Research Article

Blocking The Activity of CTGF is Capable of Preventing The Development of Early-Stage Atherosclerosis in Rat Carotid Artery

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Summary

Background: Although connective tissue growth factor (CTGF) has been demonstrated to contribute to the formation of atherosclerotic plaques, its divergent effects on atherosclerosis have been proposed. It is unclear if blocking the activity of CTGF may prevent the development of atherosclerosis.

Methods: We established a rat carotid artery atherosclerotic model which recapitulates the early-stage atherosclerotic lesion and highly expresses CTGF. We blocked CTGF expression in atherosclerotic lesion by injecting a neutralizing anti-CTGF antibody and knocked-down CTGF expression in vascular smooth muscle cells (VSMCs) using a CTGF siRNA.

Results: Blocking the activity of CTGF extensively prevented the increase in arterial stenosis rate, intima area, and tunica media, along with significant suppression of MMP-2, MMP-9, and phospho-Akt proteins in the areas of atherosclerotic lesions. CTGF siRNA suppressed the growth and migration of VSMCs in vitro.

Conclusions: CTGF contributes significantly to the development of early-stage atherosclerosis. Blocking the activity of CTGF is capable of preventing the development of early-stage atherosclerosis through a mechanism involving the inactivation of Akt and suppression of MMP-2 and MMP-9. Our result suggests a novel strategy to the prevention of atherosclerosis.

Key words: Atherosclerosis Connective tissue growth factor Vascular smooth muscle cells Matrix Metalloproteinases-2 Matrix Metalloproteinases-9 Protein kinase B

Özet

Giriş: Atherosklerotik plakların oluşumasında bağı dokusu büyüme faktörü (BDBF)’nün etkisi bilinmekle birlikte iraksak etkileri ancak tahmin edilmektedir. BDBF’nin bloke edilmesi ile atherosklerozun oluşmasının önleneceği konusu ise kesinlik kazanmamıştır.

Yöntemler: Yüksek oranda BDBF oluştururan erken-evre atheroskleroz oluşturan bir şişan karotid arter atherosklerotik modeli oluşturuldu. BDBF siRNA kullanarak vaskülerler düz kas hücrelerinde BDBF oluşumunu durdurdu ve nötralize eden anti-BDBF antikoru enjekte ederek atherosklerotik lezyonda BDBF yapımı bloke ettik.

Sonuçlar: BDBF aktivitesini bloke etmek arterial stenoz oranlarında, intima ve tunica media alanlarında artışı aşırı oranda önledi. Ayrıca atherosklerotik lezyon alanlarında MMP-2, MMP-9 bölgelerinde azalma tespit edildi.
ve phospo-Akt proteinlerinde önemli ölçüde süpersyon oluşturdu. BDBF siRNA in vitro olarak vasküler düz kas hücreinde migrasyon ve büyümeği süpersse etti.


Anahtar Kelimeler: Atheroskleroz; bağ dokusu büyüme faktörü; vasküler düz kas hücresi; Matriks Metaloproteinaz

INTRODUCTION
The development of atherosclerotic lesion is a chronic pathological process, which has been recognized as an abnormal vascular remodeling process. Excessive accumulation of extracellular matrix (ECM) and accelerated proliferation and migration of vascular smooth muscle cells (VSMCs) may contribute to the formation of atherosclerotic plaques. Connective tissue growth factor (CTGF) is a secreted protein that belongs to CCN (Cef10/cyr61, CTGF and Nov) family and is originally identified as a growth factor secreted by endothelial cells. CTGF expression supports wound healing by connective tissue formation after tissue injury and is highly induced by transforming growth factor -β (TGF-β). Previous studies have demonstrated that CTGF promotes the proliferation and migration of cultured normal VSMCs which may contributes to the development of atherosclerosis. Suppression of CTGF expression in cultured VSMCs is capable of inhibiting the growth of cultured VSMCs and reducing the accumulation of ECM. Importantly, blocking the activity of CTGF has been proposed to be a new strategy to the prevention of fibrosis. This suggests a possibility that blocking the activity of CTGF may have a therapeutic benefit on atherosclerosis. However, CTGF may also have an opposite effect on late-stage atherosclerosis by promoting the rapture of advanced atherosclerotic plaques. So, it is important to elucidate the effects of CTGF on the progression of atherosclerosis in a well-established animal model and to determine if blockage of CTGF activity may prevent the formation of atherosclerotic plaques.

To this end, we have established an atherosclerotic model highly expressing CTGF in rat carotid artery and blocked the activity of CTGF utilizing a neutralizing anti-CTGF antibody. Our results demonstrated that CTGF contributes to the development of early-stage atherosclerosis and that blocking the activity of CTGF is capable of preventing the development of early-stage atherosclerosis, thus revealing a novel strategy to the prevention of atherosclerosis.

MATERIAL AND METHODS
Reagents
Neutralizing anti-CTGF antibody was purchased from Santa Cruz Corporation (Santa Cruz, CA, USA). CTGF siRNA and its control RNA, polyclonal antibodies of CTGF, Matrix Metalloproteinases-2 (MMP-2), Matrix Metalloproteinases-9 (MMP-9), Akt, and phospho-Akt were purchased from Santa Cruz Corporation (Santa Cruz, CA, USA). Trans Messenger TM transfection reagent was purchased from QIAGEN Corporation (Hilden, GEM). BCIP/NBT reagents were purchased from Sigma (St. Louis, MO, USA). Vectastain Elite ABC kits were purchased from Vector Laboratories (Burlingame, CA, USA).

Establishment of atherosclerotic injury model
Rat carotid artery atherosclerotic model was established according to Clowes and Reidy with minus modification. Male Sprague–Dawley rats were purchased from Japan SLC, Inc (Shizuoka, Japan) and...
were housed and handled under the guidance of Institutional Animal Use and Care Committee (IACUC). The rats (300g-400g) were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg) and a 2 cm longitudinal midline incision was made through the skin. The left external carotid artery (ECA) was located using blunt dissection and ligated distally, while proximally the artery was held with an untied ligature. A small hole was made in the ECA. The rat carotid arteries were dilated and denuded of endothelium with a 2-French Fogarty balloon embolectomy catheter (Baxter Health Care) introduced into the left common carotid artery (CCA) through the ECA. Balloon injury of the CCA was performed by three passes of the partially inflated balloon. After the catheter was removed, the ECA was ligated and the skin wound sutured. The rats recovered from anesthesia 1 hour later and were allowed free access to food and water. One day after injury, those rats were intra-gastric administrated with high-fat diet emulsion (4ml each time, twice a day) and injected with vitamin D3 (5mg/kg, once a day) intraperitoneally for eight weeks. Negative control rats were given a sham operation without balloon injury and high-fat diet and vitamin D3 treatment.

Neutralizing anti-CTGF antibody administration

One day after their balloon injury, the rats were assigned randomly into two groups: antibody-injection group (CTGF antibody was injected concomitantly with the treatment of high fat diet and vitamin D3) and saline control group (saline was injected concomitantly with the treatment of high fat diet and vitamin D3). A neutralizing anti-CTGF antibody was dissolved in saline freshly and administered to the rats (4mg/kg, once a day) intraperitoneally for 7 consecutive days as previously reported\(^{[11]}\). Same volume of saline was injected into the intraperitoneal region of control rats.

Histological and morphometric examination

Eight weeks after balloon injury and subsequent treatments, the rats were euthanized by overdose injection of pentobarbital. Carotid arteries were perfusion-fixed with 10 % neutral buffered formalin. The carotid arteries were removed, fixed in natural-buffered formalin for 18 hours, embedded in paraffin, and then cut into M thickness. The sections was stained with hematoxylin and eosin atcross-sections at 4 eosin, and then subjected to morphometric examination using a microscope (HC-3001; Nikon) equipped with a computerized digital image analysis system (SCION Image, public domain software). The areas of the external elastic lamina (EEL), the internal elastic lamina (IEL), and the lumen were measured. Medial and neointimal areas were calculated as follows: medial area \((M) = \text{EEL area} - \text{IEL area}\). Neointimal area \((I) = \text{IEL area} - \text{lumen area}\). Neointima/media \((I/M) = \text{neointimal area}/\text{medial area}\). Stenosis ratio \((S) = (M+I)/\text{EEL area}\).

Immunohistochemistry

Briefly, the cut paraffin section were dewaxed and rehydrated. After hydrogen peroxide blocking, antigen retrieval, and then normal serum blocking, the section were incubated with designated primary antibodies at 4 °C overnight. The sections were then incubated with appropriate biotinylated secondary antibodies and then with Vectastain Elite avidin-biotin-peroxidase complex. 3,3'-Diaminobenzidine was serviced as the chromogen.

Primary culture of VSMCs and its Identification

Sprague–Dawley rats with asthersclerotic injury were killed by an overdose injection of pentobarbital sodium. The carotid aorta was removed immediately and washed with RPMI1640 medium containing 100 U / ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc). The artery was
incised lengthways. The intima and external coat of vessels were removed by microsurgery. The left media were filled with 1000 U/mL dispase (Godo Shusei, Tokyo, Japan) and 1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and incubated for 30 minutes at 37°C. Cells were collected by centrifugation and then cultured on the rat collagen-I coated dishes (BD Biosciences). VSMCs were identified by their characteristic valley morphology and specific expression of α-smooth muscle actin detected by immunohistochemical staining.

Inhibition of CTGF expression in vitro using CTGF siRNA

Primary VSMCs were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin. RNA interference was performed according to the manufacturer's instruction (QIAGEN TransMessenger™ Handbook). Briefly, the cells were cultured in complete growth medium to 50%-60% confluence and then incubated in the presence of the transfection complex containing 4μg siRNA for 4 hours. The cells were continually incubated in complete growth medium for 48 hours. To confirm the effects of siRNA, CTGF protein was detected by immunoblotting. VSMCs transfected with lipidosome only or without transfection were considered as mock control (MC) and negative control (NC), respectively.

Analysis of protein expression in primary cultured cells

Expressions of CTGF, MMP-2, MMP-9, Akt and phospho-Akt were examined by Immunoblotting. Extracted total protein was loaded on SDS-PAGE and performed electrophoresis. The proteins were transferred to PVDF membrane. The membranes were blocked with 5% non-fat milk for 1 hour and incubated with 1:1000 diluted primary antibodies for 1 hour at room temperature, followed by incubation with 1:2000 diluted secondary antibodies for 1 hour at room temperature. Protein bands were revealed using BCIP/NBT reagents (Amresco, USA). The expression levels were semi-quantified by ChemiGenius gel imaging and analysis system (Geneflow, UK).

Cell proliferation assay

5×10³ cells/well were plated onto 96-well culture plates and incubated for 24 hours. CTGF siRNA was transected into these cells as described previously. After 24 hour incubation, MTT (20μL, 5mg/mL) was added into the medium and then incubated for 2 hours. After the supernatant liquid was discarded, the cells were lysed by the addition of 150 μL dimethylsulfoxide (DMSO). The optical density was measured by an ELISA reader at a wave length of 570nm.

Wound healing assay

2×10⁵ VSMCs, which have been transfected with CTGF siRNA, were seeded into fibronectin coated six-well tissue culture dishes and grew to monolayer. The monolayer was then carefully wounded by sterile pipette. After washed with saline to remove the debris, the wounded monolayer was cultured in serum free RPMI1640 for 24 hours and then photographed by microscope. The distance between edges was measured.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the software SPSS (version 1.0, SPSS Sigma Stat, Chicago, USA). Continuous variables were compared by ANOVA with Bonferroni correction.

RESULTS

Balloon injury and subsequent high-fat and vitamin D3 administration caused carotid artery atherosclerosis

Carotid artery atherosclerotic model has been established in our laboratory by the approach of balloon-injury on rat carotid artery wall cooperated with high fat diet feeding and vitamin D3 injection.
Atherosclerotic plaques were successfully induced in 30 rats. Cross-sections stained with hematoxylin and eosin demonstrated atherosclerotic feature in atherosclerotic carotid arteries when compared with those of negative control. Atherosclerotic lesion was obvious with a plaque morphology showing large amount of macrophages, foam cells deposits, and fibrous caps. This closely resembles the feature of early-stage atherosclerotic lesion (Figure 1). Advanced or late-stage features of atherosclerosis such as necrosis or hemorrhage and calcium deposits at bottom of the plaque were not observed. Artery stenosis was significantly induced in carotid artery after balloon injury and subsequent high-fat diet feeding and vitamin D3 injection. No carotid artery stenosis was seen in control rats.

**CTGF, MMP-2, MMP-9 and phospho-Akt proteins were over-expressed in the areas of atherosclerotic lesion**

The expressions of CTGF, MMP-2, MMP-9, and phospho-Akt were determined by immunohistochemical staining and image analysis. Our data showed that the amounts of CTGF, MMP-2, MMP-9, and phospho-Akt proteins were significantly increased in the areas of atherosclerotic lesion than those of normal carotid arteries (Figure 2).

**Blockage of CTGF activity reduced the stenosis ratio and the size of plaques**

In order to determine if CTGF contributes to the development of atherosclerosis and if blockage of CTGF activity is capable of preventing the development of atherosclerosis, a neutralizing anti-CTGF antibody was administered concomitantly with the induction of atherosclerotic model for 7 consecutive days. The injection of neutralizing anti-CTGF antibody significantly blocked the expression of CTGF in atherosclerotic plaques. The stenosis rate and the sizes of plaques were much smaller in the carotid arteries in CTGF antibody-injected rats when compared with those in saline-injected rats (Figure 3).

**Blockage of CTGF activity suppressed the expression of MMP-2, MMP-9, and phospho-Akt**

Immunohistochemical staining and image analysis also showed that the amount of MMP-2, MMP-9, and phospho-Akt proteins in the areas of atherosclerotic lesion were significantly decreased in CTGF antibody-injected rats when compared with those in saline-injected rats. This result indicates that CTGF regulates the expression of MMP-2, MMP-9, and phospho-Akt in the development of atherosclerosis (Figure 4).

**Knockdown CTGF expression in vitro suppressed the growth and migration of primary cultured VSMCs highly expressing CTGF**

As the formation of neointima in atherosclerotic vessels, VSMCs shift from a pro-contractile phenotype into a synthetic/proliferative phenotype. In order to determine if CTGF promotes the proliferation and migration, VSMCs highly expressing CTGF were obtained from the areas of atherosclerotic lesion and were cultured shortly. CTGF siRNA was employed to suppress the expression of CTGF (Figure 5). The effect of CTGF on proliferation and migration was evaluated. As shown in Figure (6 and 7), the rates of growth and migration were significant suppressed in CTGF siRNA-treated VSMCs than those in control VSMCs.

**Knockdown CTGF expression in vitro suppressed the expression of MMP-2 and MMP-9 and the activation of AKT**

As shown in Figure 8 and 9, the expressions of MMP-2 and MMP-9 and the phosphorylation of Akt are significantly decreased in CTGF siRNA-transfected VSMCs when compared with those in negative and mock control VSMCs. These results further ascertain our in vivo finding that the over-expressions of MMP-2 and MMP-9 and the activation of Akt can be regulated by CTGF and contribute to the accelerated growth and migration of VSMCs.
Figure 1: Representative photographs of cross-sections of carotid artery stained with hematoxylin and eosin. Artery stenosis was significantly induced in rats treated with balloon injury cooperated with high fat dietary and Vitamin D3 injection (B) when compared with that in negative control rats (A). Atherosclerotic lesions were observed with obvious plaque formation showing large amount of macrophages or foam cells deposits and fibrous caps. While advanced or late stage features of atherosclerosis such as necrosis, hemorrhage, and calcium deposits at bottom of the plaques were not observed (C).

Figure 2A: CTGF, MMP-2, MMP-9, and phospho-Akt proteins were over-expressed in the area of Atherosclerotic lesion. Immunohistochemical staining shows that the expressions of CTGF, MMP-2, MMP-9, and phospho-Akt proteins were significantly increased in carotid artery wall with atherosclerotic injury when compared with those in normal carotid artery wall of control rats. Original magnification = 200. Figure 2B. CTGF, MMP-2, MMP-9, and phospho-Akt proteins were over-expressed in the area Atherosclerotic lesion. The bar curve shows the quantitative data of above protein expression. An asterisk marks indicates statistically significant difference. n=30, p < 0.05.
Figure 3: Blockage of CTGF reduced the stenosis ratio and the size of plaques
Concomitant injection of CTGF antibody during the first week of the induction of atherosclerotic injury significantly blocked the over-expression of CTGF and prevented the increase of stenosis ratio and size of plaques when compared with concomitant injection of saline. Original magnification = 200. Quantitative data shown by the bar curve was obtained by image analysis. An asterisk mark indicates a statistically significant difference. n=30, p < 0.05.

Figure 4A: Blockage of CTGF suppressed the expression of MMP-2, MMP-9, and Phospho-Akt
Immunohistochemical staining shows that concomitant injection of CTGF antibody during the first week of induction of atherosclerotic injury significantly suppressed the expression of MMP-2, MMP-9, and phospho-Akt when compared with concomitant injection of saline. Original magnification = 200. Figure 4B. Blockage of CTGF suppressed the expression of MAIP-2, MMP-9, and Phospho-Akt Quantitative data shown in the bar curve was obtained by image analysis. An asterisk mark indicates a statistically significant difference. n=30, p < 0.05.
RNA interference against CTGF suppressed the expression of CTGF in VSMC cells. Immunoblotting demonstrated that CTGF siRNA suppressed the expression of CTGF in cultured VSMCs when compared with those of negative control (NC) and mock control (MC). Quantitative data shown on the bar curve was obtained by measuring the optical density of protein bands from three separate experiments. An asterisk mark indicates a statistically significant difference, $p < 0.05$.

Figure 6: RNA interference against CTGF suppressed the growth of VSMCs. Cell growth curve shows that CTGF siRNA suppressed the growth of primary cultured VSMCs in a time (days) dependent manner. An asterisk mark indicates a statistically significant difference, $n=30$, $p < 0.05$. 

Figure 5: RNA interference against CTGF suppressed the expression of CTGF in VSMC cells.
**Figure 7:** RNA interference against CTGF inhibited the migration of VSMCs
The representative photography on the top shows that the distance between the edges is bigger in CTGF siRNA treated cells when compared with those of negative control (NC) or mock control (MC) after 24 hour growing in fibronectin-coated dishes. The bar curve on the bottom shows the quantitative data of the distances between edges from three separate experiments. An asterisk mark indicates a statistically significant difference. n=30: p < 0.05.

**Figure 8A:** MMP-2 proteins were significantly suppressed by CTGF siRNA
Immunoblotting demonstrated that CTGF siRNA suppressed the expression of MMP-2 in cultured VSMCs when compared with those of negative control (NC) and mock control (MC). Quantitative data was obtained by measuring the optical density of protein bands from three separate experiments. An asterisk mark indicates a statistically significant difference, p < 0.05.

**Figure 8B:** MMP-9 proteins were significantly suppressed by CTGF siRNA
Immunoblotting demonstrated that CTGF siRNA suppressed the expression of MMP-9 in cultured VSMCs when compared with those of negative control (NC) and mock control (MC). Quantitative data was obtained by measuring the optical density of protein bands from three separate experiments. An asterisk mark indicates a statistically significant difference, p < 0.05.
DISCUSSION

Extensive deep vascular injury with high fat diet has been shown to produce relatively complete features of atherosclerosis and has been suggested to be an ideal way to establish an experimental model for the study of atherosclerosis\(^{(11,30)}\). We have established an atherosclerotic model in rat carotid artery utilizing the method of balloon injury cooperated with concomitant high fat dietary and vitamin D3 injection. Although several studies have demonstrated the contribution of CTGF to atherosclerosis\(^{(9,26,28)}\), the precise effects of CTGF on the development of atherosclerosis is still controversial and needed to be clarified. CTGF may have divergent effects on different stages of atherosclerosis. Previous studies by other investigators indicate that CTGF may not only promotes the development of atherosclerotic lesion, but also contributes to the rapture of advanced plaque of late-stage atherosclerosis by inducing apoptosis of VSMCs\(^{(12)}\) and stimulating the expression of MMPs\(^{(17)}\), which causes the destabilization of atherosclerotic plaques. We found that CTGF expression was increased during the induction of atherosclerotic lesion and that blockage of its activity concomitantly with the atherosclerotic induction significantly prevented the increase in stenosis rate and

Figure 9: Phosphorylation of Akt were significantly suppressed by CTGF siRNA

Immunoblotting demonstrated that CTGF siRNA significantly suppressed the phosphorylation of Akt in cultured VSMCs when compared with those of negative control (NC) and mock control (MC). There is no difference in total Akt expression among those three groups. Quantitative data was obtained by measuring the optical density of protein bands from three separate experiments. An asterisk mark indicates a statistically significant difference, \(p < 0.05\).
sizes of plaques. This result not only indicates the critical contribution of CTGF to the development of early-stage atherosclerosis but also suggests a possibility that suppression of CTGF may have a therapeutic benefit on atherosclerosis. In fact, recent reports have shown that CTGF is an ideal target for hepatic fibrosis\(^{10,21}\) and that pioglitazone, a prescription medication used to treat type 2 diabetes, exert its therapeutic effect on atherosclerosis by inhibiting CTGF in advanced atherosclerotic plaques of diabetic mouse\(^{4}\). Our data, for the first time, to the best of our knowledge, demonstrates that blocking the activity of CTGF during the development of early-stage atherosclerosis in a well-established atherosclerotic model is capable of preventing the development of atherosclerosis, thus suggesting a novel preventive strategy to atherosclerosis.

Accelerated proliferation and migration of VSMCs from the media into the neointima are critical events for the formation of fibrous cap and progression of atherosclerosis\(^{16,23,32}\). It seems to be clear that MMP-2 is a major MMPs member involved in the formation of atherosclerotic plaques. MMP-2 stimulates VSMC migration\(^{2,14}\), and neointimal formation\(^{7}\). Deficiency of MMP-2 causes reduced migration of VSMCs\(^{13,15}\). In addition, MMP-2 has also been linked to increased growth of VSMC\(^{1}\). Different from MMP-2, MMP-9 may have dual roles on formation of fibrous cap and destabilization of plaques\(^{27}\). Not only increasing plaque size, MMP-9 may also cause the instability of the plaque by destructing the base of the plaque in the descending aorta\(^{24}\). Although previous study by other investigators has demonstrated that enforced CTGF expression in cultured VSMCs promoted the growth and migration of VSMCs and the production of ECM protein collagen I and fibronectin\(^{9,34}\), it is not very clear that through what mechanism CTGF function on the development of atherosclerosis. Our data showed that MMP-2 and MMP-9 were significantly elevated during the development of atherosclerosis and that blocking CTGF activity suppressed both MMP-2 and MMP-9. Knocking down CTGF in primary cultured VSMCs which were originally from the atherosclerotic carotid artery significant suppressed both MMP-2 and MMP-9. Above results clearly indicate that both MMP-2 and MMP-9 involved in prevention of early-stage atherosclerosis caused by blockage of CTGF. Akt is a kind of serine/threonine protein kinase and have been shown to play key role in VSMC migration and proliferation in response to high glucose and cytokine like tumor necrosis factor\(^{5,6}\). We found that Akt phosphorylation increased significantly during the development of atherosclerosis, which was able to be inhibited by CTGF antibody and CTGF siRNA in vivo and in vitro, respectively. This result suggests that inactivation of Akt plays a critical role in preventing the proliferation of VSMCs.

In summary, CTGF expression plays a very important role in promoting the development of early-stage atherosclerosis through a mechanism involving elevated expression of MMP-2 and MMP-9 and activation of Akt. Blockage of CTGF activity is capable of preventing the formation of atherosclerotic plaques, thus suggesting a novel strategy to the prevention of atherosclerotic lesion.

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