To block CCN1 activity, anti-CCN1 antibody (Santa Cruz Biotechnology) or rabbit control IgG were added to the cell culture at 20 μ g/ml. SP or CCN1 was added shortly after cell seeding and incubated at 37°C for 8 hours. The pattern of angiogenesis (tube formation) was observed under a phase contrast light microscope and the images were recorded.²³ Quantitative evaluation of angiogenesis was done in multiple wells of the same group to provide an average scoring of vascular patterns as suggested by the manufacturer in the following: 0, individual separated cells; 1, cells begin to migrate and align; 2, capillary tubes visible but no sprouting; 3, sprouting of capillary tubes visible; 4, closed polygon formation, and 5, complex mesh-like structure development.

Endothelial Cell Migration

HIMEC migration was assessed by a standard Transwell migration system using the Chemicon ECM508 assay kit (Chemicon, Temecula, CA).²⁴ Briefly, polycarbonate filter inserts (8- μ m pore size) serving as upper chambers were seeded with HIMECs (1×10^6 cells/ml in 150 μ l of serum-free media) for migration to lower chamber with serum-added media. Filtered (0.22 μ m, Millipore) human mucosal extracts from inflamed and unaffected areas of IBD patients (40 μ g/ml) were preincubated (30 minutes, 37°C) with 20 μ g/ml anti-VEGF antibody, anti-integrin α V β 3 antibody (Upstate Technology, Lake Placid, NY), anti-CCN1 antibody (Santa Cruz Biotechnology), or mouse IgG and then added to the lower chamber. In the case of HIMEC and NCM460-NK-1R co-culture, an additional 1×10^4 CCN1/control siRNA-transfected NCM460-NK-1R colonocytes were added into the lower chamber. SP or CCN1 was added to the lower chamber with anti-CCN1 antibody (20 μ g/ml) or anti-VEGF antibody (20 μ g/ml)

and incubated at 37°C for 16 hours. For control experiments, NCM460-NK-1R cells were seeded onto the upper chamber in the absence of HIMECs. Cells that migrated to the lower chamber were stained and lysed with buffers provided with the kit and cell migration was detected by absorbance at A_{560} .

In Vivo Matrigel Plug Angiogenesis Assay

Two hundred μ l of Matrigel (BD Biosciences, Bedford, MA) was mixed with 200 μ l of PBS containing SP (10^{-7} mol/L) or CCN1 (1 μ mol/L). Colonic mucosa from IBD patients or mouse colon from DSS colitis experiments was homogenized and centrifuged ($12,000 \times g$, 5 minutes, 4°C). Supernatants were collected and aliquots containing 20 μ g of protein/ml were mixed with anti-CCN1 or control antibodies (20 μ g/ml). The Matrigel mixtures (400 μ l) were injected subcutaneously into the laterodorsal abdominal region of 8-week-old male C57/BL6 mice. After 5 days, the mice were sacrificed; and the Matrigel plugs were removed and photographed. The hemoglobin concentration in new blood vessels within the Matrigel was measured by the hemoglobin assay (Sigma) as previously described.^{25,26}

Statistical Analyses

Results were analyzed using Prism professional statistics software program (Graphpad, San Diego, CA). Analysis of variance was used for intergroup comparisons.

Results

Increased CCN1 Expression in Inflamed Human Colon Mediates Angiogenesis

Angiogenesis has been recently suggested as an important component of IBD pathophysiology.¹³ However, there is no evidence to indicate whether the angiogenic factor CCN1 is expressed in human or animal colon and whether its expression is differentially regulated during colitis. We first determined the mRNA levels of various inflammatory and angiogenic mediators in colonic biopsies obtained from inflamed and noninflamed (control) colonic mucosa of IBD patients. As expected,²⁷ NK-1R mRNA levels in the inflamed colon of CD and UC patients were significantly elevated by 20- and 10-fold, respectively, compared to the noninvolved colonic sections from the same patients (Figure 1A). SP (TAC1) mRNA levels in the inflamed colon of CD and UC patients were also significantly elevated by 16- and 9-fold, respectively (Figure 1A). CCN1 mRNA levels in inflamed colonic mucosa of CD and UC patients were also significantly increased by 3.9- and 2.8-fold, respectively (Figure 1B). We next examined the importance of CCN1 in angiogenesis associated with IBD using human colonic mucosal extracts from IBD patients to induce migration of HIMECs in Boyden chambers. We found that human colonic mucosal extracts from inflamed IBD colon, but not from nonin-

Figure 1. CCN1 is overexpressed during colonic inflammation and mediates angiogenesis. Colon tissues of UC and CD patients were used to perform real-time RT-PCR to measure NK-1R and SP gene expression levels (A), CCN1 (B). The change of gene expression was compared to adjacent normal regions and normalized by GAPDH expression. Each group of data represented eight matching pairs of samples from human patients. *** $P < 0.001$, ** $P < 0.01$; *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ versus respective normal colon. C: Forty μ g/ml of human colonic mucosal extracts from inflamed and matching unaffected normal areas with 20 μ g/ml of control IgG, anti-VEGF, anti-CCN1, and anti- α V β 3 neutralizing antibodies were added to the lower chamber of a modified Boyden chamber. HIMECs that migrated to the lower chamber were measured as described in Materials and Methods. *** $P < 0.001$, * $P < 0.05$ versus IgG-treated IBD group.

involved areas, induced HIMEC migration (Figure 1C). Blockade of CCN1 and its receptor integrin α V β 3 by neutralizing antibodies almost abolished HIMEC migration after neutralization of the known angiogenic factor, VEGF, also inhibited HIMEC migration, but to a lesser degree (Figure 1C). This result suggested the CCN1/ α V β 3 system is important in inducing angiogenic function during IBD.

SP, via NK-1R, Modulates Angiogenesis and CCN1 Expression during Experimental Colitis

Angiogenesis occurs in the colon of animals with experimental colonic inflammation, including DSS-induced colitis.²⁸ Pharmacological antagonism of NK-1R also reduces colonic inflammation during the course of DSS-induced colitis,²⁹ whereas NK-1R expression is also increased in this model.³⁰ As also shown in Figure 2A, we observed strong CCN1 expression (green) in the colon of DSS-administered mice that was reduced by NK-1 receptor antagonist CJ-1255 treatment (Figure 2A). CCN1 staining was very low in water-treated mice and not affected by CJ-1255 treatment (Figure 2A). Using endothelial-specific vWF staining we found many dilated blood vessels in the colon 5 days after DSS administration, compared to water-treated mice (Figure 2A). To examine whether SP and its receptor

Figure 2. SP mediates angiogenesis and angiogenic factor expression *in vivo* during DSS colitis. **A:** Mice were treated with 5% DSS added to their drinking water or water alone for 5 days. The NK-1R receptor antagonist CJ-12255 was administered to block NK-1R. Colon tissues were obtained for 4,6-diamidino-2-phenylindole, CCN1, and vWF staining to visualize CCN1 expression and blood vessels. **B:** Quantitative analyses of blood vessel number per field were performed using Scion Image Analysis software. CCN1 protein was determined by Western blot analysis (**C**) and its intensity was quantified by densitometry (**D**). *** $P < 0.001$ versus PBS-treated DSS colitis group. Results are representative of four mice per group.

are involved in this response we treated animals with the NK-1R antagonist CJ-12255. NK-1R antagonism decreased blood vessel density in colonic tissues of

DSS-exposed mice (Figure 2A). In contrast, CJ-12255 did not affect vascular patterns in water-treated mice (Figure 2A). Image analyses of vWF staining demon-

Figure 3. SP induces CCN1 production from colonocytes. **A:** NCM460-NK-1R colonocytes were exposed to SP (10^{-8} mol/L) for up to 24 hours. **B:** NCM460-NK-1R cells were exposed to SP (0 to 10^{-7} mol/L) for 4 hours. Equal amounts of protein were fractionated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the levels of CCN1 in cell lysate (~45 kDa) and media (~28 kDa) and β -actin. **C and D:** NCM460-NK-1R cells were transfected with the CCN1 promoter construct, followed by exposure to different concentrations of SP at the indicated times. Cells were lysed and luciferase assays were performed to determine CCN1 promoter activity. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus respective control group. The results are representative of three experiments.

strated that CJ-12255 reduced blood vessel density by 50% (Figure 2B). Thus, SP, via NK-1R, mediates angiogenic and inflammatory responses during colitis. Western blot analyses of colonic tissues indicate that CJ-12255 significantly reduced colonic CCN1 induction by DSS (Figure 2, C and D), suggesting that SP modulates CCN1 expression *in vivo*.

SP Induces CCN1 Expression from Colonic Epithelial Cells

We next examined whether SP-induced CCN1 expression in NCM460-NK-1R colonocytes. We found that SP stimulated CCN1 protein expression in both cell lysates and cell-conditioned media at 2 to 8 hours and then decreased by 24 hours (Figure 3A). SP stimulated CCN1 expression in a dose-dependent manner starting from 10^{-8} mol/L (Figure 3B). Consistent with these results, SP also induced a time- and dose-dependent increase of CCN1 promoter activity (Figure 3, C and D). Real-time RT-PCR analysis in RNA purified from HIMECs failed to identify CCN1 expression with or without SP (10^{-7} mol/L) exposure (data not shown).

SP Mediates Tube Formation in HIMECs via CCN1 Expression in Colonocytes

Although CCN1 mediates angiogenesis in endothelial cells such as human umbilical vein endothelial cells³¹ and HIMECs³² via integrin $\alpha V\beta 3$ signaling, whether SP can mediate angiogenesis in the intestinal vasculature has not been investigated. We exposed HIMECs cultured on ECMatrix to CCN1 or SP for 8 hours and found that CCN1, but not SP, induced formation of capillary tubes that connected independent HIMEC colonies (Figure 4A). Moreover, co-culture of HIMEC and NK-1R-transfected NCM460 cells failed to induce any capillary tube formation (Figure 4A). However, stimulation of HIMEC-NCM460-NK-1R co-cultured cells with SP resulted in formation of

capillary tubes, which was inhibited by silencing of CCN1 in colonocytes (Figure 4, A and B). Successful knock-down of CCN1 in colonocytes is shown in Supplementary Figure S1A at <http://ajp.amjpathol.org>. These results indicate that SP increases expression of functional CCN1 in colonic epithelial cells, which in turn stimulates angiogenesis in HIMECs.

SP Mediates HIMEC Migration via CCN1 Expression in Colonocytes

Next we determined whether SP or CCN1 affects endothelial cell migration. For this purpose, HIMECs were seeded into the upper compartment of Boyden chambers and the migration of these cells to the lower chamber was examined after SP or CCN1 exposure. The data show that CCN1, but not SP, stimulated HIMEC migration (Figure 4, C and D) that was inhibited by anti-CCN1, but not anti-VEGF antibody. This suggests CCN1 mediates direct angiogenic effects without involvement of VEGF. Moreover, SP had no migratory effect in NCM460-NK-1R colonocytes (Figure 4C). However, co-culture of HIMECs with NCM460-NK-1R accelerated HIMEC migration in response to SP that was blocked by knockdown of CCN1, NK-1R, or anti-CCN1 antibody but not anti-VEGF antibody in colonocytes (Figure 4, C and D). CCN1 silencing reduced SP-induced CCN1 protein expression (see Supplementary Figure S1A at <http://ajp.amjpathol.org>). Also NK-1R silencing reduced NK-1R expression as well as SP-induced CCN1 protein expression (see Supplementary Figure S1B at <http://ajp.amjpathol.org>). Thus, SP-associated pro-angiogenic responses are mediated via CCN1 released from colonic epithelial cells.

The angiogenic effects of CCN1 are mediated via binding to its cell surface receptor integrin $\alpha V\beta 3$.¹⁵ However, there is no evidence indicating a role for this integrin in modulating CCN1 activity in HIMECs, whereas the effect of SP in this response has never

Figure 4. SP stimulates *in vitro* angiogenesis via CCN1 expression from colonocytes. **A:** HIMECs cultured with or without NCM460-NK-1R were seeded onto ECMatrix and incubated with SP (10^{-8} mol/L) or CCN1 (10^{-9} mol/L) for 8 hours. Angiogenesis patterns were recorded by a camera under $\times 20$ magnification. Before the experiment, NCM460-NK-1R cells were pretransfected with control/CCN1 siRNA. **B:** Angiogenesis patterns were evaluated and quantified according to the scoring system provided by the manufacturer. **C** and **D:** HIMECs were seeded onto the upper compartment and NCM460-NK-1R colonocytes were seeded onto the lower compartment of Boyden chambers and treated with SP/CCN1. Cells migrated to the lower compartment were stained, lysed, and absorbance at A_{560} was determined. $**P < 0.01$ versus HIMEC migration alone. $***P < 0.01$ versus HIMEC +/– NCM460-NK-1R with control siRNA or IgG. **E:** NCM460-NK-1R colonocytes were stimulated with SP (10^{-7} mol/L) for 6 hours. SP-conditioned media were filtered ($0.22 \mu\text{m}$) and then added to HIMEC cultures pretreated with IgG, anti-CCN1, or anti- $\alpha\text{V}\beta 3$ (LM609) for 15 minutes. Cells were then lysed and used for detection of phospho- $\beta 3$ integrin by Western blots.

been examined. To determine the function of CCN1 secreted from SP-stimulated colonocytes, we first stimulated NCM460-NK-1R with SP for 6 hours; conditioned media were then collected and added into HIMEC

culture. As shown in Figure 4E, conditioned media from SP-stimulated NCM460-NK-1R cells induced rapid integrin $\beta 3$ phosphorylation in HIMECs and this response was attenuated by the $\alpha\text{V}\beta 3$ neutralizing antibody

Figure 5. CCN1 mediates angiogenesis *in vivo*. **A:** Matrigel mixed with SP, CCN1, and mucosal extracts from DSS-treated mice or human IBD patients were isolated 5 days after implantation. **B:** New blood vessel formation in Matrigel was quantified by the hemoglobin assay as described in the Materials and Methods. Results are representative of three independent experiments.

LM609, and an anti-CCN1 antibody. Thus, SP stimulates colonocytes to release functional CCN1 that binds to integrin $\alpha V\beta 3$ and mediates integrin $\beta 3$ phosphorylation in HIMECs.

CCN1 Mediates Angiogenesis *in Vivo*

To further characterize the angiogenic effects of SP and CCN1 *in vivo*, we performed Matrigel angiogenesis assay. Matrigel plugs, containing different angiogenic factors indicated in Figure 5A, were first implanted into the flank skin space of mice, and after 5 days, plugs were removed and photographed. The data show that, unlike the control Matrigel plugs that did not show significant blood vessel formation, Matrigel plugs filled with CCN1, but not SP, induced neovascularization (Figure 5A). These results confirmed that SP is not a direct angiogenic factor. Colonic extracts from DSS-treated mice (5% DSS for 5

days) also induced angiogenesis (Figure 5A). However, angiogenesis was significantly less evident when inflamed colonic extracts from DSS-exposed mice injected with CJ-12255 were used (Figure 5A). Similarly, human colonic mucosal extracts from inflamed, but not noninvolved colon of IBD patients also induced angiogenesis in mice *in vivo* (Figure 5A). In addition, colonic mucosal extracts from DSS-exposed mice and IBD patients induced angiogenesis that was diminished by addition of an anti-CCN1 antibody in the Matrigel. Quantitation of blood vessels infiltrated into the Matrigel measured by hemoglobin assay confirmed this response (Figure 5B).

CCN1 Induces Colonic Angiogenesis

To directly demonstrate an angiogenic role of CCN1 in the colon, we transfected CCN1 or control, EGFP-over-expressing constructs into mouse colon *in vivo* followed by treatment with 5% DSS for 5 days. In CCN1-transfected mice, we observed increased CCN1 expression (red), whereas transfection of EGFP into mouse colon resulted in increased colonic expression of GFP (green) (Figure 6A), indicating successful transfection of these genes. As shown by endothelial-specific vWF immunohistochemistry (Figure 6A), CCN1-transfected, water-treated mice had more blood vessels along the submucosal layer, compared to EGFP-transfected, water-treated mice, indicating that CCN1 induced angiogenesis in the colon (Figure 6, A and B). As expected, EGFP-transfected DSS-treated mice showed higher number of vascular networks in the colonic submucosa (Figure 6, A and B), and quantification of blood vessels confirmed this response (Figure 6, A and B). All CCN1-transfected mice had increased CCN1 protein expression, compared to EGFP-transfected mice (Figure 6, C and D).

Discussion

We report here that the angiogenic factor CCN1 is significantly up-regulated in DSS colitis as well as in the colon of IBD patients and that this induction is mediated, at least in part, by SP acting in colonic epithelial cells. To our knowledge, this is the first report to indicate that SP has potent angiogenic activities in the gut by acting in colonic epithelial cells to express the pro-angiogenic factor CCN1, which in turn can promote angiogenesis by acting on intestinal endothelial cells. Our results from the CCN1 transfection experiments also support an important role of this factor in angiogenesis in the intestine.

Although some neuropeptides, such as, ie, neuropeptide Y (NPY) and gastrin,^{33,34} are associated with angiogenesis-related responses in nonintestinal tissues, there is little evidence that neuropeptides are involved in angiogenesis in the intestinal tract. Most notably, there are no studies to link neuropeptides with angiogenesis in the pathophysiology of intestinal inflammation. SP regulates vascular tone and blood flow and initiates angiogenesis in the cardiovascular system³⁵ and also mediates angiogenesis in synovial inflammation³⁶ and chronic airway inflammation.³⁷ Results presented in this report strongly

Figure 6. CCN1 induces colonic angiogenesis. **A:** Mice (C57/BL6, $n = 4$ per group) were transfected intracolonic with CCN1- or EGFP-overexpressing constructs and then treated with 5% DSS in their drinking water or water alone for 5 days. Colon tissues were obtained for 4,6-diamidino-2-phenylindole, GFP, CCN1, and vWF staining to visualize CCN1 expression and blood vessels. **B:** Quantitative analyses of blood vessel number per field were performed using the Scion image analysis software. Levels of CCN1 and β -actin were determined by Western blot analyses (**C**) and their signal intensities were quantified by densitometry (**D**). *** $P < 0.001$, ** $P < 0.01$ versus EGFP-transfected water-treated group. ** $P < 0.01$ versus EGFP-transfected DSS-treated group.

suggest that SP mediates angiogenesis during colitis, and this event is involved in the pathophysiology of IBD.

Human umbilical vein endothelial cells express NK-1R³⁸ and SP exposure induces human umbilical vein endothelial cell migration, suggesting angiogenic activity.³⁹ SP also induces COX-2 expression⁴⁰ and

neutrophil adhesion⁴¹ in these cells. Our results show that HIMECs do not respond to SP directly. Consistent with these results, real-time PCR experiments indicate that HIMECs express very low levels of NK-1R mRNA, whereas Western blot analysis failed to identify NK-1R protein in these cells (data not shown). Along these

lines, HIMECs have been previously shown to have different functional properties compared to human umbilical vein endothelial cells.⁴²

Our results indicate that SP stimulates release of CCN1 from colonocytes that could be detected in the conditioned media of these cells (Figure 3). A recent report also suggested that, to exert angiogenic activity, CCN1 is released into cell media via protease cleavage, such as tissue plasminogen activator (tPA).⁴⁵ Interestingly, SP causes a rapid and substantial local release of tPA when injected in human forearm.⁴⁶

There are some reports indicating functional interactions between CCN1 and VEGF toward angiogenesis.¹⁷ CCN1 and VEGF-C knockout mice share similar phenotypes of compromised vascular integrity, leading to embryonic death. VEGF can stimulate CCN1 expression²⁵ whereas CCN1 can induce expression of VEGF-C and thus CCN1 knockout mice have impaired VEGF-C expression.¹⁷ Although we did not perform experiments to directly examine the possibility that VEGF-CCN1 interactions are involved in the angiogenic responses in our experimental system, our data presented in Figure 4D indicate that VEGF might not play a role in SP-induced angiogenesis and endothelial cell migration.

Recent studies suggest that angiogenesis is an important component of colonic inflammation. Several angiogenic factors such as vascular endothelial growth factor, basic fibroblast growth factor, and interleukin-8 are elevated in the colon of IBD patients.¹³ However, the potential input of angiogenic factors in the development of colitis, both during the acute and the repair or healing phase are not fully understood. Angiogenesis during inflammation will increase the influx of inflammatory cells, help provide nutrients to metabolically active immune cells, and transport cytokines and chemokines in the circulation.^{45,46} For example, anti-angiogenic therapy with ATN-161, a novel integrin $\alpha(5)\beta(1)$ antagonist, inhibits DSS-induced colitis,²⁸ consistent with the notion for a proinflammatory role of angiogenesis in the development of colonic inflammation. In addition, evidence indicates that temporal expression of VEGF and angiopoietin-1 can accelerate healing during experimental colitis.⁴⁸ We did not examine whether CCN1 can also mediate mucosal healing after colitis. However, CCN1 has been shown to stimulate proliferation in human skin fibroblasts, and to activate antiapoptotic pathways in pulmonary epithelial cells.⁴⁸

In conclusion, we have identified CCN1 as an important angiogenic factor that might promote angiogenesis in experimental colitis and possibly IBD. Our data also present evidence that the neuropeptide SP via its high-affinity NK-1R mediates angiogenesis by inducing CCN1 secretion from colonocytes. These results provide a novel neuropeptide-colonocyte-mucosal endothelial cell paradigm involved in angiogenesis-related intestinal inflammation with putative implications in IBD pathophysiology.

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