Endothelial cell-derived connective tissue growth factor stimulates fibroblast differentiation into myofibroblasts through integrin $\alpha_V\beta_3$

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Abstract. Connective tissue growth factor (CTGF) is expressed at high levels in blood vessels, where it functions as a regulator of a number of physiological processes, such as cell proliferation, angiogenesis and wound healing. In addition, CTGF has been reported to be involved in various pathological processes, such as tumor development and tissue fibrosis. However, one of the main roles of CTGF is to promote the differentiation of fibroblasts into myofibroblasts, a process that is involved in disease progression. Therefore, the present study aimed to investigate the possible mechanism by which pathological changes in the microvasculature can direct the activation of fibroblasts into myofibroblasts in the context of hypoxia/reoxygenation (H/R). Human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts were used in the present study. The expression levels of CTGF were determined by western blot analysis and reverse transcription-semi-quantitative PCR. To analyze the paracrine effect of HUVECs on fibroblasts, HUVECs were infected with CTGF-expressing adenovirus and then the culture supernatant of HUVECs was collected to treat fibroblasts. The formation of α-smooth muscle actin (α-SMA) stress fibers in fibroblasts were observed by immunofluorescence staining. It was found that H/R significantly increased CTGF expression in HUVECs. CTGF was also able to directly induce the differentiation of fibroblasts into myofibroblasts. In addition, the culture supernatant from CTGF-overexpressing HUVECs stimulated the formation of α-SMA stress fibers in fibroblasts, which was inhibited by treatment with a functional blocking antibody against integrin $\alpha_V\beta_3$ and to a lesser degree by a blocking antibody against α_6 integrin. The mechanism of CTGF upregulation by H/R in HUVECs was then evaluated, where it was found that the CTGF protein was more stable in the H/R group compared with that in the normoxic control group. These findings suggest that CTGF expressed and secreted by vascular endothelial cells under ischemia/reperfusion conditions can exert a paracrine influence on neighboring fibroblasts, which may in turn promote myofibroblast-associated diseases. This association may hold potential as a therapeutic target.

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Introduction

Fibroblasts are tissue-resident stromal cells that are important for maintaining the structural integrity of tissues (1). They function to synthesize and integrate structural proteins, such as collagen and elastin, into the extracellular matrix (ECM) of connective tissues (1). In environments where this homeostasis is disturbed, such as during wound healing, chronic inflammation and cancer, fibroblasts are activated to proliferate and upregulate ECM production (2,3). Activated fibroblasts acquire various smooth muscle features, including enhanced formation of contractile stress fibers and expression of α-smooth muscle actin (α-SMA) (2,3). Cells with these characteristics are known as myofibroblasts (2,3). Although transient acquisition of this myofibroblast phenotype confers beneficial effects on normal tissue repair processes, persistence of myofibroblasts is associated with the development of diseases mediated by tissue stiffening and deformation (4). Stiff scar tissues adversely alters normal organ function (4). In addition, fibrosis is characterized by the abnormally excessive accumulation of ECM proteins, which contributes to organ failure in various chronic diseases affecting the liver, kidney, skin, lungs and the heart (5). By contrast, activated fibroblasts, especially cancer-associated fibroblasts (CAFs) in the tumor stroma, serve an important role in tumorigenesis by stimulating angiogenesis, cancer cell proliferation and invasion (6). Activated CAFs can also produce a variety of growth factors and proinflammatory cytokines, such as TGF-β, vascular endothelial growth factor, IL-6 and CXC-chemokine ligand 12, to promote tumor progression (6-8). CAFs have been reported to contribute to ECM remodeling and cancer cell invasion by secreting connective tissue growth factor (CTGF), collagen, fibronectin and elastin (9), implicating CAFs to be targets for anti-cancer therapy (6-8).

CTGF, also known as cellular communication network factor 2, is a regulatory protein that has been demonstrated to be involved in several biological processes, such as cell

proliferation, angiogenesis and wound healing (10). In addition, CTGF has been associated with a number of pathological processes, such as tumor development, cardiovascular diseases, inflammatory diseases and tissue fibrosis in major organs (10). CTGF was first discovered as a protein secreted by endothelial cells during angiogenesis under normal conditions (11). CTGF expression is generally higher in blood vessels compared with that in other organs or tissues (11). CTGF mRNA is expressed at particularly high levels in developing blood vessels and in the large blood vessels of the adult heart, suggesting that CTGF is involved in the development and maintenance of blood vessels (12). However, one of the main roles of CTGF is considered to be the promotion of myofibroblast differentiation and angiogenesis (13-15). CTGF is typically secreted into the extracellular environment, where it interacts with cell surface receptors, growth factors and the ECM (13-15). Subsequently, CTGF mediates downstream effects by binding to heterodimeric cell-surface integrin complexes, such as α_6, β_1, α_V and β_3 integrins (13-15).

The present study aimed to investigate whether CTGF from endothelial cells after hypoxia/reoxygenation (H/R) could stimulate the differentiation of neighboring fibroblasts into myofibroblasts in a paracrine manner.

Materials and methods

Cell culture conditions. HUVECs (passages 4-10; Lonza Group, Ltd.) were cultured in EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza Group, Ltd.) containing all the included supplements at 37˚C in a humidified atmosphere containing 5% CO_2. Normal human dermal fibroblasts (PromoCell GmbH) were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2. For the H/R conditions, the cells were first incubated at 37˚C for 16 h in a hypoxic incubator (Thermo Scientific 3131 Forma Incubator; Thermo Fisher Scientific, Inc.) filled with 1% O_2 and 5% CO_2, balanced with N_2, before being placed under normoxic conditions at 37˚C for 2 h for reoxygenation treatment. For the preparation of conditioned media (CM), the medium of HUVECs was changed with EBM™-2 Basal Medium (Lonza Group, Ltd.) containing 1% FBS at 37˚C for 18 h before it was collected, and filtered using a Minisart® Syringe Filter (0.25 μm; Sartorius AG).

RNA extraction and semi-quantitative reverse transcription-PCR (sqRT-PCR). Total RNA was isolated from HUVECs using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.). To remove genomic DNA, total RNA (4 μg) was treated with RQ1 RNase-Free DNase (cat. no. M6101; Promega Corporation) prior to sqRT-qPCR, according to the manufacturer's protocols. A PrimeScript™ First Strand cDNA Synthesis Kit (Takara Bio, Inc.) was used to synthesize cDNA from 2 μg total RNA in a total volume of 20 μl, according to the manufacturer's protocols. PCR was performed on 1 μl cDNA in a total volume of 25 μl using TaKaRa Ex Taq® DNA Polymerase (cat. no. RR01AM; Takara Bio, Inc.) according to the manufacturer's instructions. The following primer pair was used for human CTGF: Forward, 5'-GCATCCGTACTCCCAAAATCTC-3' and reverse, 5'-ATGTCTCTCACTCTCT GGCTTC-3' (melting temperature: 55˚C; 27 cycles at 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec). GAPDH was used for normalization: Forward, 5'-CGTGGAAGGACTCATGAC-3' and reverse, 5'-CAAATTCGTTGTCATACCAG-3' (melting temperature: 55˚C; 27 cycles at 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec). The PCR products were mixed with Ezstain DNA loading dye (cat. no. B006M; Enzynomics, Inc.) and analyzed using a 1.2% agarose gel. Semiquantification of band intensity was performed using ImageJ software (V1.8.0; National Institutes of Health) and normalized to the intensity of GAPDH.

Vector construction. The coding region of human CTGF (NM_001901) was PCR-amplified from HUVEC cDNA (1 μl) using TaKaRa Ex Taq® DNA Polymerase (cat. no. RR01AM; Takara Bio, Inc.) according to the manufacturer's instructions, cloned into the pGEM®-T Easy Vector Systems (cat. no. A1360; Promega Corporation), before being subcloned into the pShuttle-CMV vector in the AdEasy adenoviral vector system (cat. no. 240009; Agilent Technologies, Inc.) to produce CTGF-expressing adenovirus. The primers are as follows: cdsCTGF forward, 5'-GAGTCGACAGTGCCAACCATGACCGC-3' (nucleotides plus *Sal*I adapter) and cdsCTGF reverse, 5'-GACTCGAGCTGGCTTCATGCCATGTC-3' (nucleotides plus *Xho*I adapter). PCR was performed for 27 cycles at 94˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min. All PCR-amplified fragments and cloning junctions were verified by DNA sequencing performed by SolGent Co., Ltd. Adenoviral CTGF cloning was performed according to the manufacturer's protocols. Production and harvesting of adenoviruses were performed as described (16,17). The pShuttle-CMV vector containing CTGF (1 μg) was cotransformed with pAdEasy-1 vector (100 ng) into BJ5183 competent cells (20 μl; supplied in the in the AdEasy adenoviral vector system), where homologous recombination occurred. The recombinant adenoviral vector expressing human CTGF was transfected into 293A cells to obtain viral particles. 293A cells (cat. no. R70507; Thermo Fisher Scientific, Inc.) were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C. 293A cells ($5x10^6$ cells/100 mm dish) were plated 24 h before transfection. Subsequently, 10 μg recombinant adenoviral vector DNA was used for transfection with MetaFectene PRO (cat. no. T040-1.0; Biontex Laboratories GmbH) according to the manufacturer's instruction. Transfected cells were incubated at 37˚C for 7-10 days, the adenovirus-producing 293A cells were collected and the virus particles were purified. The infection into HUVECs was performed as previously described (18). The harvested adenoviruses (25 MOI) were added to cells in endothelial basal medium (Lonza Group, Ltd.) containing 1% FBS at 37˚C for 4 h, then virus-containing medium was removed and growth medium was added. After 24 h, medium was removed and the cells were washed one time with endothelial basal medium, and then incubated in endothelial basal media containing 1% FBS at 37˚C for 18 h before collecting CM.

Control HUVECs were infected with a control adenovirus generated with control shuttle vector (pShuttle-CMV-*lac*Z) and pAdEasy-1 vector.

Reagents. Recombinant human CTGF (rhCTGF; cat. no. 30R-2206; 0.5 or 1 μg/ml) was purchased from Fitzgerald Industries International, Inc. (19,20), and used to treat fibroblasts at 37˚C for 3 days. Function-blocking Anti-Integrin $\alpha_V\beta_3$ Antibody (clone LM609; cat. no. MAB1976; 10 μg/ml) and Anti-Integrin α_6 Antibody (clone NKI-GoH3; cat. no. MAB1378; 10 μg/ml) were purchased from Sigma-Aldrich; Merck KGaA (21,22). Normal mouse IgG (cat. no. NI03; 10 μg/ml) was purchased from Sigma-Aldrich; Merck KGaA. Functional-blocking antibodies were used to treat fibroblasts at 37˚C for 3 days. To analyze the half-life or synthesis rate of the CTGF protein in HUVECs, the proteasome inhibitor MG132 (cat. no. M8699; 10 μM) or the translational blocker cycloheximide (CHX; cat. no. C0934; 10 μg/ml) was used (both Sigma-Aldrich; Merck KGaA) to treat cells at 37˚C for the indicated time (2 h for MG132; 30, 60 or 120 min for CHX).

Western blotting. HUVECs were harvested and lysed using RIPA buffer (cat. no. 9806; Cell Signaling Technology, Inc.) containing Xpert Protease Inhibitor Cocktail Solution (cat. no. P3100; GenDEPOT) and Xpert Phosphatase Inhibitor Cocktail Solution (cat. no. P3200; GenDEPOT). Total protein content was determined by Pierce™ BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total protein (20-30 μg) was separated by SDS-PAGE on 12% gels and transferred onto a nitrocellulose blotting membrane (pore size, 0.45 μm; cat. no. 10600003; Amersham; Cytiva). After transfer, the membrane was incubated with 5% skimmed milk (cat. no. 232100; BD Biosciences) in PBS containing 0.1% Tween-20 for 1 h at room temperature. The membranes were then immunoblotted with a specific antibody against CTGF (1:500; cat. no. sc-14939; Santa Cruz Biotechnology, Inc.) for 16 h at 4˚C. β-actin (1:2,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.), which was used as an internal control, was incubated with the membranes for 16 h at 4˚C. For the incubation with secondary antibodies, HRP-conjugated goat anti-mouse antibody (1:5,000; cat. no. 1031-05; SouthernBiotech) or HRP-conjugated rabbit anti-goat antibody (1:5,000; cat. no. 6160-05; SouthernBiotech) was applied for 1 h at room temperature. Chemiluminescence signals were obtained with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (cat. no. 34580; Thermo Fisher Scientific, Inc.) and chemiluminescence intensity was measured using the ImageQuant™ LAS 4000 apparatus (Cytiva). The quantification of band intensity was performed using ImageJ software.

Immunofluorescence staining. Fibroblasts were grown on cover glasses ($2x10^4$ cells/well in a 12-well plate) in a monolayer and treated with either CM or rhCTGF (0.5 or 1 μg/ml) at 37˚C for 3 days. The cells were then fixed with 4% paraformaldehyde for 10 min at 4˚C, before being incubated with blocking buffer comprising of 2% bovine serum albumin (cat. no. 0332; Amresco, LLC) in 1X PBS for 1 h at room temperature. The cells were labeled with anti-α-SMA antibody (1:800; cat. no. ab7817; Abcam) overnight at 4˚C, followed by Alexa Fluor™ 488-conjugated Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody (1:200; cat. no. A-11001; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The nuclei were stained with DAPI (1:5,000; cat. no. D1306; Molecular Probes; Thermo Fisher Scientific, Inc.) for 1 h at room temperature and mounted using a fluorescent mounting medium (cat. no. S3023; DAKO; Agilent Technologies, Inc.). Fluorescent images were obtained using a Zeiss LSM 700 laser scanning confocal microscope (Carl Zeiss AG; magnifications, x100, x200 and x400). The quantification of SMA^+ area (n=5-6 fields/group) was performed using ImageJ software.

Statistical analysis. All data are expressed as the mean ± standard error of the mean from three or four independent experiments. All of the significance analysis was performed using the SigmaPlot version 14.0 software (SPSS, Inc.). The statistical differences were compared using one-way ANOVA followed by the Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CTGF treatment causes the differentiation of fibroblasts into myofibroblasts. It was reported in our previous study that endothelial cells undergo endothelial-to-mesenchymal transition (EndMT) when subjected to ischemia/reperfusion, influencing neighboring fibroblasts to actively participate in cardiac fibrosis (23). Although it was found in this previous study that CTGF from EndMT cells has a paracrine influence on fibroblast activation (23), the direct effects of CTGF on the activation of fibroblasts into myofibroblasts or its mechanism were not examined. Therefore, in the present study, the effects of hypoxia/reoxygenation (H/R) on CTGF expression in HUVECs was first assessed (Fig. 1A). It was found that H/R significantly increased the expression of the CTGF protein (Fig. 1A). Culture supernatants from both normoxic and H/R HUVECs were then obtained and were used to treat fibroblasts (Fig. 1B). The immunofluorescence of α-SMA stress fibers was examined, which indicates the generation of myofibroblasts (2). Fibroblasts treated with CM from normoxic HUVECs (N CM) showed punctate or patchy α-SMA immunofluorescence. By contrast, the treatment of fibroblasts with CM from H/R HUVEC (H/R CM) led to the formation of more intense and fibrous α-SMA immunofluorescence, typical of stress fibers (Fig. 1B). The effect of soluble CTGF on fibroblast differentiation was then examined using rhCTGF (Fig. 2). Fibroblasts were treated with 0.5 or 1 μg/ml rhCTGF, before α-SMA immunofluorescence was observed. Treatment with rhCTGF dose-dependently stimulated the formation of α-SMA stress fibers in fibroblasts, indicating that rhCTGF directly induced the differentiation of fibroblasts into myofibroblasts.

Function-blocking antibody against integrin $\alpha_V\beta_3$ abolishes the effect of CM from CTGF-overexpressing HUVECs on α-SMA fiber formation. To investigate how CTGF from endothelial cells affects neighboring fibroblasts, function-blocking antibodies against integrin α_6 and $\alpha_V\beta_3$ were used alongnside CM from CTGF-overexpressing HUVECs (Fig. 3). CTGF mediates downstream effects by binding to integrins, such

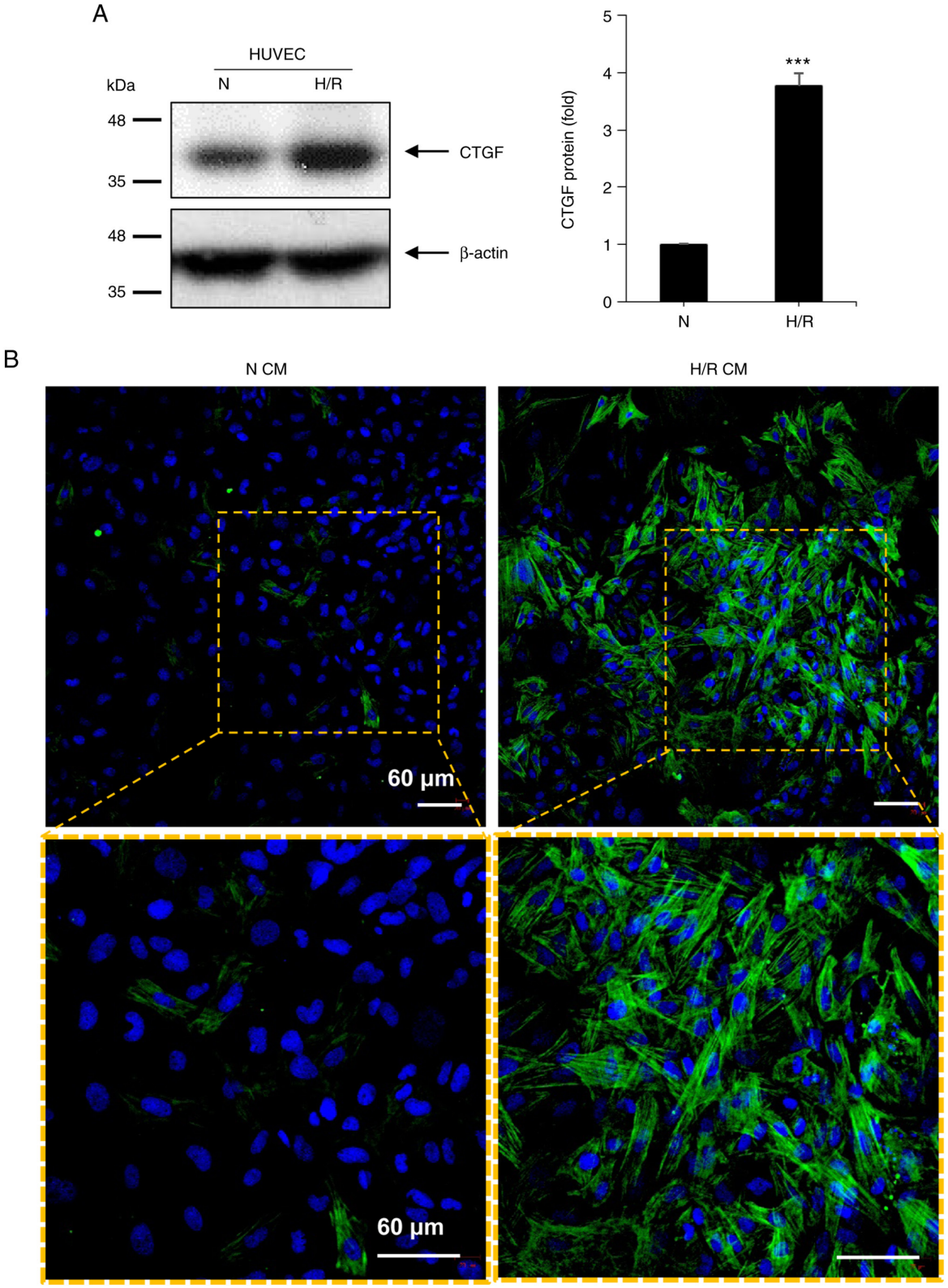

Figure 1. CM from HUVECs after H/R activates fibroblasts into myofibroblasts. (A) Western blot analysis indicated that CTGF protein expression was increased in HUVECs by H/R (left), which was semi-quantified (n=4). ***P<0.001 vs. N. (B) CM from H/R HUVECs stimulated the formation of α-smooth muscle actin filaments in fibroblasts (green). Nuclei were stained with DAPI (blue). Magnification, x100. CM, conditioned media; H/R, hypoxia/reoxygenation; N, normoxia; CTGF, connective tissue growth factor.

as α_6, β_1, α_V and β_3 (15). HUVECs were first infected with a control adenovirus (Ad-mock) or an adenovirus expressing human CTGF (Ad-CTGF), before CTGF overexpression was confirmed using western blot analysis (Fig. 3A). HUVEC CM was then collected and used to treat fibroblasts and immunofluorescence staining for α-SMA was performed (Fig. 3B). CM from CTGF-overexpressing HUVECs potently stimulated α-SMA stress fiber formation, which was significantly inhibited

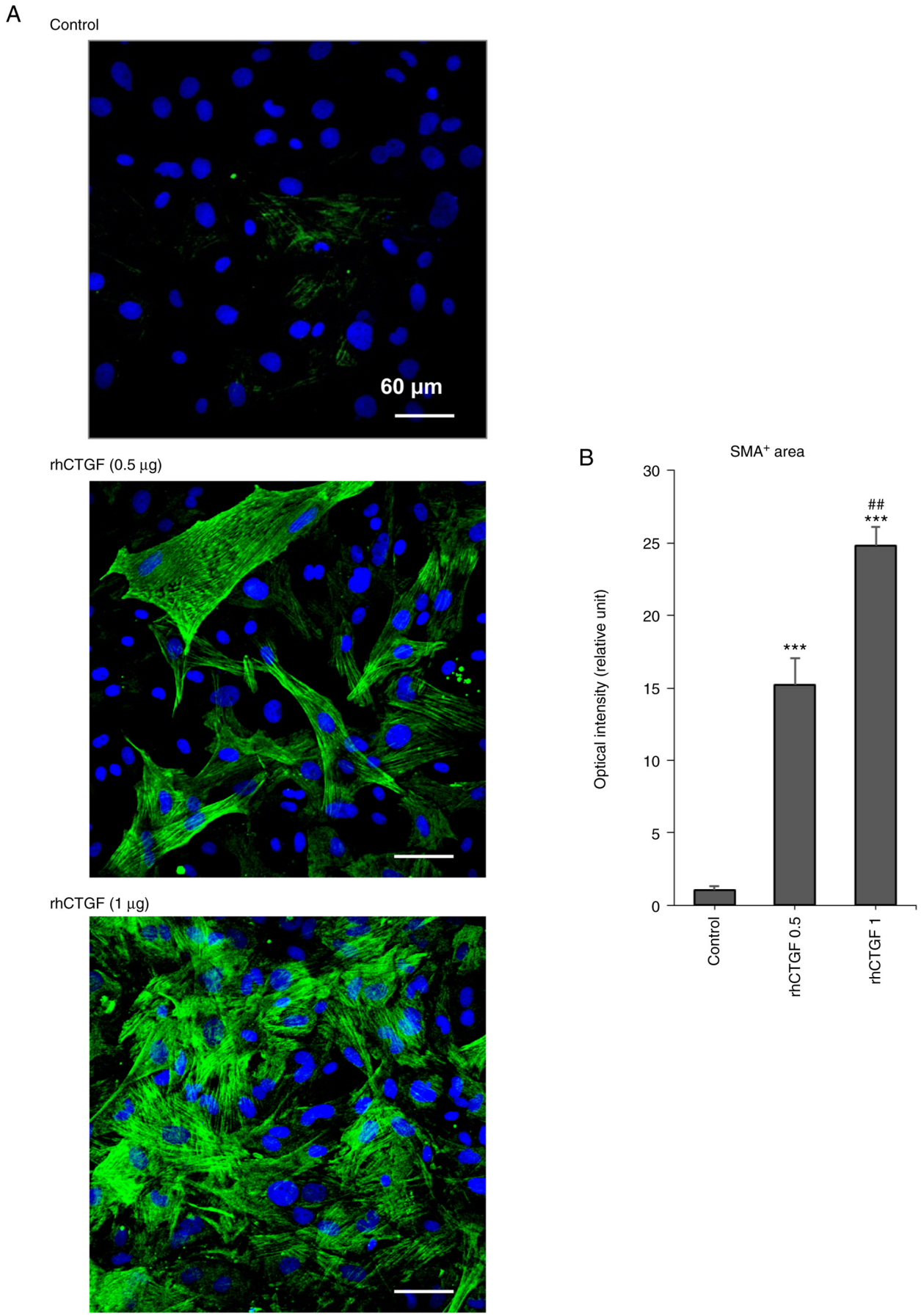

Figure 2. Treatment with rhCTGF stimulates the formation of α-SMA stress fibers in fibroblasts. (A) Fibroblasts were treated with rhCTGF (0.5 or 1 μg/ml) prior to the fluorescence staining of α-SMA was performed (green). Nuclei were stained with DAPI (blue). Magnification, x200. (B) Quantification of the SMA$^+$ area (n=5-6 per group). ***P<0.001 vs. control. $^{\#\#}$P<0.01 vs. rhCTGF 0.5. rhCTGF, recombinant human connective tissue growth factor; SMA, smooth muscle actin.

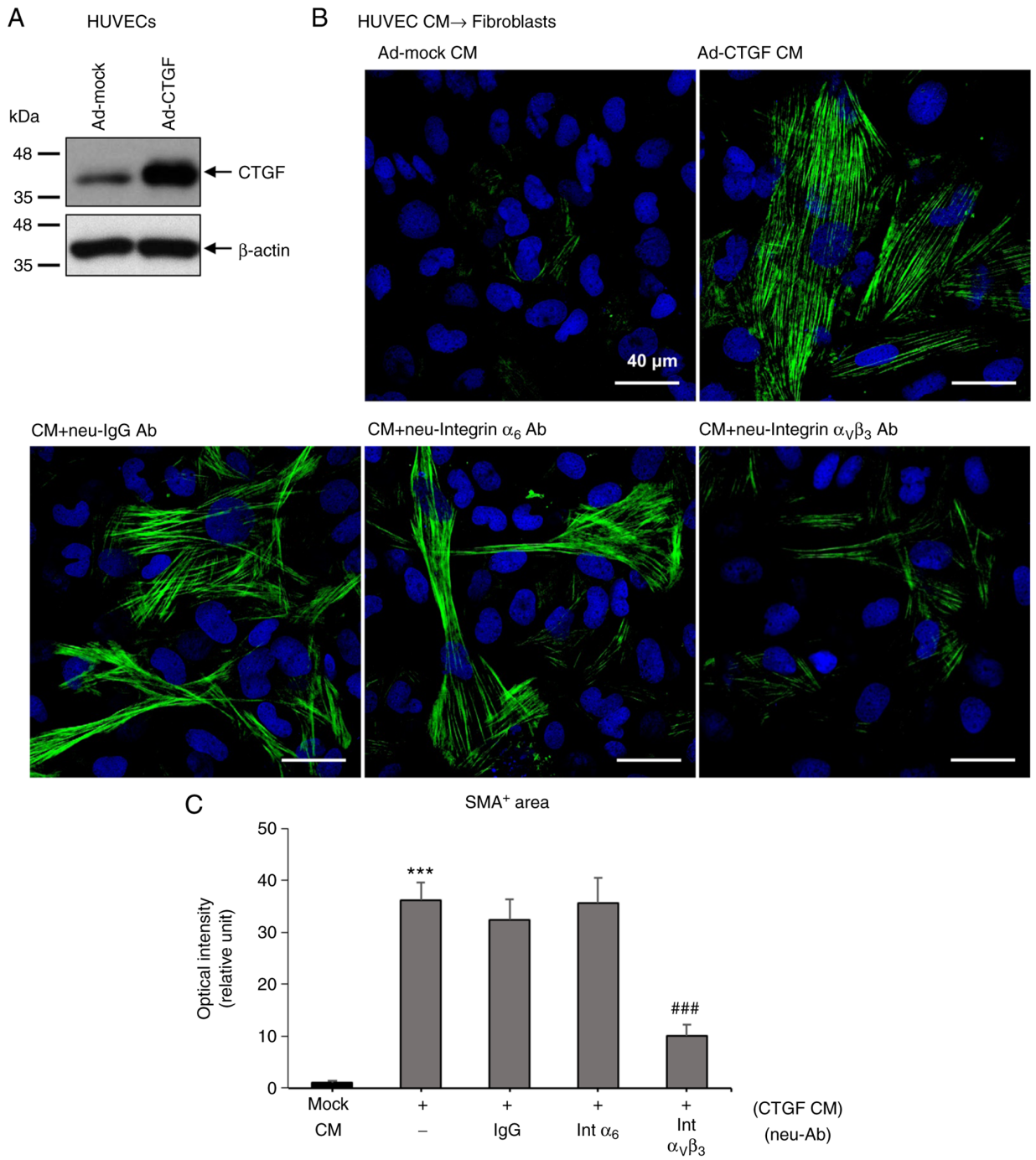

Figure 3. CM from CTGF-overexpressing HUVECs stimulates α-SMA stress fiber formation through $\alpha_V\beta_3$-integrin in fibroblasts. (A) CTGF protein expression was increased in HUVECs infected with Ad-CTGF. CTGF overexpression was confirmed using western blot analysis. Ad-mock HUVECs were infected with a control Ad containing a control shuttle vector (pShuttle-CMV-*lac*Z). (B) Function-blocking Int $\alpha_V\beta_3$ diminished the effects of CTGF CM on α-SMA fiber formation (green) in fibroblasts. Intα_6, Int $\alpha_V\beta_3$ or purified mouse IgG (each 10 μg/ml) was added to the CM directly. Nuclei were stained with DAPI (blue). Magnification, x400. (C) Quantification of the α-SMA$^+$ area (n=5-6 per group). ***P<0.001 vs. mock CM. $^{###}$P<0.001 vs. CTGF CM only. CM, conditioned medium; Ad, adenovirus; CTGF, connective tissue growth factor; IgG, immunoglobulin G; neu, neutralizing; Ab, antibody; Intα_6, anti-integrin α_6 antibody; Int $\alpha_V\beta_3$, anti-integrin $\alpha_V\beta_3$ antibody; SMA, smooth muscle actin.

by a function-blocking antibody against integrin $\alpha_V\beta_3$ (Fig. 3B). The function-blocking antibody against integrin α_6 could not block stress fiber formation (Fig. 3B and C), suggesting that CTGF from endothelial cells stimulates the differentiation of fibroblasts to myofibroblasts through integrin $\alpha_V\beta_3$.

CTGF protein stability is increased under H/R in HUVECs. Subsequently, the mechanism underlying CTGF upregulation by H/R in HUVECs was evaluated (Fig. 4). The CTGF mRNA level was first examined to test whether H/R could affect the transcription of CTGF. However, CTGF mRNA was not changed under H/R conditions (Fig. 4A), although CTGF protein expression was markedly increased by H/R (Fig. 4B). This suggests that increased CTGF protein expression was not due to any changes in CTGF mRNA levels under H/R conditions. The protein synthesis of CTGF by H/R was therefore

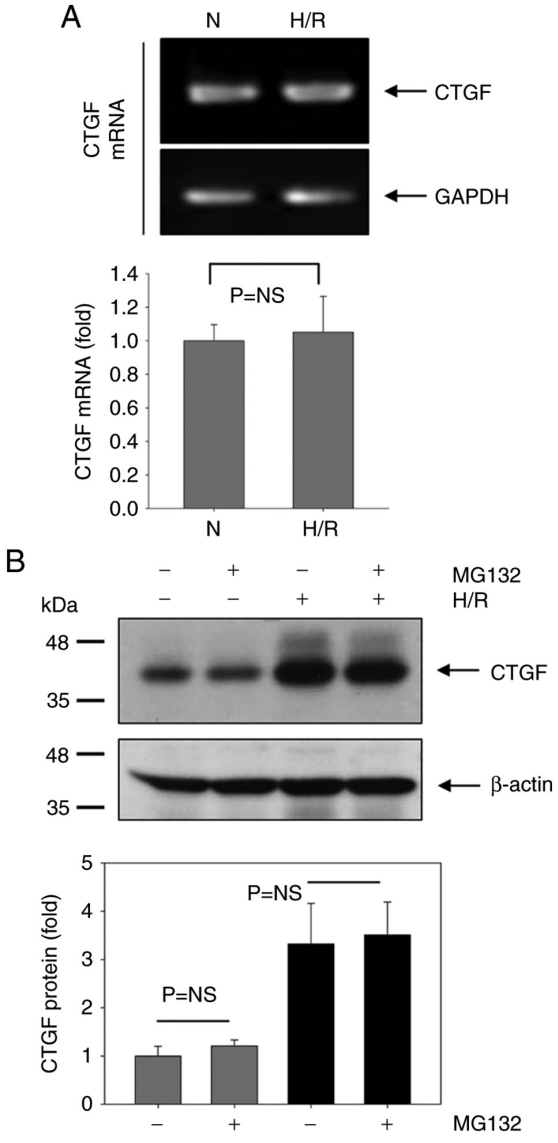

Figure 4. CTGF synthesis under normoxic and H/R conditions. (A) Reverse transcription-semi-quantitative PCR of CTGF mRNA (upper panel). The corresponding semi-quantification for CTGF mRNA levels is presented in the lower panel (n=3). (B) HUVECs were treated with the proteasome inhibitor MG132 (10 μM) and/or H/R. Western blot analysis for CTGF is shown in the upper panel whereas the corresponding quantification for CTGF protein levels is in the lower panel (n=3 each). H/R, hypoxia/reoxygenation; N, normoxia; CTGF, connective tissue growth factor; NS, not significant.

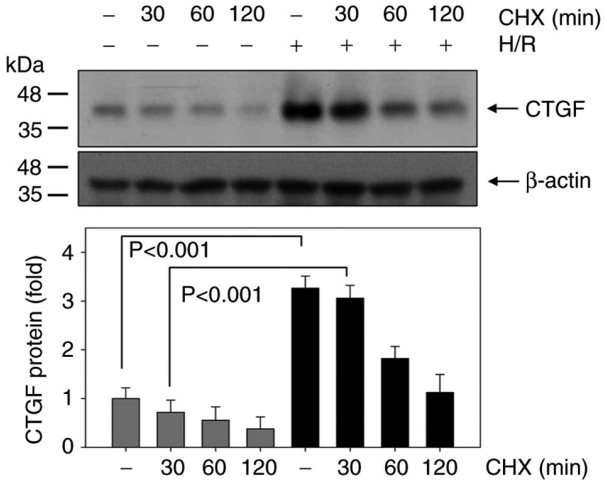

Figure 5. CTGF protein stability is increased by H/R. HUVECs were treated with normoxia or H/R, followed by treatment with CHX (10 μg/ml). Western blotting for CTGF is shown in the upper panel whereas and the corresponding semi-quantitation for CTGF protein expression is shown in the lower (n=4). CTGF, connective tissue growth factor; CHX, cycloheximide; H/R, hypoxia/reoxygenation.

tested after blocking protein degradation with the proteasome inhibitor MG132 (Fig. 4B). No significant differences in the protein synthesis of CTGF was detected between the normoxia and H/R groups with the presence or absence of MG132. Changes in CTGF stability following H/R was then examined using CHX, a protein translation blocker (Fig. 5). Notably, CTGF stability was significantly higher in the H/R group compared with that in the normoxic control group at 30 and 60 min (Fig. 5).

Discussion

The tumor microenvironment can contain fibroblasts, immune cells, blood vessels and the ECM (8). Fibroblasts are typically quiescent but can be activated into myofibroblasts during the wound-healing response (6). In addition, CAFs can directly regulate cancer cell proliferation, tumor immunity, angiogenesis, ECM remodeling and metastasis, suggesting that they can be a target for anti-cancer therapy (8). Several preclinical studies have reported CAFs to be possible targets for anti-cancer therapy in lymphoma, Lewis lung cancer, melanoma and gastrointestinal cancer (6-8). α-SMA is a marker that can be used to reflect the myofibroblast population of CAFs, such that docetaxel-conjugate nanoparticles have been shown to target α-SMA$^+$ stromal-suppressed metastases in a mouse model of breast cancer (24). Furthermore, selective depletion of myofibroblasts has been documented to attenuate angiogenesis in a pancreatic ductal adenocarcinoma mouse model. However, depletion of α-SMA$^+$ myofibroblasts in mouse pancreatic cancer can also increase the population of immunosuppressive CD4$^+$Foxp3$^+$ Tregs, leading to more invasive tumors (25). In the present study, CM from HUVECs under H/R conditions, in addition to that from CTGF-overexpressing HUVECs, was found to activate fibroblasts into α-SMA$^+$ myofibroblasts. This suggests that blood vessels can promote neighboring fibroblasts into differentiating into myofibroblasts. A clinical trial of bevacizumab targeting endothelial cell precursors with CAFs has previously been conducted; the addition of bevacizumab to the standard of care significantly improved overall survival in malignant pleural mesothelioma (26). Although targeting α-SMA$^+$ myofibroblasts therapeutically remains to be a controversial topic (24,25), targeting only CAFs, CAFs with endothelial cells or other types of cells is a promising strategy (26). However, future studies are required to define this strategy more precisely.

CTGF is mainly secreted from endothelial cells and can modulate complex biological processes during normal embryonic development and tissue repair (10). Abnormal CTGF expression profiles have been observed in several diseases, including tissue fibrosis (of the lung, heart and liver), systemic sclerosis and tumors in major organs (23,27). It has been previously reported that >30 types of human cancers are associated

with the dysregulated aberrant expression of CTGF (27). Higher CTGF expression is associated with more aggressive inflammatory colorectal cancer, whilst CTGF expression has also been found to be increased in breast cancer, chondrosarcoma and glioma (10,27). By contrast, CTGF can also function as a tumor suppressor. CTGF expression has been observed to be reduced in non-small cell lung cancer cells, where decreased CTGF expression may be involved in lung tumorigenesis (10,28). In the present study, CTGF from HUVECs stimulated fibroblast differentiation, suggesting a possible association of HUVECs with CAF generation. Further investigations on the functional roles of CTGF-expressing endothelial cells and myofibroblasts in tumors or ischemic diseases are required.

CTGF can bind to several types of receptors, such as integrins, heparan sulfate proteoglycans, lipoprotein receptor-related proteins and tyrosine kinase receptors (29). However, integrins are known to be the principal CTGF receptors (29). CTGF mediates downstream effects through α_6, β_1, α_V and β_3 integrins (15). Physiologically, CTGF enhances the lactogenic differentiation of mammary epithelial cells by binding to integrins α_6 and β_1, and to a lesser degree β_3 integrin (15). In addition, CTGF can activate β_1 integrin signaling in primary skin fibroblasts (30) and pancreatic stellate cells through $\alpha_V\beta_3$ (13,31). In the present study, CM from CTGF-overexpressing endothelial cells stimulated fibroblast differentiation, which was inhibited by a function-blocking antibody against integrin $\alpha_V\beta_3$, but not by a function-blocking antibody against integrin α_6.

The present study has a number of limitations. Although it was demonstrated that H/R increased CTGF expression in HUVECs and had a direct effect on the differentiation of fibroblasts to myofibroblasts through integrin $\alpha_V\beta_3$, the mechanism of integrin-mediated fibroblast differentiation by H/R endothelial CM was not explored. Integrin-linked kinase (ILK) is a key mediator of integrin signaling that interacts with the cytoplasmic domain of β-integrins (32). Therefore, ILK may be a downstream candidate in this case. It has previously been reported that integrin $\alpha_V\beta_3$ is involved in the stress fiber formation through signaling molecules, such as focal adhesion kinase (FAK), PKCα and RhoA (33,34). In human aortic smooth muscle cells, osteoprotegerin, a ligand for integrin $\alpha_V\beta_3$, mediated the phosphorylation of FAK and actin cytoskeleton reorganization (34). Integrin $\alpha_V\beta_3$ triggers the formation of focal adhesions and stress fibers through the activation of the transforming protein RhoA in astrocytes (33). Another limitation of the present study is that the binding of CTGF onto integrin $\alpha_V\beta_3$ on fibroblasts was not confirmed. CTGF-overexpressing HUVEC CM facilitated the differentiation of fibroblasts into myofibroblasts, which was inhibited by a functional blocking antibody against integrin $\alpha_V\beta_3$. Therefore, it is highly likely that CTGF will bind to $\alpha_V\beta_3$. However, more specific assays, such as a CTGF adhesion assay (15), are required to investigate the possible interaction between CTGF and integrin $\alpha_V\beta_3$. The previous report by Morrison *et al* (15) demonstrated the interaction between CTGF and integrin complexes using an adhesion assay. HC11 epithelial cells adhered onto CTGF-coated wells, where function-blocking antibodies against both α_6 and β_1 interrupted this CTGF-mediated epithelial cell adhesion (15). Further studies are required to determine the underlying mechanism(s) of CTGF/integrin $\alpha_V\beta_3$-mediated fibroblast differentiation, especially the stress fiber formation in fibroblasts.

Furthermore, another limitation of the present study is that the proteasomal degradation and stability of CTGF was analyzed using cellular homogenates. Since CTGF is a secreted protein, it is not sufficient to analyze proteasomal degradation and stability using cell lysates. Interestingly, whilst CTGF is a secreted protein, CTGF can also strongly bind to heparin and other matrix components, rendering it detectable in the supernatants or in cellular homogenates, depending on the cell type investigated (35,36). Although CTGF (38 kDa) was readily detected in cell lysates, it was not detectable in the conditioned medium (35). Upon stimulation of CTGF with serotonin, enhanced levels of CTGF protein were detected in the cellular homogenates, whereas no protein was detectable in cell culture supernatants (37). Therefore, the regulatory mechanisms associated with proteasomal degradation or stability of CTGF, especially secreted CTGF, under H/R and additional conditions require additional in-depth investigations.

To conclude, the supernatants of CTGF-overexpressing HUVECs stimulated fibroblast differentiation, which was significantly inhibited by a function-blocking antibody against integrin $\alpha_V\beta_3$. These findings suggest that communication between CTGF-secreting endothelial cells and neighboring fibroblasts can lead to the development of myofibroblast-associated diseases, which may be a potential therapeutic target for the treatment of cancer or ischemic diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL was responsible for designing the research, conducting the experiments, performing the data analysis and writing the manuscript. SL confirms the authenticity of all the raw data. SL has read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

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