The matrix protein CCN1 (CYR61) promotes proliferation, migration and tube formation of endothelial progenitor cells

Yang Yu\textsuperscript{a,1}, Yu Gao\textsuperscript{b,1}, Hong Wang\textsuperscript{a}, Lan Huang\textsuperscript{a,⁎}, Jun Qin\textsuperscript{a}, Ruiwei Guo\textsuperscript{a}, Mingbao Song\textsuperscript{a}, Shiyong Yu\textsuperscript{a}, Jianfei Chen\textsuperscript{a}, Bin Cui\textsuperscript{a}, Pan Gao\textsuperscript{a}

\textsuperscript{a}Institute of Cardiovascular Diseases of PLA, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, People’s Republic of China
\textsuperscript{b}Department of Rehabilitation, Southwest Hospital, Third Military Medical University, Chongqing 400038, People’s Republic of China

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ABSTRACT

Neovascularization and re-endothelialization relies on circulating endothelial progenitor cells (EPCs), but their recruitment and angiogenic roles are subjected to regulation by the vascular microenvironment, which remains largely unknown. The present study was designed to investigate the effects of mature ECs and matrix protein CCN1 on the properties of EPCs. In a coculture system, effects of ECs on proliferation, migration and participation in tube-like formation of EPCs were evaluated, and functional assays were employed to identify the exact role of CCN1 in EPCs vitality and function. We demonstrated that ECs, as an indispensable part of the cellular milieu, significantly promoted the proliferation, migration and tube formation activities of EPCs, and more importantly, CCN1 was potentially involved in such effects of ECs. Expression of CCN1 in EPCs was significantly increased by serum, VEGF, ECs-cocultivation and ECs conditioned medium. Moreover, Ad-CCN1-mediated overexpression of CCN1 directly enhanced migration and tube formation of EPCs, whereas silencing of endogenous CCN1 in EPCs inhibits cell functions. Furthermore, CCN1 induced the expressions of chemokines and growth factors, such as MCP-1 and VEGF, suggesting a complex interaction between those proangiogenic factors. Our data suggest that matrix protein CCN1 may play an important role in microenvironment-mediated biological properties of EPCs.

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Introduction

A growing body of evidence indicates that bone marrow-derived endothelial progenitor cells (EPCs) are mobilized, recruited and prominently contributed to neovascularization and re-endothelialization after vascular injury, in contrast to conventional assumption that postnatal neovascularization is attributed to the migration and proliferation of preexisting mature endothelial cells (ECs)\textsuperscript{[1–4]}. In particular, EPCs not only directly incorporate into blood vessels, replacing the defective or injured mature ECs, but also secrete a variety of cytoprotective or proangiogenic factors in a paracrine manner to promote the survival and proliferation of ECs. However, up to now, investigations have emphasized a strong impact of microenvironment on the biological properties of EPCs\textsuperscript{[5–7]}, though the matrix microenvironment and interactions between local ECs and EPCs that may control EPCs proliferation and functions remain poorly understood.

CCN1 is a secreted matrix protein belonging to the emerging CCN family, which also includes CCN2/CTGF, CCN3/Nov, CCN4/WISP-1, CCN5/WISP-2 and CCN6/WISP-3\textsuperscript{[8]}. CCN1 is expressed by
all types of vascular cells, and has been implicated in diverse cellular processes such as adhesion, migration, proliferation and survival [9,10]. Moreover, noticeably, a potential role of CCN1 in angiogenesis and vascularization has been demonstrated by accumulating evidence. Targeted knockout of CCN1 gene in mice results in embryonic lethality due to placental vascular insufficiency and compromised vessel integrity [11]. Besides its ability to induce tumor angiogenesis, CCN1 is able to stimulate neovascularization in rat cornea [12] as well as rabbit ischemic hindlimb [13]. In addition, CCN1 can be rapidly induced by vascular endothelial cell growth factor (VEGF), and promote proliferation, migration and tube formation of ECs in vitro [14]. However, whether and what impact CCN1 may play on EPCs is largely unknown at present. Interestingly, more recent observations showed that recombinant CCN1 and supernatants from CCN1-stimulated CD34+ cells effectively promoted proliferation of ECs [15], and exogenous CCN1 could promote adhesion and migration of human CD34+ progenitor cells and mesenchymal stem cells [16], suggesting a potential role of CCN1 in matrix microenvironment which may affect properties of EPCs.

In this study, we found that expression levels of CCN1 were increased in EPCs and ECs after stimulation with serum and VEGF. Our co-culture experiments revealed that surrounding ECs was involved in the regulation of proliferation, migration and tube formation of EPCs, and what is more, CCN1 was significantly contributed to the paracrine effects of ECs on EPCs. Forced expresssion exogenous CCN1 was shown to induce secretion of VEGF and MCP-1, and enhanced proliferation, migration and tube formation of EPCs. RNA interference (RNAi)-mediated knockdown of CCN1 expression diminished migration and tube formation of EPCs. These findings suggest that matrix protein CCN1 promotes proliferation, recruitment and function of EPCs, and may play an important role in microenvironment-mediated biological properties of EPCs.

Materials and methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. All cell culture plates were obtained from Costar, whereas culture medium and serum were obtained from Gibco (Grand Island, NY, USA). FITC-conjugated antibodies against rat CD133, rat CD34, rat VEGFR-2 and corresponding isotype control IgG were from Bios (Beijing, China), while antibodies against rat CD44 and rat CD45 were from BD (BD Biosciences, San Jose, CA). Rabbit polyclonal antibody against rat CCN1 and blocking antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human VEGF was from R&D Systems, Inc. (Minneapolis, MN) and Dil-AcLDL was from Biomedical Technologies, Inc. (Stoughton, MA).

Recombinant adenoviral vectors expressing CCN1

To evaluate the role of CCN1, adenovirus vector expressing CCN1 was generated using the AdEasy system. Briefly, full-length rat CCN1 cDNA was generated by RT-PCR using total RNA from Sprague–Dawley (SD) rat heart and the following primers: sense 5’-aggagatctatgagctcagcaccatacag-3’ and antisense 5’-gtcaagctt-cttgagcccgtacaattttgtcgaca-3’ (nucleotides 186 to 1325, GenBank accession number NM_031327). The CCN1 cDNA was first TA-cloned into pMD19-T simple vector and then subcloned into pAdTrack-CMV, resulting in pAdTrack-CCN1. The shuttle vector was used to generate recombinant adenovirus Ad-CCN1 according to the manufacturer’s protocol. All PCR-amplified fragments and cloning junctions were verified by DNA sequencing (Sangon, Shanghai, China). An adenovirus encoding green fluorescent protein (GFP; Ad-GFP) was used as control. All adenoviruses were replication deficient and used at 20 multiplicity of infection (mois) for 24 h without apparent cytotoxicity.

RNAi-mediated silencing of CCN1 expression

The target sequences were selected by the web-based small interfering RNA (siRNA) hairpin engine at www.genscript.com and www.ambion.com, which showed no homology to any other sequences by a blast search. The synthesized template oligonucleotides consisting of sense target sequence, antisense target sequence, loop structure and a transcription stop signal were then annealed and subcloned, respectively, into shRNA expression vector pGenesil1-U6 (Genesil, Wuhai, China) to obtain the final construct pGenesil1-CCN1. According to the preliminary experiments on rat renal fibroblasts (line NRK) about CCN1 RNAi efficiency, following sequences were used in this study: CCN1 siRNA, 5’-gatccgcaactcaacgaggactgctctagacacagtctcctctg- ggttggcttcttttgctcaga-3’ , negative control siRNA 5’-gcacgtgttttcagagccctatgacatcgtctcctcctctcct- gagttgcttttttgtcgaca-3’ , positive control siRNA 5’-gtacgactatca taaagctgatgtttgtcgaca-3’ and GAPDH-A siRNA as control 5’-gatcgtgttaggtttgtcgaca-3’. Transfection of the siRNA was carried out using Lipofectamine 2000 reagent with a molar ratio about DNA: lipid = 1:3 (Invitrogen, CA, USA). 24 h after transfection, cells were collected and used for functional assays (see below).

EPCs isolation and characterization

All animal procedures have been approved by the Care of Experimental Animals Committee of the Third Military Medical University. Culture and characterization of EPCs was performed previously in our laboratory [17]. BM was harvested by flushing the femurs and tibias of SD rats (male, 180 to 220 g, Chongqing). BM-derived mononuclear cells were isolated by density gradient centrifugation (Lymphoprep 1.083, Tianjing, China) at 400×g for 20 min. After purification with three washing steps, cells were resuspended in low glucose DMEM supplemented with 10% FCS and 10 ng/mL VEGF, plated on gelatin-coated cell culture flasks and incubated at 37 °C under 5% CO2. Twenty-four hours later, nonadherent cells were aspirated and transferred to a new gelatin-coated flask in order to remove rapidly adherent hematopoietic cells and mature endothelial cells. Another 48 h later, nonattached cells were removed and adherent cells were continuously cultured. Only those adherent cells were used in further experiments.

To confirm the EPCs phenotype, cells were incubated with acLDL-Dil (10 mg/ml) for 4 h, fixed with 4% paraformaldehyde and then incubated with FITC-labeled lectin (UEA-1, 10 mg/ml) for 1 h. Dual-stained cells positive for both acLDL-Dil and UEA-1 were identified as EPCs. Nearly all adherent cells (>95%) were double
positive cells. Additionally, flow cytometry (FACS) analysis was performed using antibodies against rat CD45, CD44, CD133, CD34, VEGFR-2 and the corresponding isotype control antibodies.

**Coculture of EPCs with endothelial cells (ECs)**

Endothelial cells from rat thoracic aorta were isolated in parallel with the endothelial progenitor cells by collagenase digestion using the standard protocol as described previously [18]. Isolated endothelial cells were cultivated in DMEM with 10% FCS and 10 ng/mL VEGF. For coculture experiment, passages 2–5 EPCs transfected with Ad-CCN1, Ad-GFP and non-transfected EPCs, together with ECs were pre-conditioned in serum/VEGF free medium for 24 h in order to minimize CCN1 basal secretion stimulated by serum or cytokines. Then, EPCs were subcultured in 6-well plates and ECs were placed over the EPCs monolayers in 0.4 mm pores transwell inserts, allowing the diffusion of soluble factors without direct cell contact. Cells were cocultured in the presence or absence of anti-CCN1 antibody in a total volume of 2 ml DMEM with 10% FCS. In some experiments, the coculture medium was replaced with 2 ml conditioned medium collected from cultivated ECs and the transwell inserts without ECs were set up. As controls, ECs and EPCs were cultured alone as described for coculture experiment. After 48 h, the cells and cell supernatants were collected for further analysis. All experiments were performed in triplicate.

**Cell proliferation assay**

EPCs were harvested from the cultures and replaced into 96-well plate (2 × 10^5 cells/mL) in triplicates. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays according to the protocol of the manufacturer. Prior to the optical density (490 nm) reading, 15 μL MTT solution and 200 μL DMSO were sequentially added to each well. All groups of experiments were performed in triplicate.

**Cell migration assay**

EPCs migration was evaluated using a modified Boyden’s chamber assay. EPCs (1 × 10^5) in 100 μL serum-free DMEM were placed in the upper chamber. The lower chamber contained either ECs suspensions or ECs conditioned medium. For controls, DMEM plus 10% FCS supplemented with or without 100 ng/mL SDF-1 were filled in the lower chamber as positive and negative controls, respectively. To test the effects of CCN1 on cell migration, EPCs were transfected with either Ad-CCN1 or siRNA-CCN1, and anti-CCN1 antibody was added. After 6 h in culture, cells on the upper side of the 8-μm filters were removed and the filters were washed with PBS. Then, the underside cells were fixed in methanol and stained with hematoxylin stain. Migration activity was evaluated as the mean number of migrated cells in 5 random high power fields per chamber under a microscope (Leica, Germany).

**Tube formation assay**

Matrigel of the same batch was thawed and laid into 24-well culture plates at 37 °C for 1 h to allow solidification. EPCs transfected with Ad-CCN1, Ad-GFP, or siRNA-CCN1 were harvested, resuspended and placed on the matrigel, with non-transfected EPCs as a control. The effect of ECs was studied using a coculture model. ECs seeded on inserts or ECs-conditioned medium were added to the wells, in the presence or absence of CCN1 blocking antibody. After incubation at 37 °C for 18 h, EPCs tube formation was observed microscopically and the total length of such tube like structures was measured by Leica Qwin V3.1 software.

**RNA extraction and reverse transcriptase-PCR (RT-PCR)**

Total RNA was extracted from EPCs and ECs by using TRIzol (Invitrogen), followed by cDNA synthesis using oligo (dt) and M-MLV reverse transcriptase (Takara). Then, cDNA amplification and semi-quantitative PCR were performed using the following primers: CCN1: 5’gccgtcaccctttctcactg 3’(forward) and 5’-gccttcattgcttcag-3’(reverse); VEGF: 5’-cctccgaaaccatgaactttctgcctc-3’(forward) and 5’-cagcctggtttcgcgcctgtc-3’(reverse); MCP-1: 5’-ctcaggatgctgcatctg-3’(forward) and 5’-tgagaaatggttaat-3’(reverse); GAPDH: 5’-accacagtccatgccatcac-3’(forward) and 5’-ctcaccgggtctgctga-3’(reverse). For quantitative RT-PCR analyses, the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and SYBR Green PCR Master Mix (Toyobo, Japan) were used with specific primers as follows: CCN1: the same as above; VEGF: 5’-aatctggaatcctagctc-3’(forward) and 5’-tccatcticctggcttcctg-3’(reverse); MCP-1: 5’-aattcggccatcactgct-3’(forward) and 5’-gaggttctaggaat-3’(reverse); GAPDH: 5’-aaccgctctgactccac-3’(forward) and 5’-acccatcctctttgct-3’(reverse). All primers were synthesized by Invitrogen (Shanghai, China) and were of high purity salt-free quality.

**Western blot analysis**

Culture supernatants were collected and concentrated approximately 20-fold with a centricon (Millipore, Bedford, MA). Proteins from both cell supernatants and lysates were measured using the Bradford method. The same amount of proteins were loaded in each lane, separated by 10–15% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk, and then the membrane-bound
proteins were probed with primary antibodies against CCN1 and GAPDH followed by secondary horseradish peroxidase-conjugated antibodies. Protein bands were visualized by chemiluminescent detection (ECL) (Amersham Biosciences) and quantified by a gel image analysis system.

**Enzyme-linked immunospecific assay (ELISA)**

Protein levels of VEGF and MCP-1 in the cell supernatants were determined by ELISA kit (R&D Systems), according to the manufacturer's instructions. Samples were measured in triplicate and
were properly diluted to ensure that measured values were within the concentration range of the standard curve.

**Statistical analysis**

Data from independent experiments were expressed as mean ± S.D. of at least three experiments. Comparisons between groups were analyzed by two-tailed Student’s t test or ANOVA, as appropriate. p values < 0.05 was considered statistically significant.

**Results**

**CCN1 expression is up-regulated in EPCs and ECs**

After 4–7 days of culture (typical culture period before coculture and further experiments), adherent EPCs were characterized by immunofluorescence and flow cytometry (FACS) analysis. The majority of cells (>90%) stained positive for Dil-AcLDL and lectin, and expressed endothelial/stem cell markers, including CD34, VEGFR-2, and CD133, but not CD44 or CD45 (Fig. 1a). However, ECs only express the marker of endothelial cells like vWF.

We thereafter investigated the expressions of CCN1 in primary EPCs and ECs. Fig. 1b showed that CCN1 was present at fairly low levels in quiescent EPCs but was rapidly up-regulated upon stimulation with serum, either the mRNA by RT-PCR or the protein by immunoblotting. In addition, cocultivation with ECs and with ECs conditioned medium also induced a significant increase of CCN1 expression in EPCs. For comparison, VEGF, a strong growth factor of EPCs, did not show any predominant effect on the expression of CCN1, comparable with that cocultured with ECs and ECs-conditioned medium. Similar results were obtained from ECs as well as EPCs (Fig. 1c).

**CCN1 is involved in effects of ECs on EPCs proliferation, migration and tube formation**

We previously observed that coculture with ECs promoted mesenchymal stem cells proliferation and differentiation, in a milieu-dependent manner [19]. To determine whether the mature ECs could affect EPCs in the same way, we used a previously described coculture system and the proliferation, migration and tube formation of EPCs were detected, respectively.

The proliferation of EPCs was significantly accelerated after coculture with ECs or ECs-conditioned medium compared with unstimulated groups, and was comparable with that of VEGF (Fig. 2a). In addition, no statistically significant differences were found in the EPCs treated with ECs and ECs-conditioned medium.

Next, we investigated the ability of mature ECs to promote the migration of EPCs, using in vitro migration assay, with SDF-1 as a positive control. Fig. 2b shows a notable enhancement in migration of EPCs when cocultured with ECs or supernatants from cultured ECs. Lastly, matrigel angiogenesis assay was performed with EPCs, and VEGF served as a positive control. The total length of tube-like structures after 18-h coculture were measured and compared with controls. It is noteworthy that ECs and supernatants taken from ECs significantly increased tube formation of EPCs (Fig. 2c). Taken together, all these results indicated that mature ECs promoted EPCs proliferation, migration and angiogenesis in vitro, as expected.

It is documented that the extracellular matrix (ECM) and cytokines plays important regulations in cells viability and functions. To probe the potential role of CCN1 in the regulation of EPCs proliferation, migration and angiogenesis, we first measured CCN1 levels in the conditioned medium and then added neutralizing antibody against CCN1 in the coculture systems. As shown in Fig. 2d, CCN1 was present at high levels in conditioned medium of ECs and EPCs. And interestingly, the proliferation, migration and tube formation augmented by ECs-couple or ECs supernatants were attenuated significantly in the presence of anti-CCN1 antibody (p < 0.01), while no obvious changes were seen in the control groups (data with control IgG not shown). These results suggested that the effect of mature ECs on EPCs proliferation and functions was at least partially through the matrix protein CCN1. Moreover, ccn1 might not be restricted to the ECM, but somehow acted as paracrine factors between mature and progenitor endothelial cells.

**CCN1 induces VEGF and MCP-1 release of ECs and EPCs**

In the present study, we observed that the matrix protein CCN1 could be secreted by either ECs or EPCs, and that was important for the paracrine effects of mature ECs on the proliferation and functions of EPCs. Furthermore, to explore which factors in response to CCN1 might be involved in the effects of ECs, we constructed recombinant adenovirus Ad-CCN1 and transduced it into ECs for detection the potential downstream factors. Using RT-PCR, we showed that the overexpression of CCN1 caused a 3.7-fold induction of VEGF mRNA and 4.0-fold induction of MCP-1 mRNA in ECs compared with the Ad-GFP transfected ECs (Figs. 3a, c). Both VEGF and MCP-1 are important factors during vascular repair and angiogenesis processes, and specifically, this Ad-CCN1-mediated induction of VEGF mRNA and MCP-1 mRNA was inhibited by a CCN1-blocking antibody (p < 0.05) whereas the control antibody was not effective at all (data not shown). Then, using ELISA analysis, we also confirmed the same expression pattern of VEGF and MCP-1 at the protein levels (Figs. 3b, d).
Moreover, the presence of VEGF and MCP-1 in EPCs stimulated with ECs supernatant was measured. Supernatant from Ad-CCN1-transfected ECs significantly increased the expression of VEGF and MCP-1 at both mRNA and protein levels in EPCs compared with the control, which was reversed by the CCN1 antibody \( (p<0.05) \), indicating a CCN1-dependent mechanism for the paracrine induction of MCP-1 and VEGF by EPCs.

**Overexpression of CCN1 enhances migration and tube formation of EPCs**

Since the matrix protein CCN1 plays an evident role in ECs paracrine effects on EPCs proliferation and functions, it may exert direct effects on EPCs. To further investigate the role of CCN1 in EPCs proliferation and functions, we constructed an adenoviral...
vector that expressed CCN1 (i.e., Ad-CCN1) exogenously using the AdEasy system. Adenovirus-mediated CCN1 expression was confirmed by Western blot analysis (data not shown). EPCs transfected with either Ad-CCN1 or control Ad-GFP were subsequently subjected to separate assays to examine their proliferation, migration and angiogenesis activities. As depicted in Fig. 4, overexpression of exogenous CCN1 extensively improved the migration of EPCs, and in fact, the average migrated cell number of the EPCs increased by approximately 4-fold compared to that of the control cells. More remarkably, the tube formation ability of EPCs was increased to about 250% as compared with Ad-GFP transfected EPCs \( (p < 0.01) \). In contrast, the proliferation of EPCs was not enhanced, even a minor decrease was observed in cells transfected with Ad-CCN1, though it has no significant meaning in compare with untransfected control. Nevertheless, the transfection efficacy in our study was about 50–60% assessed by immunofluorescence, and our findings suggested that CCN1 played an important role in regulating the EPCs migration and angiogenesis.

Fig. 3 — CCN1 induces VEGF and MCP-1 release of ECs and EPCs. (a, c) RNA from ECs subjected to Ad-CCN1 transduction and EPCs stimulated with ECs supernatants (ECs-SN) were isolated, reverse transcribed and amplified by PCR using specific primers for MCP-1, VEGF and GAPDH. Representative images of semi-quantitative PCR are shown in the top panel and the graphical representation of mRNA levels normalized to those of GAPDH are shown in the bottom panel of a and c. Quantitative evaluation is expressed as a percentage of control (in the absence of stimuli, serum free). (b, d) Supernatants from ECs transfected with Ad-CCN1 and EPCs stimulated with ECs-SN were collected and subjected to ELISA analysis, using VEGF and MCP-1 specific kits. Expression of MCP-1 and VEGF was increased by Ad-CCN1 and supernatants of Ad-CCN1 transfected ECs, and the increase was damped in the presence of 20 μg/ml CCN1 antibody (CCN1 Ab), whereas a control antibody was not effective at all (data not shown). Data are mean±S.D.\( (n=9) \), \*\( p \) value <0.05.
EPCs migration and tube formation is inhibited by shRNA-mediated knockdown of CCN1

Although the overexpression of exogenous CCN1 directly enhanced EPCs migration and tube formation, the actual role of endogenous CCN1 remained to be fully elucidated. Because the basal expression of CCN1 in quiescent EPCs was barely detectable, and it was dramatically increased in response to stimulations such as serum and VEGF, we therefore used a U6 promoter-driven shRNA (i.e., pGenesil1-CCN1) to silence the CCN1 gene in EPCs, and culture medium containing 20% FCS and 10 ng/mL VEGF was used as stimulation. Introduction of pGenesil1-CCN1 was shown to specifically knockdown CCN1 expression as measured by Western blot and RT-PCR (not shown). To determine whether the RNA interference-mediated silencing of CCN1 expression would affect serum+VEGF-induced EPCs proliferation, migration and tube formation, we transduced the pGenesil1-CCN1 into EPCs. However, introduction of the pGenesil1-CCN1 caused an approximate 50% loss of CCN1 expression in EPCs. And significantly, the EPCs exhibited a decrease in cell proliferation, migration and tube formation compared to negative control siRNA-transfected cells (p < 0.01) (Fig. 4). The results were reproducible in at least three independent batches of experiments. Thus, these results indicated that knockdown of endogenous CCN1 significantly reduced the proliferation, migration and tube formation of EPCs, suggesting an important role in EPCs of endogenous CCN1.

Discussion

Accumulating evidence indicates that neovascularization does not exclusively rely on the migration and proliferation of local ECs, but also includes the recruitment of bone marrow-derived circulating stem cells. Interest focuses on EPCs which considerably contribute to postnatal neovascularization and re-endothelialization in response to vascular injury during physiological and pathological processes [2]. These EPCs can be recruited and incorporated into injured vessel wall and then promote local angiogenesis by direct cellular differentiation and/or by releasing growth factors acting in a paracrine fashion [3]. However, it is documented that the recruitment and function of EPCs are tightly regulated by the neovascular microenvironment [5,7,20]. In particular, local vas-

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**Fig. 4** – Overexpression of CCN1 directly enhances migration and tube formation of EPCs, whereas silencing of endogenous CCN1 in EPCs inhibits cell proliferation, migration, and tube formation activities. (a) EPCs were transfected with or without Ad-GFP, Ad-CCN1, negative control siRNA or CCN1-siRNA, in the presence or absence of anti-CCN1 antibody (CCN1-Ab), and then followed by MTT assays. Three separate experiments were done in triplicates. (b) EPCs migration in response to Ad-GFP, Ad-CCN1, negative control siRNA or CCN1-siRNA, in the presence or absence of anti-CCN1 antibody was detected using the Boyden Chamber Model. EPCs cultured in 10% low glucose DMEM without any treatment were used as control for overexpression experiment, while EPCs cultured in low glucose DMEM supplemented with 20%FCS and 10 ng/mL VEGF were used as control for RNA interference experiment. (c) Tube formation by EPCs transfected with or without Ad-GFP, Ad-CCN1, negative control siRNA or CCN1-siRNA, in the presence or absence of anti-CCN1 antibody, were performed as described in Materials and methods, attached to Matrigel-coated 24-well culture plates and incubated at 37 °C for 18 h. EPCs images were captured and analyzed by Leica Qwin system as depicted in the legends to Fig. 2. Values are presented as the mean±S.D. of total length per field (*p<0.05 versus control; #p<0.05 versus Ad-CCN1).
cular cells and especially secreted factors, such as VEGF, SDF-1, FGF, PDGF and many more, play important roles in the regulation of EPCs and thus in neovascularization. Although the application of a single factor has entered the clinical arena, i.e., VEGF, large scale trials have not yielded expected beneficial results [21–23]. Thus, other potential factors and interactions between these factors remain to be further elucidated.

In the present study, we explored the role of novel matrix protein CCN1 in the regulation of EPCs proliferation and function. CCN1, belonging to the emerging CCN family, is a secreted, extracellular matrix associated proangiogenic factor. CCN1 is expressed by all types of vascular cells in response to a variety of physical and chemical stimuli such as growth factors, proteases, ischemia, hypoxia, and shear stress [24–27]. In addition, aberrant expression of CCN1 is associated with several diseases such as wound healing [28], atherosclerosis [29] and restenosis following percutaneous transluminal coronary angioplasty (PTCA) [30], supporting that CCN1 may play an important role in the self-renewal program, specifically EPCs-involved vascular regeneration. Here, we showed that CCN1 was present at a fairly low or undetectable level in quiescent EPCs, but was rapidly up-regulated upon stimulation with serum, VEGF, ECs-cocultivation and EC-conditioned medium. In accordance with other observations, we also identified the inducible expression of CCN1 in ECs. It is tempting to speculate that matrix CCN1 may act on EPCs in autocrine and paracrine manners, contributing to neovascularization under physiological and pathological conditions.

Our previous work demonstrated that mesenchymal stem cells co-cultured with mature ECs underwent milieu-dependent differentiation towards ECs [19]. In addition, observations from others showed that co-culture with ECs efficiently supported hematopoietic cells proliferation and trafficking [31] and was able to promote proliferation, migration and differentiation of neural progenitor cells [32]. In this study, we showed that mature ECs, the important cellular component of vascular microenvironment, also impacted on the biological properties of EPCs. The proliferation, migration and angiogenesis activities of EPCs were all significantly enhanced by either coculture with ECs or ECs-conditioned medium. More importantly, no obvious differences were found between treatments of ECs and ECs supernatant that emphasized the critical paracrine role played by mature ECs. Herein, we sought to investigate the role of CCN1 thereafter. First, we blocked CCN1 in the co-culture system with an anti-CCN1 antibody and found that ECs-augmented EPCs migration and tube formation were significantly attenuated by the antibody, suggesting a CCN1 dependent mechanism of ECs effects. Next, we constructed an adenoviral vector expressing rat CCN1 and transduced it into EPCs. Exogenous overexpression of CCN1 directly enhanced the migration and tube formation of EPCs as expected, whereas no effect was observed on cell proliferation. Then, we knocked down endogenous CCN1 by small interfering RNA, and further confirmed that RNAi-mediated knockdown of endogenous CCN1 inhibited the serum and VEGF-induced proliferation, migration and tube formation of EPCs. This was consistent with the above observations.

However, the effect of CCN1 on cell proliferation and migration is controversial [32–36]. CCN1 enhanced both migration and proliferation in vascular ECs and fibroblasts, and suppressed apoptosis in breast cancer cells, while CCN1 expression was associated with cell death in neuronal progenitor cells and CCN1-induced apoptosis in fibroblasts and endometrial cancer cells. Moreover, recent findings showed that CCN1 inhibited mesangial cells migration induced by PDGF-BB, but did not affect cell proliferation [37]. What impact CCN1 may play on EPCs is currently unknown. In our study, we demonstrated that CCN1 promoted the proliferation and migration of EPCs, using both loss and gain function assays. Our results raised a possibility that, under stimulation of vascular injury, CCN1 secreted by local ECs and other vascular cells might recruit circulating EPCs to sites of injury and facilitate regeneration and neovascularization. The seeming inefficacy of Ad-CCN1 transfection on the proliferation of EPCs could possibly be attributed to the cytotoxicity of adenoviral vectors. However, combined with previous observations by others, our results support the cell type-specific activities of CCN1. Well, the underlying mechanism involved in these properties is not entirely elucidated and is still under investigation.

Furthermore, our study observed that transfection of Ad-CCN1 into ECs increased the release of growth factors and chemokines such as VEGF and MCP-1, and the supernatant from transfected ECs also resulted in an increase of VEGF and MCP-1 levels in EPCs. It was intriguing to observe such effects of CCN1, as both VEGF and MCP-1 were thought to be important mediators of EPCs recruitment, proliferation and eventually regeneration and neovascularization. However, as an immediate early gene, CCN1 was induced by growth factors and cytokines such as VEGF, which has been proven by our study and studies from others. A previous experiment using microarray analysis identified that in human umbilical vein endothelial cells (HUVECs), VEGF induced more than two-fold increase of 139 cDNAs, and CCN1 was among them [14]. Kuiper and colleagues [38] reported that VEGF induced CCN1 expression in retina in vivo, as well as in cultured retinal endothelial cells and pericytes in vitro. An additional study reported that stimulation of osteoblasts with VEGF resulted in up-regulation of CCN1 mRNA and protein [39]. In fact, on the contrary, CCN1 has been known to regulate VEGF expression in human skin fibroblasts [28] and bladder smooth muscle cells [40]. Additionally, CCN1-deficient mice exhibited a substantial down-regulation of VEGF, which contributed to placental vascular insufficiency and reduced vessel integrity [11]. In present study, our results showed that VEGF (and serum) up-regulated CCN1 in ECs and EPCs, and vice versa, strengthening the hypothesis that a paracrine loop may exist between CCN1 and VEGF that regulates EPCs-mediated neovascularization and re-endothelialization. After vascular injury, CCN1 is induced by VEGF and other stimuli in local vascular milieu, especially in ECs. Then, matrix-associated CCN1 in turn up-regulates synthesis of VEGF, MCP-1 and some other factors, directly and/or indirectly promotes proliferation, recruitment, and angiogenesis activities of circulating EPCs. EPCs recruited into local vessels may equally synthesize and secrete CCN1 and VEGF in a circuit, thereafter stimulate migration and proliferation of adjacent ECs or support more circulating EPCs, further enhancing the vascular repair process in an amplifying-cytokine network. Furthermore, limited induction of CCN1, albeit to various extents, may be shown during pathological process as a result of vascular cells dysfunction, suggesting that inadequate CCN1 impacts on the vascular repair process and that targeting CCN1 may be a valuable approach in future therapy.

However, we cannot exclude other factors besides VEGF and MCP-1 are induced in EPCs and ECs by CCN1, and the signaling intermediates responsible for the functional interplay between those factors were not addressed in this study. To date, several
possibilities have emerged concerning the mechanism for CCN1 effects on EPCs [41–45]. First, as an extracellular matrix protein, CCN1 might exert its actions on EPCs via mediating the matrix microenvironment and release the matrix-binding cytokines and chemokines. Second, CCN1 behaved as a ligand for heparin sulfate proteoglycan and various integrins, and thus it might directly induce factors such as VEGF and MCP-1 through “outside-in signaling” transduction in EPCs and ECs. Third, CCN1 could functionally synergize with other factors such as VEGF and MCP-1 to enhance EPCs proliferation, recruitment and angiogenesis activities in an autocrine and paracrine fashion. Therefore, the interplay between the actions of CCN1 and other paracrine factors seems complex in microenvironment that affects on EPCs properties and remains to be more extensively investigated in further studies.

In conclusion, the present study provides evidence that CCN1, a novel extracellular matrix-associated angiogenic factor, is induced by serum and VEGF in both ECs and EPCs, and mediates the milieu-dependent activities of EPCs, such as proliferation, migration, and tube formation. In addition, CCN1 up-regulates VEGF and MCP-1 levels in both ECs and EPCs, suggesting a paracrine loop mediated by cytokines and chemokines induced by CCN1 may amplify the proangiogenic effects of CCN1. These findings demonstrate an important role of CCN1 in neovascularization and re-endothelialization by mediating EPCs proliferation, recruitment, and angiogenesis. Thus, future studies are warranted to elucidate the complex mechanism and therapeutic potential of CCN1 in the angiogenesis and tissue regeneration process mediated by EPCs.

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2008.08.001.

REFERENCES


