Up-Regulation of Connective Tissue Growth Factor in Endothelial Cells by the Microtubule-Destabilizing Agent Combretastatin A-4

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Abstract

Incubation of microvascular endothelial cells with combretastatin A-4 phosphate (CA-4P), a microtubule-destabilizing compound that preferentially targets tumor vessels, altered cell morphology and induced scattering of Golgi stacks. Concomitantly, CA-4P up-regulated connective tissue growth factor (CTGF/CCN2), a pleiotropic factor with antiangiogenic properties. In contrast to the effects of other microtubule-targeting agents such as colchicine or nocodazole, up-regulation of CTGF was only detectable in sparse cells, which were not embedded in a cell monolayer. Furthermore, CA-4P induced CTGF expression in endothelial cells, forming tube-like structures on basement membrane gels. Up-regulation of CTGF by CA-4P was dependent on Rho kinase signaling and was increased when p42/44 mitogen-activated protein kinase was inhibited. Additionally, FoxO transcription factors were identified as potent regulators of CTGF expression in endothelial cells. Activation of FoxO transcription factors by inhibition of phosphatidylinositol 3-kinase/AKT signaling resulted in a synergistic increase in CA-4P-mediated CTGF induction. CA-4P-mediated expression of CTGF was thus potentiated by the inhibition of kinase pathways, which are targets of novel antineoplastic drugs. Up-regulation of CTGF by low concentrations of CA-4P may thus occur in newly formed tumor vessels and contribute to the microvessel destabilization and antiangiogenic effects of CA-4P observed *in vivo*. (Mol Cancer Res 2009;7(2):180–8)

Introduction

Combretastatin A-4 (CA-4) is a microtubule-depolymerizing agent with *in vivo* selectivity against the tumor vasculature (1). CA-4 phosphate (CA-4P), the prodrug of CA-4, is rapidly cleared from the blood and shows reversible binding kinetics to tubulin, leading to disruption of microtubular structures (2). Destabilization of microtubules and subsequent alterations of the actin cytoskeleton are the basis of morphologic alterations, which lead to enhanced permeability of CA-4P-treated endothelial cell monolayers. In contrast to other microtubule-disrupting agents such as nocodazole or colchicine (3), the effects of CA-4P seem to be specific for endothelial cells as found in neovessels, that is, characterized by irregular structure, poor connection to pericytes, and loose interconnections (2).

In the micromolar range, CA-4P induced membrane blebbing in postmitotic human umbilical vein endothelial cells (4), whereas much lower concentrations induced mitotic arrest and cell death in single growing endothelial cells (5). Changes in endothelial cell permeability were specifically observed in nonstabilized human umbilical vein endothelial cell monolayers reminiscent of instable tumor neovessels (6). At the molecular level, this was attributed to the disruption of endothelial cell-specific VE-cadherin junctions and to interference with the β-catenin/AKT signaling pathway (6). It is assumed that the early CA-4P-mediated alterations in endothelial cell morphology ultimately lead to cell death when the cells go through mitosis. Changes in cell morphology, however, may also directly affect gene expression (7). This aspect has not yet been investigated in relation to CA-4P and may affect proteins other than cell cycle regulators.

Connective tissue growth factor (CTGF/CCN2) is a multifunctional protein associated with fibrotic disorders, regulation of angiogenesis, and development of certain tumors. As a matricellular protein, it is secreted from the cells and then retained in the extracellular matrix. By binding to other growth factors, CTGF acts in a context-dependent manner, which may explain discrepant results in terms of its function. Overexpression of CTGF in tumor cells may facilitate angiogenic microcapillary formation in a paracrine manner (8). This proangiogenic effect of CTGF was recently confirmed in von Hippel-Lindau mutant renal carcinoma cells (9). By contrast, myc-mediated down-regulation of CTGF correlated with enhanced neovascularization in experimental tumors (10). Furthermore, reduced microvessel density was detected within human tumors overexpressing CTGF indicative of antiangiogenic properties of CTGF (11). It was also shown that angiogenesis was not impaired in CTGF knockout mice,

indicating that endogenous CTGF is not necessary for vessel formation (12). Vascular endothelial growth factor (VEGF), one of the most important angiogenic factors, was identified as interaction partner of CTGF (13). By forming a complex with VEGF-165, CTGF interfered with VEGF-induced angiogenesis *in vivo* and *in vitro* (13). When CTGF was cleaved by metalloproteinases, active VEGF was released, establishing CTGF as negative regulator of VEGF-mediated angiogenesis (14, 15). It is interesting to note that CTGF can be induced by VEGF (16), suggesting a negative feedback loop involved in the regulation of angiogenesis.

CTGF is coded for by an immediate-early response gene and, apart from VEGF (16), is rapidly induced in endothelial cells by soluble mediators such as transforming growth factor-β (17) or bioactive lipids (18). Furthermore, CTGF expression in endothelial cells is regulated by mechanical stimuli. CTGF is down-regulated in human umbilical vein endothelial cells exposed to laminar flow reminiscent of the situation in healthy vessels, where CTGF is not detectable in endothelial cells (19). On the contrary, the expression of CTGF is increased in human umbilical vein endothelial cells exposed to nonuniform shear stress (19, 20). Adaptation of endothelial cells to flow is accompanied by activation of integrins, small GTPases of the Rho family, and restructuring of focal adhesions and the actin cytoskeleton (21). Nonuniform shear stress-induced up-regulation of CTGF has been shown to involve activation of Rho proteins (22). In our previous study, G-actin was identified as negative regulator of CTGF expression downstream of RhoA activation, providing evidence for a direct link between alteration of the actin cytoskeleton and CTGF expression (23).

Disruption of microtubules results in activation of RhoA signaling via the microtubule-associated swap over factor GEF-H1 (24). In an earlier study in fibroblasts, we showed RhoA-dependent CTGF induction when the microtubules were disrupted by colchicine or nocodazole (25, 26). Therefore, we hypothesized that CA-4P by depolymerizing microtubules might regulate CTGF synthesis in endothelial cells. In the present study, we present evidence that CTGF is up-regulated specifically in subconfluent endothelial cells. Inhibition of
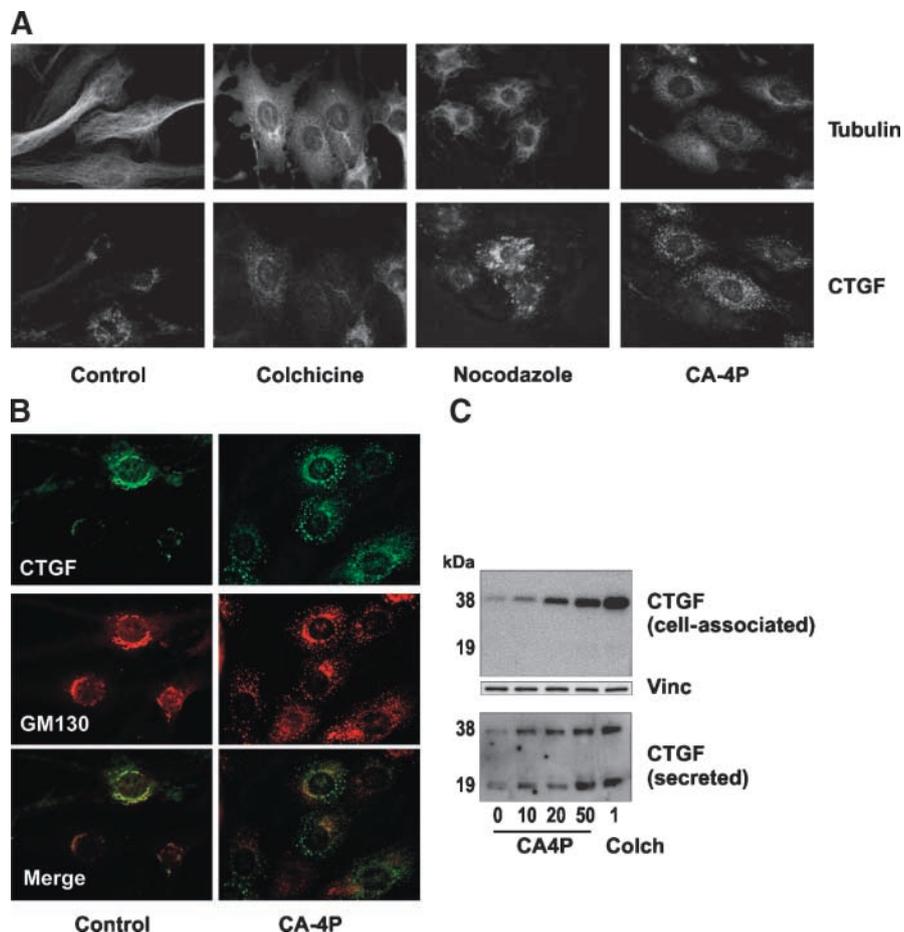

FIGURE 1. Localization and expression of CTGF in endothelial cells. **A.** Mouse microvascular endothelial cells (glEND.2) were treated with CA-4P (50 nmol/L), colchicine (1 μmol/L), or nocodazole (1 μmol/L) for 2 h. Tubulin and CTGF were detected by immunocytochemistry. Representative photographs: original magnification, 1:100. **B.** glEND.2 cells were treated with CA-4P (50 nmol/L) for 2 h. Cells were double-stained for CTGF and the Golgi marker GM130 (original magnification, 1:100). **C.** glEND.2 cells were treated with increasing concentrations of CA-4P (10-50 nmol/L) or colchicine (1 μmol/L) for 5 h. CTGF was detected in cellular homogenates or concentrated supernatants. Vinculin was used as a control for equal loading and blotting of cellular protein. Equal volumes of cell culture supernatants were concentrated by acetone precipitation. Representative of three independent experiments.

kinase signaling, particularly p42/44 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling, further increased CTGF expression, linking up-regulation of CTGF to different antitumor strategies.

Results

Cellular Localization of CTGF in CA-4P-Treated Endothelial Cells

The effects of CA-4P, the water-soluble form of CA-4, were analyzed in a mouse microvascular endothelial cell line (glEND.2), which has been used previously to characterize the relationship between alterations of the cytoskeleton and CTGF expression (23). Treatment with nanomolar concentrations of CA-4P led to a time- and concentration-dependent disruption of microtubular structures. As an example, cells treated with 50 nmol/L CA-4P for 2 h are shown in Fig. 1A and compared with cells exposed to nocodazole and colchicine at 1 μmol/L. The effect of CA-4P was reversible with restored formation of microtubules detectable after 24 h. Destabilization of microtubules induced dramatic changes in cell structure, which were still visible after 24 h, CA-4P-treated cells being round compared with more elongated control cells, although microtubules were reorganized (data not shown).

In control cells, low levels of CTGF immunoreactivity were detectable in a perinuclear localization. CTGF is a secreted protein and is localized to the Golgi in cells with ongoing biosynthesis (23) as confirmed by colocalization with the *cis*-Golgi marker GM130 (Fig. 1B; ref. 27). Concomitant with the disruption of the microtubules by CA-4P, CTGF appeared in a punctuate pattern distributed in the cytoplasm. The pattern was reminiscent of scattered Golgi ministacks, a known consequence of microtubule disruption. However, there was hardly any colocalization of GM130 and CTGF. The vesicular appearance of CTGF was thus most likely due to retention of CTGF in endoplasmic reticulum export sites as described for other secreted proteins (28). It was interesting to note that treatment with CA-4P resulted in redistribution of the Golgi apparatus in all cells, whereas CTGF expression was stimulated only in part of the cells, indicating more complex regulatory mechanisms as outlined below.

Increased expression of CTGF by CA-4P was quantified by Western blot analysis, providing evidence for a concentration-dependent up-regulation of cell-associated protein (Fig. 1C). Disruption of microtubules and reorganization of the Golgi did not impair CTGF secretion. Analysis of cellular supernatants showed that CTGF escaped the intracellular vesicular structures (Fig. 1C). In addition to the full-length protein, a faster migrating band of an apparent molecular weight of 19 kDa was detected in cell culture supernatants. CTGF is a modular protein that has been shown to be cleaved by metalloproteinases (15), resulting in a 19 kDa COOH-terminal fragment, which was recognized by the antibody used.

To further analyze the effect of CA-4P on CTGF expression in endothelial cells, glEND.2 cells were cultured on a basement membrane gel to allow formation of organized structures (Fig. 2). Incubation with 20 nmol/L CA-4P for 2 h barely altered cell morphology and did not disrupt the tubular structures. CTGF was not detectable in unstimulated cells but
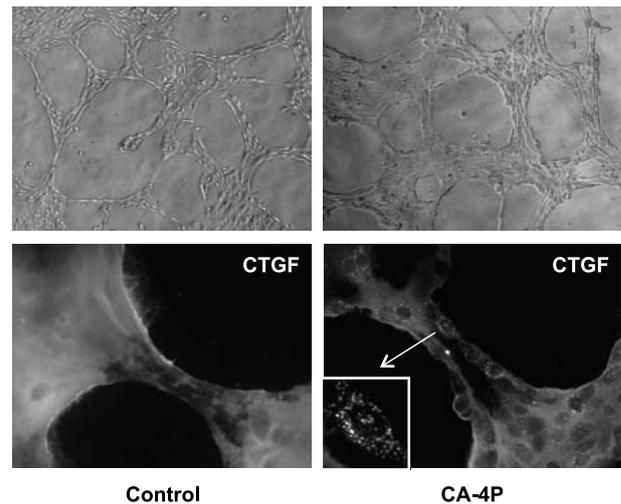

FIGURE 2. Up-regulation of CTGF in tubular structures. glEND.2 cells were cultured on basement membrane gels overnight and then stimulated with 20 nmol/L CA-4P for 2 h. Top, representative areas of tube-like structures (original magnification, 1:4); bottom, CTGF was detected by indirect immunofluorescence (original magnification, 1:40). Inset, magnification of a CTGF-expressing cell.

was up-regulated by CA-4P in all cells, appearing in a vesicular pattern (Fig. 2, *bottom*). CTGF was thus up-regulated in a model system where endothelial cells form structures in a soft matrix but are not stabilized by interaction with surrounding pericytes.

Cell Density-Dependent Regulation of CTGF by CA-4P

The molecular effects of CA-4P on endothelial cells have been shown to depend on cell density *in vitro* (5, 6). Therefore, we incubated microvascular endothelial cells seeded at different densities with CA-4P or colchicine (Fig. 3A). Colchicine at 1 μmol/L induced CTGF expression within 2 h independently of cell density. Comparable results were obtained with lower concentrations of colchicine or with nocodazole, which binds reversibly to tubulin (Supplementary Fig. S1A; data not shown). CA-4P, in contrast, showed a marked cell density-dependent effect. Strong up-regulation was observed in sparse endothelial cells, whereas CTGF was not induced in confluent cells (Fig. 3A). Even when cells are seeded at the same density, their growth behavior is variable. Therefore, up-regulation by CA-4P could not be quantified exactly. When cells were seeded at a density of 20,000 per cm^2, 50 nmol/L CA-4P up-regulated CTGF, on average, 2.5-fold within 2 h (2.6 ± 0.6, mean $\pm$ SD; $n = 7$) but, in some experiments, could reach 10-fold induction.

Induction was time- and concentration-dependent, 10 nmol/L CA-4P being sufficient to up-regulate CTGF within 5 h (Fig. 1C). In contrast to the cell density-dependent up-regulation of CTGF, reorganization of the Golgi apparatus was observed in all cells independently of the cell density (data not shown).

The difference between colchicine and CA-4P in terms of cell density-dependent up-regulation of CTGF was confirmed in another microvascular endothelial cell line obtained from

mouse myocardium. Colchicine up-regulated CTGF independently of cell density, whereas up-regulation by CA-4P was reduced at higher density (Fig. 3B; quantification in Supplementary Fig. S1B).

When the cells were preincubated with 1 μmol/L Taxol (paclitaxel) to stabilize microtubular structures, CTGF up-regulation was prevented (Fig. 3C), indicating that induction of CTGF by CA-4P was dependent on the destabilization of microtubules. Furthermore, microtubule disruption by CA-4P was linked to RhoA-Rho kinase signaling. Rho kinase activation leads to alterations of the actin cytoskeleton, which we have

FIGURE 3. Cell density-dependent expression of CTGF. **A.** glEND.2 cells were seeded at different densities that resulted in sparse (low density; 20,000 per cm^2), medium (40,000 per cm^2), or dense (high density; 80,000 per cm^2) cell layers. Cells were treated with CA-4P (50 nmol/L) or colchicine (1 μmol/L) for 2 h. CTGF expression was detected in cellular homogenates. **B.** MyEND cells were seeded at low and medium cell densities and stimulated with CA-4P (50 and 25 nmol/L) or colchicine (1 and 0.5 μmol/L) for 2 h. CTGF was detected by Western blotting. **C.** glEND.2 cells were seeded at low cell density (20,000 per cm^2) and preincubated with Taxol (10 μmol/L) for 30 min before stimulation with CA-4P (50 nmol/L) for 2 h. **D.** glEND.2 cells (low cell density; 20,000 per cm^2) were pretreated with the Rho kinase inhibitor Y27632 (10 μmol/L) for 30 min and then stimulated with CA-4P (50 nmol/L) for 5 h or colchicine (1 μmol/L) for 2 h. To compare different experiments, CTGF expression of stimulated cells was set to 100%. Mean ± SD of $n = 3$ (CA-4P) and $n = 4$ (colchicine) experiments. $P < 0.05$, ANOVA with Tukey-Kramer multiple comparison test.

shown earlier to induce CTGF expression (23, 25). Here, inhibition of the Rho kinase by the inhibitor Y27632 strongly reduced CA-4P-induced CTGF expression (Fig. 3D). A similar effect was observed in colchicine-treated cells (Fig. 3D).

Negative Regulation of CA-4P-Induced CTGF Expression by p42/44 MAPKs

To get further insight into the signaling mechanisms underlying CA-4P-induced CTGF expression, we tested pharmacologic inhibitors of various signaling pathways, which have been described previously to regulate CTGF expression: SB203580 (5 and 1 μmol/L) was used to inhibit p38 MAPKs, forskolin (10 μmol/L) to activate protein kinase A and/or Epac/Rap signaling, PP2 (2 μmol/L) to inhibit Src family kinases, and EGTA (1 mmol/L) to affect Ca^{2+}-dependent signaling pathways. None of these treatments significantly reduced the CA-4P-induced expression of CTGF. Based on data obtained with colchicine-treated fibroblasts (26), an involvement of Src family kinases was expected but not detectable after treatment of cells with Src family kinase inhibitors (Fig. 4A). In earlier studies, inhibition of p42/44 MAPK interfered with CTGF expression (e.g., refs. 29, 30). In this study, interference with the basal p42/44 MAPK activity by pretreatment of the cells with the specific p42/44 MAPK inhibitor UO126 (10 μmol/L) unexpectedly increased CA-4P-mediated CTGF expression (Fig. 4B). Interestingly, up-regulation of CTGF by MAPK inhibition was only detectable after prolonged incubation (5 h), whereas no effect was seen after 2 h, suggesting an indirect interaction between p42/44 MAPKs and RhoA signaling because both signaling pathways were modulated in <1 h.

PI3K/AKT-FoxO Signaling Pathway in CTGF Induction

Cell density-dependent effects of CA-4P have been attributed previously to interference with PI3K/AKT signaling (6). Therefore, we analyzed the activation status of the PI3K/AKT signaling pathway by measuring the level of phospho-AKT. Phospho-AKT was reduced in dense cells compared with nonconfluent cells (~45% reduction; Fig. 5A). Incubation of nonconfluent cells with CA-4P did not significantly alter the phosphorylation status of AKT within the first hour of incubation (Fig. 5A, 30 min incubation). After 2 h, the ratio of phospho-AKT/AKT was reduced to 88.4 ± 10.0% ($n = 5$), which was not significant. These data indicated that CA-4P did not lead to rapid alterations of AKT activity, which might have accounted for CTGF induction.

However, inhibition of PI3K/AKT signaling by the PI3K inhibitor LY294002 was sufficient to increase CTGF expression. Incubation with LY294002 reduced phosphorylation of AKT in a concentration-dependent manner (see below; Fig. 6A). Concomitantly, a significant time- and concentration-dependent increase in CTGF expression was observed (Fig. 5B).

Inhibition of PI3K/AKT signaling is linked to the activation of FoxO transcription factors. Therefore, we hypothesized that FoxO1 and FoxO3a, the main FoxO proteins in endothelial cells (31), might be involved in the up-regulation of CTGF in glEND.2 cells. FoxO1 and FoxO3a were down-regulated using specific small interfering RNA (siRNA). As shown in Fig. 5C, a combination of both siRNAs was necessary to interfere
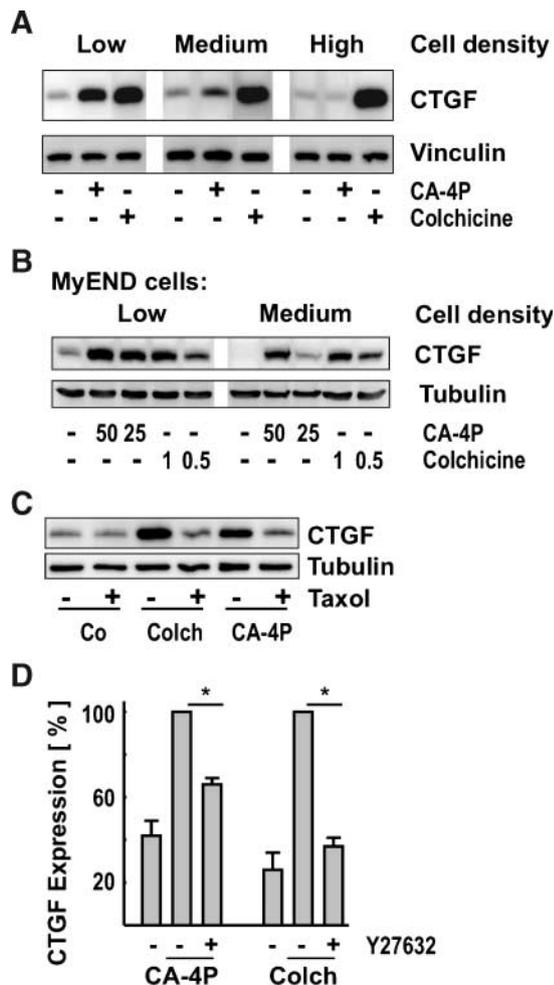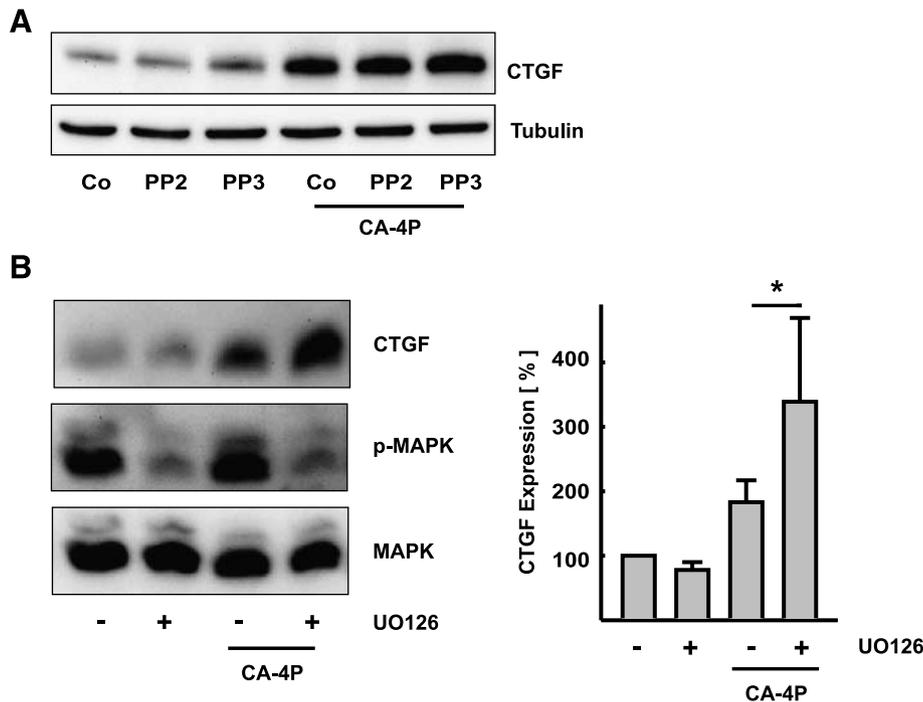
FIGURE 4. Increased CA-4P-mediated up-regulation of CTGF on inhibition of p42/44 MAPK. **A.** gIEND.2 cells were preincubated with 5 μmol/L PP2 or its inactive analogue PP3 for 30 min and then stimulated with 50 nmol/L CA-4P for 2 h. CTGF was detected by Western blot analysis. **B.** gIEND.2 cells were preincubated with the MAPK inhibitor UO126 (10 μmol/L) for 30 min and then stimulated with CA-4P for 5 h. CTGF, phospho-MAPK, and MAPK were detected on the same blot. Results of $n = 5$ experiments. CTGF expression of control cells was set to 100%. **, $P < 0.01$, ANOVA with Tukey-Kramer multiple comparison test.

efficiently with CTGF up-regulation by LY294002. By contrast, CA-4P-mediated up-regulation of CTGF was not significantly affected by siRNA directed against FoxO1/FoxO3a (data not shown). Regulation of CTGF by PI3K/AKT signaling was also observed in cardiac microvascular endothelial cells (MyEND). Incubation with the PI3K inhibitor LY294002 increased CTGF by ∼ 5-fold with 2 h (4.3 ± 1.3, mean ± SD; $n = 3$; $P < 0.05$). This increased induction of CTGF was reduced when the cells were treated with siRNA against FoxO1/FoxO3a (Fig. 5D).

Synergistic Induction of CTGF by CA-4P and Inhibition of PI3K/AKT Activity

Coincubation of endothelial cells with CA-4P (50 nmol/L) or colchicine (1 μmol/L) plus LY294002 further increased CTGF expression compared with either stimulus alone (Fig. 6A). At 10 μmol/L LY294002, phosphorylation of AKT was almost completely inhibited in the presence or absence of CA-4P or colchicine. Under these conditions, the increase of CTGF expression by the combination of stimuli was higher than calculated from the individual values (Fig. 6B, *white columns* representing the calculated additive stimulation). These results indicated activation of different signaling pathways by CA-4P and colchicine compared with LY294002. The mutual interaction between Rho kinase signaling and FoxO signaling was also seen when cells were pretreated with the Rho kinase inhibitor Y27632 before stimulation with LY294002. A partial but significant reduction of CTGF expression was observed in these conditions (Fig. 6C).

Discussion

CA-4P is characterized by its tumor vessel specificity and its rapid reduction of tumor blood flow followed by a more delayed death of vascular endothelial cells (2, 32). Up-regulation of CTGF, which we observed in endothelial cells within hours after treatment with CA-4P, represents a novel aspect of CA-4P action, that is, the rapid induction of proteins with antiangiogenic properties. Up-regulation of CTGF by the microtubule-disrupting agents nocodazole or colchicine has been reported before (25); however, only the induction by CA-4P proved to be restricted to cells that formed a loose network and were not embedded in a dense cell monolayer. Furthermore, CTGF was up-regulated by CA-4P in cells forming tube-like structures on basement membrane gels. These cells were not stabilized by pericytes and may thus be reminiscent of endothelial cells in newly formed tumor vessels. We have shown earlier that CTGF expression is sensitive to the consistence of the cellular microenvironment by comparing fibroblasts cultured on rigid surfaces with cells embedded in collagen gels (26). Morphologic alterations are expected to be more dynamic in nonconfluent microvascular endothelial cells or in endothelial cells cultured on basement membrane gels compared with endothelial cells, which are embedded in a monolayer and cultured on a rigid surface. It is very likely that these differences contribute to the selective up-regulation of CTGF by CA-4P. Translated to the *in vivo* situation, we would expect preferential up-regulation of CTGF in less organized tumor vessel rather than in stabilized regular vessels. Further *in vivo* studies will be necessary to confirm this hypothesis.

CTGF is a matricellular protein that is secreted but remains associated with the cells and the extracellular matrix. Therefore, on induction by CA-4P, most of CTGF was detected as cell-associated protein and only a small part was released into cell culture supernatants. It was interesting to note that the COOH-terminal cleavage product of CTGF was only detected in the cell culture supernatants, indicating the occurrence of

extracellular cleavage of the protein. In accordance with these findings, CTGF has been shown to be a target of matrix metalloproteinase-2 (15), which cleaves this growth factor in the interdomain linker, between the von Willebrand factor type C and the thrombospondin type 1 repeat domains. However, there was no evidence for an increased matrix metalloproteinase-2 activity in the presence of CA-4P, which

might have functionally counteracted the CA-4P-mediated up-regulation of CTGF.

Depolymerization of microtubules by CA-4P was related to activation of RhoA-Rho kinase signaling as shown by the inhibitory effect of Y27632 on CA-4P-mediated CTGF induction. A detailed analysis of the role of Rho kinase in nocodazole-induced cell contractility has recently been published (24). We
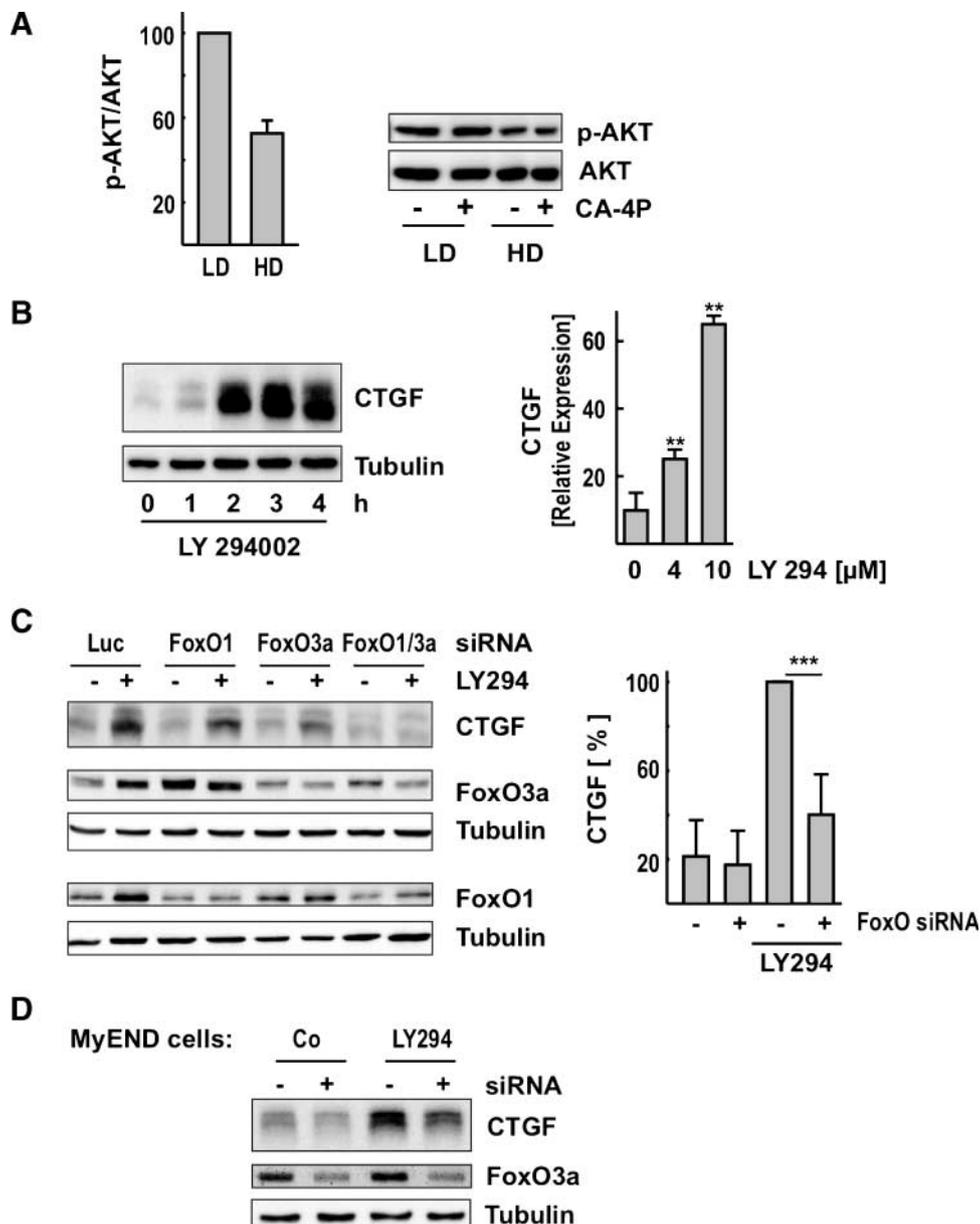

FIGURE 5. Up-regulation of CTGF in response to inhibition of PI3K/AKT signaling. **A.** glEND.2 cells were seeded at high (HD; 80,000 per cm^2) or low (LD; 20,000 per cm^2) densities. After 24 h, phospho-AKT and AKT were detected by Western blot analysis in cellular homogenates. Results of three experiments. The ratio of phospho-AKT/AKT in low-density cells was set to 100%. Example of cells stimulated with or without CA-4P (50 nmol/L) for 30 min. **B.** Cells were treated with PI3K inhibitor LY294002 (10 μmol/L) for the times indicated. CTGF expression was detected by Western blot analysis. Mean $\pm$ SD of $n = 3$ experiments (4 or 10 μmol/L LY294002, 2 h). In each experiment, the signal intensity of all bands was summarized and set to 100%. $P < 0.01$, compared with control, ANOVA with Dunnett's multiple comparisons test. **C.** glEND.2 cells were pretreated overnight with FoxO siRNA (50 nmol/L FoxO1 and 50 nmol/L FoxO3a siRNA) or siRNA against luciferase. The cells were subsequently stimulated with LY294002 (10 μmol/L) for 2 h. CTGF was detected by Western blot analysis. Results of $n = 5$ experiments. CTGF expression in cells treated with siRNA against luciferase was set to 100%. **, $P < 0.001$, compared with cells treated with luciferase siRNA, ANOVA with Tukey-Kramer multiple comparison test. **D.** MyEND cells were pretreated overnight with FoxO siRNA (50 nmol/L FoxO1 and 50 nmol/L FoxO3a siRNA) or siRNA against luciferase and then stimulated with LY294002 (10 μmol/L) for 2 h. FoxO3a and CTGF were detected in cellular homogenates. FoxO1 was barely detectable (data not shown).
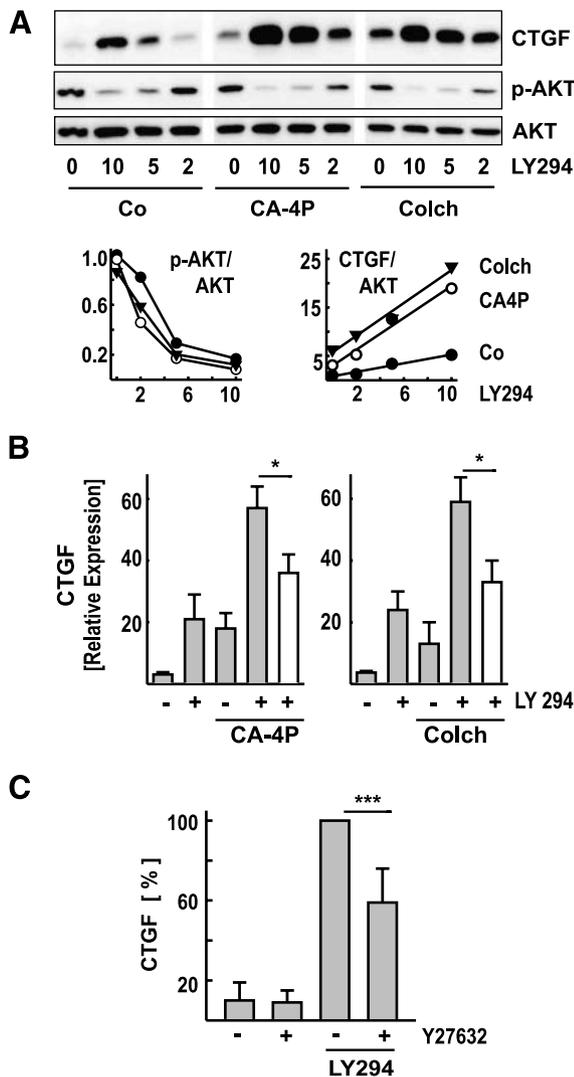
FIGURE 6. Synergistic up-regulation of CTGF by CA-4P and PI3K inhibition. **A.** glEND.2 cells were pretreated with the PI3K inhibitor LY294002 (2, 5 or 10 μmol/L) for 30 min and then incubated with CA-4P (50 nmol/L) or colchicine (1 μmol/L) for 2 h. CTGF, phospho-AKT, and AKT were analyzed in cellular homogenates and detected on the same blot. In the graphical analyses, expression of phospho-AKT/AKT or CTGF in control cells was set to 1. ●, control cells; ○, CA-4P; ▼, colchicine. **B.** Cells were treated as in **A** with 10 μmol/L LY294002. Gray columns, CTGF expression as determined by Western blot analyses. Total expression in each experiment was set to 100%. Mean ± SD ($n = 3$). Open columns, calculated additive stimulation of LY294002 plus CA-4P or colchicine. $P < 0.05$, paired Student's t test, calculated additive versus measured values. **C.** glEND.2 cells were pretreated with the Rho kinase inhibitor Y27632 (10 μmol/L) for 30 min and then stimulated with LY294002 for 2 h. CTGF protein was detected by Western blotting. CTGF expression in the presence of LY294002 was set to 100%. $n = 4$. $P < 0.001$, ANOVA with Tukey-Kramer multiple comparison test.

observed a further increase in CTGF expression, when p42/44 MAPK was inhibited, indicative of Rho kinase signaling being restrained by p42/44 MAPK activity. This finding supported earlier data of Mavria et al. (33) who showed that activation of Rho kinase resulted in detachment of isolated endothelial cells and retraction of sprouting vessels, which was counteracted by active p42/44 MAPK. Downstream of Rho kinase, we have previously described G-actin as a negative regulator of serum

response factor, which activates CTGF transcription by binding to a serum response element in the CTGF promoter (23). In a recent publication, Muehlich et al. showed that phosphorylation of MKL1, a binding partner of G-actin and activator of serum response factor, is phosphorylated by p42/44 MAPK and thereby exported from the nucleus to the cytosol (34). Inhibition of p42/44 MAPK may thus lead to an extended activation of serum response factor and increased up-regulation of CTGF. Therapeutically, CA-4P and other vascular disrupting agents are considered as comedication to be used with other drugs targeting tumor cells directly. Drugs targeting epidermal growth factor signaling to impair tumor cell growth will also reduce p42/44 MAPK activity, which is a downstream mediator of epidermal growth factor receptor activation (35).

The PI3K/AKT pathway is one of the most commonly altered pathways in human malignancy, and small-molecule inhibitors of this pathway are being developed for tumor therapy (36). Therefore, it was of interest that CTGF was synergistically up-regulated when CA-4P treatment was combined with PI3K/AKT inhibition. By itself, CA-4P did not inhibit phospho-AKT activity at time points early enough to be related to CTGF induction. In line with our results, Vincent et al. observed reduced phospho-AKT only after several hours of treatment (6).

Downstream of PI3K/AKT, we identified FoxO proteins as inducers of CTGF expression. FoxO transcription factors are important regulators of angiogenesis and maintenance of vascular homeostasis (37, 38). However, the function of FoxO proteins is highly context-specific and may differ even between endothelial cells obtained from different vascular beds (31). This may explain discrepancies in previously published results concerning the role of AKT signaling and CTGF expression. For example, VEGF-dependent induction of CTGF in bovine retinal endothelial cells was inhibited rather than induced by interference with AKT signaling. However, the downstream mediators of AKT signaling were not investigated in that study (16). In accordance with our present results, microarray data of Daly et al. showed that, on overexpression of constitutively active AKT in human umbilical vein endothelial cells, CTGF mRNA was down-regulated, whereas overexpression of constitutively active FoxO1 resulted in CTGF up-regulation (39).

FoxO proteins as transcription factors have been shown to bind to coactivator and corepressor complexes. There are several putative FoxO binding sites in the core promoter of CTGF (40), allowing interactions with other transcription factors. The molecular details of FoxO interaction with transcription factors activated by CA-4P are presently investigated.

The effective concentrations of CA-4P leading to CTGF expression were in the nanomolar range, which are much lower than the maximally tolerated micromolar plasma concentrations (41). Therefore, we suggest that up-regulation of CTGF may also occur *in vivo* in tumor neovessels. There is recent evidence that CTGF is involved in pericyte detachment and anoikis (42), which may contribute to microvessel destabilization. Furthermore, the published data implicate that CTGF may counteract VEGF-driven angiogenesis. Full-length CTGF has been shown to form an inhibitory complex with VEGF-165 (13), which can be cleaved by matrix metalloproteinase-2 to release VEGF (15). Increased local concentrations of CTGF may thus shift the balance between these factors toward inhibition of tumor

angiogenesis. Our present data suggest that CTGF may play a role in the early phase of microvessel destabilization observed in tumors treated with CA-4P.

Materials and Methods

Materials

Cell culture materials were purchased from PAA Laboratories. FCS was from PAN Biotech. LY294002 from Biomol and UO126 and Y27632 were from Calbiochem (Merck). CA-4P was kindly provided by OXiGENE.

Cell Culture

The murine glomerular microvascular endothelial cell line (glEND.2) was kindly provided by R. Hallmann. Cells were characterized by positive staining for typical endothelial cell markers MECA-32 and CD31 and the lack of staining for mesangial cell markers such as α-smooth muscle actin and α8-integrin as well as epithelial cells markers such as WT-1 and cytokeratin (43). The cells were cultured as described (23). The microvascular endothelial cell line MyEND was obtained from mouse myocardium and characterized by positive staining for VE-cadherin, α-, β-, and γ-catenin, ZO-1, and Claudin-5 as well as von Willebrand factor (44). The cells were cultured as described (45).

Western Blot Analysis

Cells were lysed in buffer containing 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 100 mmol/L EDTA, and 10% glycerol for the detection of CTGF or FoxO proteins. To detect hypoxia-inducible factor-1α, the cells were lysed using 6.65 mol/L urea, 10% glycerol, 10% SDS, 1 mol/L Tris-HCl (pH 6.7), 5 mmol/L DTT, and protease inhibitors Complete EDTA-free (Roche Diagnostics).

Proteins were separated by 10% or 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (CTGF: Macherey-Nagel). As indicated by the manufacturer, the antibody used to detect FoxO1 (Cell Signaling) interacted primarily with dephosphorylated FoxO1. Therefore, homogenates were prepared in the absence of phosphatase inhibitors and incubated for 1 h at 37°C before SDS gel electrophoresis. Furthermore, nitrocellulose membranes (Protran nitrocellulose membrane; Whatman) were proven to be more effective than polyvinylidene difluoride membranes to detect the weak endogenous FoxO1 signals.

The following antibodies were used: rabbit polyclonal anti-FoxO1 (Cell Signaling), rabbit polyclonal anti-FoxO3a (Cell Signaling), mouse polyclonal anti-phospho-AKT (Cell Signaling), rabbit polyclonal anti-AKT (Santa Cruz Biotechnology), goat polyclonal anti-CTGF (Santa Cruz Biotechnology), rabbit polyclonal anti-vinculin (Santa Cruz Biotechnology), donkey anti-goat IgG (Santa Cruz Biotechnology) conjugated to horseradish peroxidase, mouse monoclonal anti-tubulin antibody E7 developed by Klymkowsky (Developmental Studies Hybridoma Bank, University of Iowa), and peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG secondary antibodies (Amersham Biosciences).

Immunoreactive proteins were visualized by the enhanced chemiluminescence detection system (ECL-Plus; Amersham Biosciences). Immunoreactive bands were quantified using the luminescent image analyzer (LAS-1000 Image Analyzer; Fujifilm) and AIDA 4.15 image analyzer software (Raytest). To correct for equal loading and blotting, all blots were redetected with antibodies directed against tubulin or vinculin. For quantification purposes, the ratio of the specific protein band and a control protein was calculated. To compare different blots, bands were either normalized to control cells or the sum of all bands belonging to one experiment was set to 100%.

Immunocytochemistry

glEND.2 cells were fixed with 3.5% paraformaldehyde in Dulbecco's PBS (140 mmol/L NaCl, 2.68 mmol/L KCl, 1.47 mmol/L KH_2PO_4, and 8.1 mmol/L Na_2HPO_4) for 10 min and afterwards permeabilized by 0.2% Triton X-100 in Dulbecco's PBS for 10 min. Cells were incubated with primary antibodies against CTGF (1:500), GM130 (1:500; mouse monoclonal antibody; BD Biosciences), and tubulin (1:5,000; Developmental Studies Hybridoma Bank) overnight and with secondary antibody (1:500; Alexa Fluor 555 anti-rabbit; Invitrogen) for 45 min. After mounting, slides were viewed by using a Nikon fluorescent microscope. Digital images were recorded using MetaVue software (Universal Imaging).

siRNA Transfection

To down-regulate FoxO1 or FoxO3a expression, endothelial cells were transfected with FoxO1 siRNA (sense 5′-GCGGG-CUGGAAGAAUUCAA-3′; 50 nmol/L), FoxO3a siRNA (sense 5′-GCUCUUGGUGGAUCAUCAA-3′; 50 nmol/L), or luciferase siRNA (100 nmol/L) 3 h after seeding using HiPerFect (Qiagen) according to the manufacturer's instructions. All siRNAs were designed and synthesized by Eurogentec. Experiments were done 24 h after transfection.

In vitro *Capillary Morphogenesis Assay*

Basement membrane extract (12.85 mg/mL; R&D Systems) was spread over each well (50 μL) of a 24-well plate and polymerized for 30 min at 37°C. glEND.2 cells were suspended in medium containing 10% FCS and seeded on basement membrane extract at 75×10^3 per well. After 13 h incubation, the cells were treated with CA-4P and then fixed with 3.5% paraformaldehyde in Dulbecco's PBS for 30 min. Tube formation was examined by phase-contrast and fluorescence microscopy.

Statistics

Data are presented as mean ± SD. To compare multiple measurements, ANOVA with Tukey-Kramer multiple comparison test or Dunnett's post hoc test was used (Prism GraphPad Software). The paired Student's *t* test was used to compare any two conditions. $P < 0.05$ was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest.

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