Connective Tissue Growth Factor Is Overexpressed in Complicated Atherosclerotic Plaques and Induces Mononuclear Cell Chemotaxis In Vitro

Iwona Cicha, Atilla Yilmaz, Michael Klein, Dieter Raithel, David R. Brigstock, Werner G. Daniel, Margarete Goppelt-Struebe, Christoph D. Garlichs

Objective—Atherosclerotic blood vessels overexpress connective tissue growth factor (CTGF) mRNA, but the role of CTGF in atherosclerosis remains controversial. To assess the hypothesis that CTGF is involved in atherosclerotic plaque progression, we investigated CTGF protein expression and distribution in the different types of plaque morphology.

Methods and Results—Serial cross-sections of 45 human carotid plaques were immunohistochemically analyzed for the presence of CTGF protein, neovascularization (von Willebrand factor), macrophages (CD68), and T cells (CD3). The lesions were categorized according to American Heart Association (AHA) classification as fibrous (type IV and V) or complicated plaques (type VI). The levels of CTGF were significantly higher in complicated compared with fibrous plaques (P = 0.002). CTGF accumulated particularly in the rupture-prone plaque shoulder and in the areas of neovascularization or infiltration with inflammatory cells. Macrophage-like cells stained positive for CTGF protein in plaques. Subsequent in vitro studies showed that although monocyte-derived macrophages do not produce CTGF on stimulation with transforming growth factor-β, lipopolysaccharide, or thrombin, they take it up from culture medium. Furthermore, CTGF induces mononuclear cell chemotaxis in a dose-dependent manner.

Conclusion—CTGF protein is significantly increased in complicated compared with fibrous plaques and may enhance monocyte migration into atherosclerotic lesions, thus contributing to atherogenesis. (Arterioscler Thromb Vasc Biol. 2005;25:1008-1013.)

Key Words: connective tissue growth factor ■ atherosclerosis ■ plaque development ■ chemotaxis

Atherosclerosis is a chronic multifactorial disease characterized by the accumulation of lipids, fibrous tissue, and inflammatory cells in the large arteries. Whereas the earliest type of lesion consists mainly of lipid-laden foam cells and some T cells, the feature of advanced lesions is the accumulation of lipid-rich necrotic debris, encapsulated by a fibrous cap consisting of extracellular matrix produced by smooth muscle cells (SMCs). In the process of plaque development, complex cellular interactions between cells of the vessel wall and the immune system result in thinning of the fibrous cap, growing lipid core, increased inflammatory activity, and neovascularization. These processes lead to plaque instability and may result in plaque rupture, which is a common pathogenetic feature in a majority of acute manifestations of atherosclerosis, such as acute coronary syndrome and stroke.1

Connective tissue growth factor (CTGF), a potent angiogenic, chemotactic, and extracellular matrix—inducing growth factor, is produced by a wide variety of cells, including endothelial cells (ECs), SMCs, and fibroblasts. At low levels, CTGF supports wound healing by connective tissue formation after tissue injury and plays a role in angiogenesis and skeletal development.2 However, overexpression of CTGF gene was implicated in progression of many chronic inflammatory-fibroproliferative disorders, such as glomerulosclerosis, pulmonary fibrosis, and cirrhosis.3

As reported by Oemar et al.,4 CTGF mRNA is expressed at very high levels in atherosclerotic but not in normal human blood vessels. CTGF-producing cells in plaques, mainly nonproliferating SMCs and ECs, were detected predominantly along the shoulders of the fibrous cap. However, CTGF protein expression in relation to plaque morphology has not been investigated, and the role of this growth factor in atherosclerosis remains controversial. CTGF may reduce the likelihood of plaque rupture through extracellular matrix–mediated stabilization of the fibrous cap. On the other hand, it may contribute to plaque destabilization by inducing SMCs to undergo apoptosis.5 It was also shown that CTGF acts as an adhesion factor for activated platelets and monocytes via...
integrins αIIb/β3 and αM/β2, respectively.6,7 These findings suggest that this protein may be involved in promoting platelet and monocyte adhesion to dysfunctional endothelium, and in the formation of platelet-rich thrombi at ruptured atherosclerotic lesions.

In this study, we investigated the occurrence and distribution of CTGF protein in atherosclerotic plaques and correlated it with different types of plaque morphology. Moreover, we associated the CTGF levels with known risk factors for atherosclerosis and with patients’ medication. We report here a significant increase in the mean number of CTGF-expressing cells in complicated (type VI) compared with fibrous (type IV and V) plaques. Furthermore, CTGF is shown to induce chemotaxis in human mononuclear cells in vitro, suggesting an active role of this protein in atherosclerosis.

Materials and Methods
The Materials and Methods section is available online at http://atvb.ahajournals.org.

Results
CTGF Protein Is Overexpressed in Advanced Atherosclerotic Plaques
According to their morphologies, the plaques were defined as fibrous (AHA type IV and V; n=21) or complicated plaques (type VI; n=24). To confirm the previous report of Oemar,4 the cross-sections of 5 of 45 carotid specimens from regions bordering with morphologically normal vessel were used to mimic initial stages of plaque development. These cross-sections of preatheroma morphology contained very few CTGF-positive cells in immunohistochemical analysis (24.2±5.1 versus 76.9±5.9 in plaques [type VI-VI]; P<0.01). In accordance with the study by Oemar,4 this finding was subsequently confirmed using in situ hybridization method, which detected increased CTGF mRNA expression mainly in SMCs and luminal ECs of advanced plaques, whereas in cross-sections of preatheroma morphology, CTGF expression was limited to scarce ECs at the lumen (Figure 1A). In advanced plaques, levels of CTGF protein were significantly higher in complicated (type VI) lesions (93.9±8.3; n=24) compared with fibrous plaques (57.5±6; n=21; P=0.002; Figure 1B and 1C).

The presence of CTGF protein was compared with clinical data and the current medication of the patients (Table). There were no significant correlations between mean CTGF-positive cell numbers and smoking, diabetes mellitus, hyperlipidemia, or hypertension. In plaques of patients with acute cerebral symptoms, there was a trend for increased levels of CTGF protein compared with asymptomatic patients (84.8±8.1, n=27 versus 66.8±8.5, n=18; P=0.13). Treatment with aspirin, angiotensin-converting enzyme (ACE) inhibitors, clopidogrel, or angiotensin II type 1 receptor antagonists had no effect on the CTGF expression in plaques. A reduction in the level of CTGF was observed in plaques from patients treated with statins (56.6±5.4, n=11 versus 85.9±7.7 in untreated patients, n=34; P=0.013). However, the prevalence of fibrous plaque morphology and of concomitant treatment with ACE inhibitors was much higher in this group and might contribute to the decreased CTGF level in these patients.

Distribution of CTGF in Advanced Atherosclerotic Plaques
In the cross-sections of the vessel regions exhibiting preatheroma morphology, CTGF-positive cells localized mainly to lumen endothelium and thickened intima, whereas no CTGF was observed in media or in contralateral intima (data not shown). In advanced plaques, CTGF-positive staining was localized mainly to the plaque shoulders, fibrous cap, and the borders of the lipid core (Figure 2A). The plaque shoulder with the stronger degree of inflammation was defined as plaque shoulder-1, and the other as plaque shoulder-2. As shown in Figure 2B, significant increases in the numbers of CTGF-positive cells were observed between complicated and fibrous plaques in the following regions: fibrous cap (81.8±10.5 versus 43.4±10; P=0.005), plaque shoulder-1 (160.4±16.3 versus 104±13.8; P=0.03), border regions of the lipid core (117.9±16 versus 68.7±8.3; P=0.02). The trend for increased occurrence of CTGF-positive cells was also observed in the contralateral intima and the plaque shoulder-2 of complicated plaques; however, it was not statistically significant (P<0.07). There were no differences between the numbers of CTGF-positive cells in the media of complicated versus fibrous plaques.

CTGF Presence Is Associated With Intimal Neovascularization and Inflammation
The numbers of CTGF-positive cells were greatly dependent on the plaque vulnerability, with CTGF-expressing cells localized particularly in the areas of complications (ie, intimal neovascularization [Figure 3A], endothelial erosion, mural
thrombi, or the sites of heavy infiltration with inflammatory cells). In particular, average numbers of CTGF-positive cells were significantly higher in plaques undergoing neovascularization (88.4±8.5; n=25) compared with plaques without neovascularization (62.7±6.8; n=20; P=0.03).

In all analyzed plaques, macrophage and T-cell infiltration occurred coincidentally and was most pronounced in plaque shoulder-1 and in the areas bordering the lipid core. Macrophages always outnumbered lymphocytes, but both cell types were significantly more abundant in complicated (type VI) than in fibrous plaques (types IV and V). Mean macrophage cell numbers in complicated plaques were 86.3±5.6 versus 59.5±6.4 in fibrous plaques (P=0.005). A similar increase in T-cell number of complicated plaques was observed (21.2±1.6 versus 13.6±1.8; P<0.002). The mean numbers of T cells and macrophages correlated positively with CTGF expression in plaques (r=0.915, P<0.001 for macrophages [Figure 3B]; r=0.737, P<0.001 for T cells [data not shown]).

Immunohistochemical analysis showed that CTGF protein was often expressed by cells of macrophage-like morphology. Because the results of double-immunostaining of the same cell type are often unclear because of the color overlap, we performed the staining on serial 2-μm sections, which allows identification of single cells. The sections were stained either for anti-CD68 as macrophage/foam cell marker or for anti-SM actin as SMC marker. CTGF-positive cells were detected on the parallel sections. The results of the staining (Figure 3C, top) clearly demonstrate that the majority of CD68-positive cells are also positive for CTGF. On the other hand, a subpopulation of CD68-negative cells of SMC-like morphology from the same area also showed strong CTGF expression. These results were confirmed by staining with SMC marker, which showed that some but not all SMCs express CTGF (Figure 3C, bottom).

CTGF Is Taken Up by Macrophages In Vitro and Acts As Chemoattractant for Peripheral Blood Mononuclear Cells

Immunohistochemical analysis showed that the presence of CTGF protein in plaques was often associated with macrophages/foam cells (Figure 3C). However, macrophages did not express CTGF mRNA in Northern blot analysis4 or in situ hybridization11,12 (data not shown). Therefore, we investigated whether stimulated macrophages can produce CTGF in vitro. For this purpose, monocyte-derived macrophages were incubated with transforming growth factor-β (TGF-β) or thrombin, both known to induce CTGF in other cell types13,14 or with lipopolysaccharide (LPS). The stimulation of macrophages with TGF-β, thrombin, or LPS had no inducing effect on CTGF expression (Figure 4A). However, macrophages cultured with medium containing CTGF could take up CTGF from the medium, as demonstrated by Western blotting (Figure 4B). After 2 hours of culture, Myc-tagged CTGF was observed in washed cell pellets, whereas in the negative control samples without added recombinant CTGF, no CTGF bands were detectable either in cell pellets or in culture media. Detected CTGF was clearly not a result of platelet contamination because its molecular weight corresponded to the size of the recombinant CTGF containing a tag (42 versus 38 kDa of endogenous CTGF). The exogenous origin of CTGF was further confirmed by Western blot analysis using anti-Myc tag antibody (Figure 4B, right).

To prove the physiological relevance of CTGF–monocyte/macrophage interactions we performed a chemotaxis assay. As shown in Figure 4C, CTGF (10 to 100 ng/mL) induced
peripheral blood mononuclear cell (PBMC) migration in a dose-dependent manner. The mean number of migrated cells in the control wells without chemoattractant (56.1 $/-10^3$ cells) was set as a baseline. There was a statistically significant increase in the number of migrated nonadherent cells already at 10 ng/mL of CTGF ($P=0.005$ versus control wells). At 100 ng/mL CTGF, a 3-fold increase in migrated cell numbers was observed ($P=0.001$ versus control). To prove that the observed induction of PBMCs migration is CTGF specific, we performed further experiments adding to the lower wells heparin, which is known to bind CTGF.$^7$ Because the highest chemoattractant activity was observed at 100 ng/mL of CTGF, this concentration was used in the subsequent assays. In the presence of heparin (10 $\mu$g/mL), the chemotactic response to CTGF was reduced by 41% ($n=3; P=0.002$ versus CTGF alone; Figure 4D). Furthermore, the preincubation of PBMCs with heparin (10 $\mu$g/mL; 20 minutes at 25°C) suppressed CTGF-induced chemotaxis by 47% ($n=3; P=0.002$ versus untreated cells; Figure 4D), implying that cell surface heparan sulfate proteoglycans are required for CTGF-mediated PBMC migration.

### Discussion

The present study reports increased CTGF expression in complicated versus fibrous plaques, as well as colocalization of CTGF protein with inflammatory infiltrates and intimal neovascularization in advanced atherosclerotic lesions. Additionally, our study demonstrated the strong chemotactic effect of CTGF on PBMCs in vitro. These findings support the hypothesis that CTGF may play an active role in atherosclerosis by promoting monocyte migration into lesions and inducing intimal angiogenesis.

Chronic inflammatory responses in atherosclerosis lead to increased leukocyte accumulation and production of metalloproteinases, often resulting in degradation of the fibrous cap and plaque rupture. Clinically, in most patients, fatal coronary/cerebral events occur as a result of erosion or more often through uneven thinning and rupture of the fibrous cap.$^1$ On the basis of post mortem analyses of culprit plaques, the following criteria for defining vulnerable plaques were listed: large lipid core, thin fibrous cap, remodeling, active plaque inflammation, and superficial platelet aggregation.$^{15}$ Angiogenesis and superficial calcified nodules were also included as markers of vulnerability. The presence of 1 or a combination of these factors in the lesion may warrant higher risk of plaque complications. In light of these data, the finding that CTGF protein is overexpressed in complicated plaques and correlates positively with the numbers of inflammatory cells within the plaque may underscore the pathophysiologic importance of this growth factor in the progression of atherosclerosis. CTGF gene expression in ECs and SMCs is strongly upregulated by various cytokines (TGF-β, platelet-...
induces a dose-dependent chemotactic response in PBMCs. Values from control wells (no chemotacticant in the lower chamber) were set as a baseline. Monocyte chemoattractant protein-1 (MCP-1; 50 ng/mL; positive control) or CTGF at 10 to 100 ng/mL was placed in the lower chamber, and the cells were allowed to migrate for 1 hour at 37°C. Results are expressed as mean ± SEM; number of experiments given in brackets. To compare the data from different groups, 1-way ANOVA was used; **P < 0.001; *P < 0.05 vs control wells; ‡P < 0.01 vs 100 ng/mL CTGF. D, CTGF-induced chemotaxis is inhibited by heparin. CTGF-induced chemotaxis values (CTGF; 100 ng/mL) were set as 100%. CTGF plus heparin, 100 ng/mL CTGF plus 10 μg/mL heparin were added to lower wells; PBMCs plus heparin, cells were preincubated with 10 μg/mL heparin for 20 minutes at 25°C and allowed to migrate toward 100 ng/mL CTGF. Results are expressed as mean ± SEM of 3 independent experiments. Paired t test was used to compare the data between groups; *P = 0.002.

Figure 4. CTGF interaction with monocytes/macrophages. A, Macrophages do not produce CTGF on stimulation with TGF-β (5 ng/mL; 24 hours), thrombin (5 U/mL; 24 hours [T24] or 6 hours [T6]), or LPS (0.1 μg/mL; 24 hours). Autologous CTGF released from platelet was used as a positive control (P); c indicates unstimulated cells. B, Western blot analysis of macrophages cultured in the presence or absence of CTGF (0.4 μg/mL) shows CTGF uptake from culture medium. Recombinant-purified CTGF at 0.4 μg/mL was used as a positive control (c); a indicates macrophages; m, culture medium. Results are representative of 3 independent experiments. C, CTGF uptake of this protein into platelet α-granules. In the present study, macrophages cultured in presence of CTGF were able to take up CTGF from the culture medium, as demonstrated by Western blot analysis. Wahab et al 28 demonstrated that CTGF is internalized from the mesangial cell surface in endosomes; however, it is unclear whether macrophages take up CTGF by phagocytosis or via a specific receptor. Activated human monocytes were shown previously to adhere to CTGF via integrin αMβ2, but it remains to be investigated whether CTGF is also internalized as a ligand of this integrin. Also, the functional meaning of CTGF uptake is unknown so far. Importantly, integrin outside-in signaling in monocytes/macrophages often involves upregulation of proinflammatory cytokines such as interleukin-1β. CTGF was shown to upregulate matrix metalloproteinase expression in ECs. 29 It is thus possible that it can induce similar response in macrophages. Further detailed studies will be necessary to characterize the functional interactions between monocytes and CTGF and specific intracellular signal transduction pathways in these cells in response to CTGF.
In our study, we demonstrated that CTGF induces a dose-dependent chemotactic response in PBMCs. This effect may be physiologically relevant in chronic conditions of atherosclerosis. Platelet adhesion and mural aggregates of platelets are ubiquitous in the initiation and generation of atherosclerotic lesions. Platelets precede monocytes in adhesion to dysfunctional endothelium and, on activation, release their granules, which contain cytokines and growth factors, among them CTGF. CTGF acting in concert with other chemooactivants may thus lead to the enhanced adhesion and migration of monocytes across the endothelium. In this study, CTGF-dependent chemotaxis was inhibited by pretreatment of cells with heparin, pointing to the involvement of heparan sulfate proteoglycans in the mononuclear cell response to CTGF. These data should stimulate further studies to identify other receptor(s) and signaling pathways responsible for CTGF-mediated monocyte migration.

In conclusion, this study demonstrated that CTGF protein is increased in complicated versus fibrous atherosclerotic plaques. Furthermore, the in vitro results show that by stimulation of chemotaxis, CTGF could contribute to mononuclear cell recruitment in the artery wall. Further research will be required to elucidate the contribution of CTGF to plaque development and to investigate the mechanisms of CTGF–monocyte interactions in atherosclerosis.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft grant GRK 750. We thank Dr Oliver Pulig and Herbert Rohrmueller for help with in situ hybridization, and Dr Juliane Heusinger-Ribeiro for kind help providing CTGF. Technical assistance of Katja Schubert is gratefully acknowledged.

References

Connective Tissue Growth Factor Is Overexpressed in Complicated Atherosclerotic Plaques and Induces Mononuclear Cell Chemotaxis In Vitro
Iwona Cicha, Atilla Yilmaz, Michael Klein, Dieter Raithel, David R. Brigstock, Werner G. Daniel, Margarete Goppelt-Struebe and Christoph D. Garlichs

Arterioscler Thromb Vasc Biol. 2005;25:1008-1013; originally published online March 10, 2005;
doi: 10.1161/01.ATV.0000162173.27682.7b
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/5/1008

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

Antibodies and reagents

A highly-specific affinity-purified rabbit anti-human CTGF IgG (1.92 µg/ml), recognizing a peptide domain not present in other CCN family proteins, was prepared as previously described (8). Mouse monoclonal antibodies against macrophages (anti-CD68, PG-M1; prediluted), T-cells (anti-CD3, 1:50), and SMCs (anti-SM actin; 1:200) were from DakoCytomation, Hamburg, Germany. To detect neovascularization, endothelial cells were stained with a mouse monoclonal antibody against human von Willebrand factor (vWF; 1:25, DakoCytomation). For Western blotting, goat polyclonal antibody against human CTGF, and the anti-goat IgG conjugated to horseradish-peroxidase (both from Santa Cruz, Heidelberg, Germany) were used. Mouse monoclonal anti-Myc-tag antibody (1:1000) was purchased from Cell Signaling Technology, Beverly, USA.

Thrombin, heparin, and lipopolysacharide (LPS) were obtained from Sigma, Munich, Germany. Transforming growth factor-β (TGF-β) and monocyte chemotactic protein-1 (MCP-1) were from Tebu-bio, Offenbach, Germany. Purified recombinant human CTGF (myc-tagged; produced in HEK cells using a vector containing the full-length CTGF construct provided by Dr. Michael Bauer), was a kind gift from Dr. J. Heusinger-Ribeiro, Medical Clinic IV, University of Erlangen-Nuremberg. Phosphate-buffered saline (PBS, pH 7.4) was purchased from Biochrom AG, Berlin, Germany. Cell culture reagents, RPMI 1640 medium, penicillin, streptomycin, and foetal calf serum (FCS), were from PAA Laboratories, Pasching, Austria. The reagents for in situ hybridization (proteinase K, RNase A, blocking reagent for nucleic acid hybridization and detection, and alkaline phosphatase-conjugated anti-dioxygenin (DIG) antibody) were obtained from Roche Diagnostics, Mannheim Germany.
Patients and arterial specimens

Carotid specimens containing the entire intima and a part of the media were obtained from 45 consecutive patients undergoing carotid endarterectomy. Preoperative duplex scanning, magnetic resonance imaging, or angiography of the carotid arteries, were performed. Endarterectomy specimens were fixed in formalin and decalcified in EDTA buffer for 4 days. Apart from the cross-sections of 45 plaques, the cross-sections of the arteries at the border of the plaque and morphologically normal vessel were obtained from 5 out of 45 specimens used in this study. These cross-sections, exhibiting only minor thickening, were used to mimic initial stages of plaque development and are referred to as “cross-sections of preatheroma morphology”.

According to AHA classification, the plaques used in this study were grouped into fibrous (type IV (n=10) and type V (n=11), total n=21) and complicated (type VI, n=24) on the basis of their morphological characteristics (9). There were no significant differences in the clinical data of patients from both study groups (see Table 1). By the time of the surgery the majority of patients were receiving aspirin therapy. N=11 of patients were treated with HMG-CoA reductase inhibitors (statins). The numbers of patients receiving other medication are given in Table 1. The study was approved by local ethics committees and informed consent was obtained from all patients.

Immunohistochemical staining

Paraffin-embedded serial cross-sections of 4 µm thickness were placed on silane-coated slides, dewaxed in xylene, rehydrated in ethanol and washed with Tris-buffered saline (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5) containing 0.1% Tween 20 (TBS-T). Citric acid (10 mmol/L, pH 6.0) was used as antigen retrieval solution. The sections were stained using DakoCytomation catalyzed signal amplification (CSA) System™. Briefly, quenching the
endogenous peroxidase activity with H$_2$O$_2$ for 5 min was followed by blocking with serum-
free protein block for 5 min, and 15 min incubation with primary antibody. After washing
with TBS-T, the secondary biotinylated antibody was applied for 15 min, followed by
sequential 15 min incubations with secondary biotinylated link antibody, streptavidin-biotin-
peroxidase complex, biotinyl tyramide (amplification reagent), and streptavidin peroxidase.
Finally, the specimens were stained for 60-90 sec with 3,3’-diaminobenzidine
tetrahydrochloride (DAB) and counterstained with haematoxylin (DakoCytomation).
Irrelevant isotype-matched antibodies were used to obtain negative controls.

**Image quantification**

Digital images of atherosclerotic plaques were obtained by a CCD-camera (Nikon
DXM-1200, Duesseldorf, Germany) at a magnification x20. The sizes of the lipid core area
(mm$^2$) and of the total plaque area (mm$^2$) were measured by computer-aided planimetry
(Lucia Software V. 4.21, Laboratory Imaging Ltd., Nuremberg, Germany). The lipid core
ratio (LCR) was calculated as lipid core area/plaque area x 100. The thickness of the fibrous
cap was measured at its narrowest site.

For immunohistochemical analyses, images of different plaque regions were taken at
a magnification of x150. The numbers of CTGF-expressing cells, macrophages, and T-cells
were digitally counted in representative areas of 0.25 mm$^2$ in the following plaque regions:
plaque shoulders, subendothelial fibrous cap, necrotic core (at the border of lipid core), and
the plaque surrounding media. In each of these regions, the area of highest density of positive
cells and highest quality of staining was chosen for counting. The mean numbers of CTGF-
expressing cells, macrophages, and T-cells were calculated out of the cell numbers counted in
the different plaque regions. In addition, independently from the regions defined above, the
number of CTGF-positive cells was measured in the complication areas, i.e.: areas of
neovascularization, endothelium erosion, and/or sites of heavy infiltration with inflammatory cells.

**In situ hybridization**

Digoxigenin-labelled RNA probes were prepared using the DIG RNA labelling kit (Roche), with DNA fragments of human CTGF and human 28s as templates. Paraffin sections of 12 plaques were dewaxed and rehydrated. Subsequent slide preparation and hybridization was by standard techniques as previously described (10). DIG was detected using alkaline phosphatase-conjugated anti-DIG antibody, and visualised with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate for 16 to 64 hours.

**Macrophage culture**

Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated by density gradient centrifugation using Vacutainer cell preparation tubes (Becton Dickinson, Heidelberg, Germany). Monocytes were separated by adhesion for 1 h at 37°C. Differentiation of adherent cells into macrophages was carried out for 6 days in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FCS and 10% autologous serum. After day 6 differentiated macrophages were cultured for 24 h in the presence or absence of 5 ng/mL TGF-β, 0.1 µg/mL LPS, 5 U/mL thrombin. To investigate whether macrophages take up CTGF, the cells were incubated for 2 h with 0.4 µg of CTGF per 1 ml of medium. After treatment, the media were collected and the harvested cells were washed with PBS. The pellets and the media were frozen until analysis.

**Western blot analysis**

The lysis of cell pellets and subsequent Western blot analysis was carried out as described before (11). CTGF was detected using goat anti-human CTGF antibody (1:250) for
2 h, and visualised using enhanced chemiluminescence system (ECL-Plus, Amersham, Freiburg, Germany).

Migration assay

The effect of CTGF (10-100 ng/mL) on PBMCs migration was assessed in a 48-well microchamber (NeuroProbe, Gaithersburg, MD, USA). Briefly, the lower wells were filled with 28 µL serum-free RPMI 1640. MCP-1 (50 ng/mL) was used as a positive control. The upper wells were filled with 56 µL of freshly isolated PBMCs (1.5x10^6 cells/mL), and separated from the lower compartment by 8 µm-pore, collagen-coated polycarbonate membrane (Nucleopore, NeuroProbe). After incubation for 1 h at 37°C, migrated non-adherent cells from the lower wells were counted using a Bürker-Türk counting chamber. All samples were run in hexaplicate and averaged. For collagen-coating, the filter membranes were incubated in 20 µg/mL collagen-1 at 4°C overnight.

Statistical analysis

Data were expressed as mean ± SEM, unless stated otherwise. P<0.05 was considered statistically significant. The clinical data of the study patients were compared by Fisher Exact Test. The Mann-Whitney Rank Sum Test was used to compare the mean numbers of positive-cells between the different study groups. The correlation between the mean CTGF-positive cell number and inflammatory cell number in atherosclerotic plaques was analyzed by Spearman Rank Order Correlation test. The differences in PBMCs migration in response to CTGF were analyzed using one way ANOVA.