Regulation of Proangiogenic Factor CCN1 in Cardiac Muscle

Impact of Ischemia, Pressure Overload, and Neurohumoral Activation

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Background—CCN1, a potent proangiogenic factor, is induced in the vasculature by tissue injury, angiotensin II (Ang II), and growth factor stimulation. Because these conditions occur in myocardial ischemia and pressure overload, we investigated the regulation of CCN1 in cardiomyocytes in vitro and in the heart in vivo.

Methods and Results—Ang II, signaling via the angiotensin type 1 (AT1) receptor, and α1-adrenergic stimulation with phenylephrine induced CCN1 expression in ventricular cardiomyocytes isolated from 1- to 3-day-old rats. Cell culture supernatant of Ang II–treated cardiomyocytes induced migration of smooth muscle cells, which was abolished by neutralizing antibody to CCN1. Ang II– and phenylephrine-mediated induction of CCN1 expression in cardiomyocytes was completely abolished by inhibition of MEK/extracellular signal–regulated kinases (ERK) or protein kinase C (PKC). Likewise, mechanical stretch induced CCN1 expression in cardiomyocytes, an effect that was prevented by AT1 receptor blockade or PKC inhibition. Similarly, pressure overload in vivo upregulated myocardial CCN1 expression levels via AT1 receptor– and PKC-dependent mechanisms. After myocardial infarction in mice, CCN1 expression was strongly induced in both ischemic and remote left ventricular myocardium. Marked CCN1 protein expression was noted in cardiomyocytes of patients with end-stage ischemic cardiomyopathy but was absent in nonfailing human myocardium.

Conclusions—Pressure overload, ischemia, and neurohormonal factors, such as Ang II or α1-adrenergic stimuli, induce myocardial expression of CCN1, a potent proangiogenic factor, supporting the notion that CCN1 may play an important role in the adaptation of the heart to cardiovascular stress.

Key Words: angiogenesis • myocardial infarction • ischemia • pressure • signal transduction

Myocardial angiogenesis is a fundamental adaptive mechanism of the heart to cardiovascular stress. The ability to promote new vessel formation within the myocardium helps to improve myocardial perfusion and to meet increased oxygen demands after ischemic injury or during hypertrophic growth. In this respect, vascular endothelial growth factor (VEGF) plays an important role in stimulating neovascularization in the myocardium exposed to ischemia or pressure overload.1-2 However, the therapeutic potential of VEGF to induce sustained cardiac neovascularization is limited, and its application in humans has yielded equivocal long-term results.3-4 Several other potent proangiogenic factors have recently been identified, such as CCN1 (formerly named CYR61)5 or placental growth factor, which may act in concert with or independent from VEGF to drive sprouting of new vessels, thus possibly providing novel tools for therapeutic application.6,7 CCN1 is an immediate early gene encoding a heparin-binding protein that integrates into the extracellular matrix and promotes integrin-mediated migration and adhesion of endothelial cells, vascular smooth muscle cells, fibroblasts, and monocytes.5,9 Potent proangiogenic properties of CCN1 were demonstrated, for example, in a rabbit ischemic hindlimb model7 and in a rat cornea model.10 We have recently demonstrated that angiotensin II (Ang II) strongly induces CCN1 expression in vascular smooth muscle cells in vitro and in vivo, implicating a role of CCN1 for neovascularization of the atherosclerotic plaque.11 However, it is unclear whether and to which extent CCN1 is expressed in the heart, particularly under pathological conditions. In the present study, we postulated that mechanical and/or ischemic stress as well as neurohormonal stimulation may induce CCN1 expression in cardiomyocytes and in the heart, thus poten-
tially providing a novel paracrine pathway controlling myocardial angiogenesis.

Methods

Substances
Telmisartan was a gift from Boehringer-Ingelheim (Ingelheim, Germany). Tumor necrosis factor-α (TNF-α) and leukemia inhibitory factor (LIF) were purchased from Genzyme. PD98059, chelerythrine, and calphostin were obtained from Calbiochem. Phorbol 12-myristate 13-acetate (PMA) and all other reagents were obtained from Sigma.

Animal Experiments
Experimental myocardial infarction (MI) was induced in male C57BL/6 mice (aged 12 weeks; bred in our lab) as described previously.12 Transverse thoracic aortic constriction (TAC) was performed according to the method of Rockman et al.13 Sham-operated and unoperated mice were served as controls. Arterial blood pressure recordings were performed with a 1.4F micromanometer conductance catheter (SPR-719; Millar Instruments) inserted via the right carotid artery.12 Telmisartan (1 mg/kg per day) and hyaladrazine (42 mg/kg per day) were administered in the drinking water starting 5 days before MI or TAC. Chelerythrine (5 mg/kg) was dissolved in 45% dimethyl sulfoxide and was injected intravenously 20 minutes before TAC. Controls received 45% dimethyl sulfoxide only. Norepinephrine (1.28 mg/kg) and Ang II (0.64 mg/kg) were injected intraperitoneally. Systolic blood pressure was measured with the use of a noninvasive computerized tail-cuff system (BP-2000, VisiTech Systems). After the mice were killed, hearts were excised, and ischemic and nonischemic (remote) left ventricular (LV) tissues were dissected and snap-frozen in liquid nitrogen. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the local authorities.

Patients
LV myocardium was obtained from patients undergoing heart transplantation due to end-stage ischemic cardiomyopathy (NYHA functional classes III or IV; n=5). For comparison, LV tissue samples from nonfailing donor hearts that could not be transplanted for technical reasons were studied (n=4). Tissue samples were fixed in buffered 4% paraformaldehyde and embedded in paraffin or were snap-frozen in liquid nitrogen. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the local authorities.

Cell Culture
Cardiomyocytes were isolated from 1- to 3-day-old Sprague-Dawley rats as described and seeded on gelatin-coated tissue culture plates or FlexWell plates. Cardiomyocytes cultured for 24 hours in DMEM/medium 199 (4:1; 10% horse serum, 5% FCS) were switched to DMEM/medium 199 (4:1) and stimulated with various agents 24 hours later. Inhibitors were applied 60 minutes before stimulation. Cardiomyocytes were stretched with the use of a FlexerCell Strain Unit (FX-4000; FlexCell). Biaxial cyclic stretch (15%, 0.5 Hz) was applied. Controls were cultured on FlexWell plates but without mechanical stretch.

Chemotaxis Assay
Chemotaxis assay was performed in modified Boyden chambers with polycarbonate filter membranes (pore size 8 μm). In brief, 25000 to 30000 cells (human coronary artery smooth muscle cells) in serum-free SmGM2 medium were added to the upper well of the Boyden chamber. Supernatant of cardiomyocytes was added to the lower well of the Boyden chamber, and migration was allowed for 4 hours. All experiments were performed in triplicate. Cell migration was quantified densiometrically.15

RNA Extraction, Northern Blot, and RT-PCR
Northern blot analyses were performed as previously described.11 Expression was normalized to G3PDH. In human LV tissue samples, CCN1 abundance, normalized to G3PDH, was determined by RT-PCR as described.11

Western Blotting
Protein extracts were prepared in RIPA buffer; cytosolic and membrane fractions were isolated in 20 mmol/L Tris, pH 7.4, and separated by centrifugation (18,000g, 1 hour). Media supernatants were concentrated (≥20-fold) with the use of Micron YM-30 columns (Millipore). Protein extracts were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed with anti-human CCN1 antibody (Santa Cruz, sc-8561) or skeletal muscle α-actin (Biomedia). Specific bands were visualized by enhanced chemiluminescence (BioRad). Equal loading was confirmed by Ponceau-S staining.

Immunohistochemistry
Immunohistochemistry was performed with the use of antibodies against CCN1 (Santa Cruz), myosin heavy chain (MHC) (Alexis), and von Willebrand factor (vWF) (DAKO).11 To control for nonspecific binding, CCN1 antibody was preabsorbed with its blocking antibody (Santa Cruz, sc-8561) at a ratio of 1:20 in PBS overnight.

Statistical Analyses
Data are presented as mean±SEM. Differences between groups were evaluated by Student t test or ANOVA, as appropriate. Statistical significance was defined as P<0.05.

Results

G-Protein–Coupled Receptor Agonists, TNF-α, and Mechanical Stretch Induce CCN1 Expression in Cardiomyocytes
Ang II, the α/β-adrenergic agonist norepinephrine, the α1-selective agonist phenylephrine, and TNF-α increased CCN1 mRNA abundance in cardiomyocytes (Figure 1A). However, LIF (10−5 mol/L) did not alter CCN1 expression levels (not shown). Rapid induction of CCN1 mRNA was detected in isolated cardiomyocytes after mechanical stretch (Figure 1A). Ang II or phenylephrine increased CCN1 mRNA levels in cardiomyocytes in a concentration-dependent manner (Ang II: 10−7 mol/L, 256%±11%; 10−8 mol/L, 187%±9%; 10−9 mol/L, 166%±22%; phenylephrine: 10−9 mol/L, 306%±14%; 10−8 mol/L, 210%±11%; 10−7 mol/L, 161%±12%). Furthermore, Ang II and phenylephrine increased CCN1 protein expression levels in cardiomyocytes and promoted release into culture media (Figure 1B). Intrapерitoneal injection of Ang II or norepinephrine in mice induced LV CCN1 mRNA expression 3.6±0.6-fold (Ang II) and 5.3±0.9-fold (norepinephrine), with both peaking at 30 minutes (n=3 experiments; data not shown), providing evidence that neurohumoral factors can regulate cardiac CCN1 levels in vivo.

Ang II Mediates Smooth Muscle Cell Migration in Part via CCN1
It has been shown that CCN1 acts as a chemotactant to induce cell migration.8,9 A directional vascular smooth muscle cell migration assay in a micro chemotaxis Boyden chamber was performed with the use of a supernatant of cardiomyocytes treated with Ang II alone or absorbed overnight with CCN1 antibody. After 4 hours, cell migration in...
response to supernatant of Ang II–treated cardiomyocytes was significantly enhanced compared with migration in response to supernatant of untreated cardiomyocytes or supernatant of Ang II–treated cardiomyocytes previously absorbed with CCN1 antibody (Figure 1C). Treatment of supernatant with nonspecific IgG had no effect.

**CCN1 Expression Is Controlled by PKC and MEK/ERK-Dependent Signaling Pathways in Cardiomyocytes**

Effects of phenylephrine and Ang II in cardiomyocytes are mediated via Gq coupled receptors activating multiple signal transduction pathways, including PKC and extracellular signal-regulated kinases (ERK). Inhibition of PKC by calphostin or chelerythrine prevented the increase in CCN1 mRNA in cardiomyocytes in response to phenylephrine or Ang II stimulation (Figure 1D, 1E). Similarly, inhibition of MEK/ERK1/2 by PD98059 abolished phenylephrine- and Ang II–mediated CCN1 expression in cardiomyocytes (Figure 1D, 1E). Effects of Ang II on CCN1 abundance were inhibited by the angiotensin type 1 (AT1) receptor antagonists telmisartan (Figure 1E) and losartan (not shown). Direct stimulation of PKC by PMA induced CCN1 expression in cardiomyocytes, an effect that was attenuated by PD98059 (Figure 1F). Inhibitors alone had no effects on CCN1 mRNA levels in cardiomyocytes.

**Effect of Mechanical Stretch and Pressure Overload on CCN1 Expression in Cardiomyocytes In Vitro and In Vivo via the AT1 Receptor and PKC**

CCN1 induction of cardiomyocytes in response to mechanical stretch was suppressed by telmisartan and calphostin (Figure 1G), indicating a critical involvement of the AT1 receptor and PKC in the stretch-mediated upregulation of CCN1 in cardiomyocytes.
Mice subjected to TAC exhibited a significant increase in LV systolic pressure (TAC: 167±25 mm Hg; controls: 123±7 mm Hg; P<0.05). One hour after TAC, LV CCN1 mRNA levels increased transiently by 10-fold (CCN1 expression levels reverted to control levels 6 hours after TAC; data not shown), and this increase was blunted by telmisartan (Figure 2A). Telmisartan did not affect systolic blood pressure proximal to the site of aortic banding compared with untreated animals (not shown), suggesting that Ang II, signaling via the AT1 receptor, controls CCN1 expression via blood pressure–independent mechanisms in the acutely pressure-overloaded heart. Similar to our observations in isolated cardiomyocytes, chelerythrine attenuated the increase in LV expression levels of CCN1 after TAC in vivo (Figure 2B).

Induction of Cardiac CCN1 Expression After MI
CCN1 mRNA levels rapidly increased in the ischemic and remote myocardium, peaking between 1 hour and 6 hours after MI, and were associated with increased CCN1 protein expression (Figure 3A, 3B). Treatment of mice with telmisartan or with the direct vasodilator hydralazine reduced systolic blood pressure before MI (controls: 123±3 mm Hg; telmisartan: 102±5 mm Hg; hydralazine: 91±7 mm Hg; P<0.05 for telmisartan and hydralazine versus control). Treatment with telmisartan or hydralazine prevented the induction of CCN1 mRNA expression in the remote LV 6 hours after MI (Figure 3C). By contrast, expression of CCN1 in the ischemic LV was not affected by either treatment (Figure 3C), indicating that ischemia promotes CCN1 expression via AT1 receptor– and blood pressure–independent mechanisms.

Sustained CCN1 Protein Expression in Cardiomyocytes After MI
Increased CCN1 protein levels 48 hours after MI were detected in cardiomyocytes, as demonstrated by anti-MHC staining of serial sections (Figure 4A, 4B). LV myocardium obtained 48 hours or 6 months after sham operation displayed only weak CCN1 staining (Figure 4C, 4H). Six months after MI, the remote LV, mainly cardiomyocytes and blood vessels, showed elevated expression of CCN1 protein (Figures 3B, 4E). By contrast, the infarct scar displayed only faint CCN1 staining (Figures 3B, 4F), mainly in surviving cardiomyocytes and in blood vessels (Figure 4F).

Enhanced CCN1 Expression in Human End-Stage Ischemic Cardiomyopathy
CCN1 protein was robustly expressed in LVs of patients with end-stage ischemic cardiomyopathy (Figure 5A, 5E), mainly in cardiomyocytes and blood vessels, as revealed by anti-MHC (Figure 5B) and anti-vWF (Figure 5C) staining of serial sections. By contrast, fibrotic areas displayed weak CCN1 staining (Figure 5E). LV myocardium obtained from nonfailing donor hearts displayed faint CCN1 staining (Figure 5D). In accordance with the immunohistochemistry data, RT-PCR analyses revealed a 3.5±1.6-fold increase (P<0.05) in CCN1 transcript levels (not shown), and Western blot analysis demonstrated elevated CCN1 protein expression (Figure 5H) in ischemic cardiomyopathy versus nonfailing LVs (ischemic cardiomyopathy, n=5; nonfailing LVs, n=4).

Discussion
Therapeutic concepts to foster neovascularization in ischemic heart disease have received major attention. Although VEGF has been advocated to represent a potent means for therapeutic angiogenesis,4 clinical trials applying VEGF in patients with ischemic cardiomyopathy have not yet yielded consistent beneficial results.3,4 The limitations of VEGF to provide sustained neovascularization were recently highlighted by Dor et al.,17 who demonstrated that VEGF overexpression in the adult mouse heart may result in the formation of a nonfunctional capillary tree. This may be related to recent observations that several other potent proangiogenic factors, such as placental growth factor6 or CCN1,7 act in concert with...
VEGF to continuously drive sprouting and maturing of new vessels. Thus, although the application of a single proangiogenic factor has entered the clinical arena, mechanisms of cardiac neovascularization in response to cardiac stress, ie, the regulation, temporal and spatial expression, and interaction of different proangiogenic factors, remain poorly understood.

In the present study, we evaluated the regulation of the proangiogenic factor CCN1 in the heart in response to ischemia, pressure overload, and neurohormonal activation to gain pathophysiological insights into cardiac angiogenesis during various forms of cardiac stress and to provide a basis for possible future therapeutic applications. We present evidence that MI, pressure overload, and neurohormonal stimulation result in a marked induction of CCN1 expression in the myocardium and that the induction of CCN1 by these different stimuli emerges predominantly in cardiomyocytes, in both murine and human myocardium.

Ang II, signaling via the AT1 receptor, α1-adrenergic agonists, and TNF-α were identified as potent inducers of CCN1 expression in cardiomyocytes. Rapid induction of CCN1 mRNA expression was followed by CCN1 protein expression. Injection of Ang II and norepinephrine in mice rapidly induced LV CCN1 expression, providing direct evi-
CCN1 expression in cardiomyocytes. Importantly, mechanical stretch was not only required but also sufficient for the induction of PKC and the MEK/ERK signaling pathway. Activated PKC in cardiomyocytes was critically dependent on activation of the gp130 receptor,18 did not augment CCN1 expression, demonstrating that CCN1 induction is not a general response to growth factor stimulation in cardiomyocytes.

Results in the release of Ang II from cardiomyocytes, which in turn stimulates a hypertrophic response in an autocrine fashion. Consistent with these studies, the stretch-induced upregulation of CCN1 in cardiomyocytes was abolished by AT1 receptor blockade. Inhibition of PKC blunted the stretch-induced upregulation of CCN1 in cardiomyocytes, further supporting the critical importance of PKC-dependent pathways in the control of CCN1 expression in cardiomyocytes.

To validate these observations in the in vivo context, mice were subjected to pressure overload by TAC. Acute pressure overload resulted in a dramatic increase in CCN1 expression, which was significantly attenuated by AT1 receptor blockade and PKC inhibition, supporting the notion that both the AT1 receptor and PKC are involved in mechanical stress–induced expression of CCN1 in vitro and in vivo.

MI was associated with a rapid induction of CCN1 expression in the ischemic and remote regions of the heart. AT1 receptor blockade and nonspecific reduction of blood pressure by hydralazine reduced CCN1 mRNA expression in the remote region of the LV but did not affect CCN1 expression in the ischemic area, suggesting that different mechanisms control CCN1 expression in the ischemic and remote (but volume/pressure-overloaded) LV. Consistent with our findings in cardiomyocytes in vitro and after TAC in vivo, our observations indicate that CCN1 expression in the remote LV is regulated primarily by hemodynamic-dependent factors. As shown by immunohistochemistry, CCN1 protein expression was augmented in cardiomyocytes and in blood vessels, both early after infarction (48 hours) and in chronically infarcted LVs (6 months). Importantly, similar observations were made in patients with end-stage ischemic cardiomyopathy, whereas nonischemic donor hearts displayed little expression of CCN1, suggesting a pathophysiological role for CCN1 in patients with coronary artery disease.

CCN1 has previously been shown to activate distinct cell types involved in angiogenesis and arteriogenesis, eg, macrophages, endothelial cells, fibroblasts, and myoblasts.8,9,20 These unique properties, combined with the widespread expression of CCN1 after cardiac injury, as shown in the present study, suggest that CCN1 may orchestrate myocardial angiogenesis and remodeling of the vascular bed in response to myocardial stress. It has previously been proposed that CCN1 integrates its activities on macrophages, endothelial cells, and fibroblasts to regulate angiogenesis, inflammation, and matrix remodeling in the context of cutaneous wound healing.21 Because our studies showed that CCN1 is produced by cardiomyocytes on injury, it is conceivable that, similar to its actions in wound healing, CCN1 may act as an extracellular matrix–associated proangiogenic factor in the myocardium. Although our studies were not designed to evaluate the proangiogenic effects of CCN1 in ischemic and pressure-overloaded myocardium, there is evidence that CCN1 may exert proangiogenic effects under similar conditions. For example, it was recently demonstrated that overexpression of CCN1 in the ischemic hindlimb model improves angiogenesis and collateral blood flow to an even larger extent than VEGF.7 Our finding that cardiomyocytes are a major source of CCN1 suggests that CCN1, in concert with VEGF and other proangiogenic factors, may be part of an important paracrine program promoting angiogenesis in the injured myocardium.

Figure 5. Myocardial expression and distribution of CCN1 in patients with end-stage ischemic cardiomyopathy analyzed by immunohistochemistry and Western blot. A, Representative section of a patient with ischemic cardiomyopathy stained with CCN1 antibody, with serial sections stained with anti-MHC as a marker for cardiomyocytes (B) or endothelial cell marker anti-vWF (C). Nonfailing LVs displayed weak CCN1 staining only (D). Weak staining for CCN1 in fibrotic scar compared with cardiomyocytes is shown in E, with serial sections stained with anti-MHC in F. A control slide (LV section from a patient with ischemic cardiomyopathy) was stained with CCN1 previously incubated with its blocking peptide (G). H, Representative Western blot confirming enhanced expression of CCN1 in LV tissue homogenates of patients with ischemic cardiomyopathy (ICM) compared with nonfailing (NF) donor hearts; protein levels were normalized to skeletal muscle α-actin (actin) content. Bars=100 μm (magnification in A through D, ×400; magnification in E through G, ×200).
heart. This notion is supported by our observation that supernatant of cardiomyocytes treated with Ang II induces migration of smooth muscle cells and that this chemoattractant effect is abolished by selectively inactivating CCN1 with its neutralizing antibody. However, future experiments are warranted to assess the angiogenic and therapeutic potential of CCN1 in the ischemic and pressure-overloaded myocardium.

Acknowledgments
This study was supported by the Leducq Foundation and the German Academic Exchange Service (DAAD). We gratefully acknowledge the expert technical assistance of Silvia Gutzke and Birgit Brandt.

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*Circulation.* 2004;109:2227-2233; originally published online April 26, 2004; doi: 10.1161/01.CIR.0000127952.90508.9D

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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