Increased MMP-2 Expression in Connective Tissue Growth Factor Over-expression Vascular Smooth Muscle Cells*

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Connective tissue growth factor (CTGF) is abundantly expressed in the vascular smooth muscle cells (VSMC) of atherosclerotic lesions but not in normal vessels. CTGF is able to promote VSMC proliferation and migration and influences the composition of extracellular matrix. The mechanisms for controlling these events remain unclear. We studied the effects of CTGF on matrix metalloproteinases (MMPs) by introducing a CTGF over-expression construct into VSMC. We found that the over-expression of CTGF significantly increased the activity of MMP-2 in VSMC conditioned medium. MMP-2 activity was similarly increased by exogenous CTGF treatment, and this effect could be blocked by an anti-CTGF antibody. We also showed that the increased MMP-2 activity was due to an increase in MMP-2 mRNA levels in VSMC. We further studied the mechanisms involved in the regulation of MMP-2 mRNA levels and found that the AP-2 transcription factor is responsible for most of the CTGF-induced MMP-2 transcription. Because MMP-2 is an important factor directly involved in controlling cell movement and the turnover of extracellular matrix, our study may provide a mechanism for CTGF-promoted VSMC migration.

The matrix metalloproteinases (MMPs) are a family of peptidases that, upon activation, can degrade a variety of extracellular matrices (ECMs) as well as other proteins (1). More than two dozen MMPs have been identified so far. Based on their substrate specificities they can be divided into a number of groups, which include collagenases, stromelysins, gelatinases, and membrane-type MMPs. The MMPs are secreted as pro-enzymes and can only be activated by cleavage of a short peptide from their N-terminal ends by plasmin as well as by one of their own members, membrane-type 1 matrix metalloproteinase (MT-MMP-1) (2). MMP activity is tightly coordinated at several levels including transcriptional regulation, activation of latent zymogen, and interaction with endogenous inhibitors (1, 3). MMP-2 (gelatinase A, type IV collagenase) is one of the MMP members that has been extensively studied and has been found to play an important role in increasing smooth muscle cell (VSMC) migration, tissue remodeling, and tumor metastasis (4, 5). In studying occlusive vascular disease, much attention has been focused on increased MMP-2 expression and its ability to promote VSMC migration to the intima from the original media after either inflammatory or mechanical injury to the vascular wall (6, 7). In normal vessels, most of the VSMC reside in the media and are embedded in and also immobilized by the ECM. During VSMC migration, the surrounding ECM barriers must first be degraded and removed by MMPs. MMP-2 is one of the major ECM-degrading proteases and was shown specifically to degrade basement membrane, which consists mainly of collagen type IV, laminin, and proteoglycans (8, 9). MMP-2 is expressed abundantly in atherosclerotic and restenotic lesions, and the inhibition of its activity by synthetic compounds greatly reduces neointima formation (10). MMP-2 expression in VSMC is up-regulated by vascular injury and low blood flow conditions and is down-regulated by cellular differentiation and high blood flow (11). The mechanism for this regulation, however, is unclear.

Connective tissue growth factor (CTGF) is a 38-kD immediate early gene product and belongs to a recently described and structurally related CCN (CTGF/Cyr16/Nov) gene family. CTGF is suggested to play an important role in the ECM remodeling that occurs in normal physiological processes such as embryogenesis, implantation, and wound healing as well as in fibrosis and scarring (see review in Ref. 12). However, recent advances have shown that CTGF is involved in diverse autocrine or paracrine actions in many cell types such as vascular endothelial cells, epithelial cells, neuronal cells, VSMC, and cells of supportive skeletal tissues (see review in Ref. 13). We previously found that CTGF promotes VSMC proliferation, migration, and production of ECM in tissue culture (14). In this study, we have found that CTGF also increases the activity of MMP-2 in VSMC conditioned medium, and this increase is the result of rising level of MMP-2 mRNA. The mRNA increase can be blocked by cycloheximide, indicating that protein synthesis is required for this effect. We further found that CTGF has no major effects on either tissue inhibitor of metalloproteinase-2 (TIMP-2) or MMP-9. Moreover, the CTGF regulation on MMP-2 seems to be mediated by the transcription factor AP-2. Because MMP-2 is intimately involved in promoting VSMC migration during neointima formation, our results suggest that CTGF-promoted VSMC migration is mediated by MMP-2.

MATERIALS AND METHODS

Cell Culture, CTGF Plasmid, and Other Reagents—VSMC were isolated from abdominal aortas of Sprague-Dawley rats as described (15). Cells were cultured in 20% fetal calf serum/RPMI with penicillin, streptomycin, and fungizone in a humidified atmosphere at 37 °C and 5% CO2. Cells between passages 7 and 13 were used in our experiments. CTGF expression plasmid construction and cell transfection methods were described previously (14). Recombinant CTGF protein and anti-CTGF antibody were gifts from Dr. Michael Fech (Hoffmann-La Roche) and were described previously (14). MMP-2 cDNA probe was a gift from Dr. Michael Pech (Hoffmann-La Roche).
was isolated according to the vendor’s instructions. RNA samples were cleared by centrifugation at 14,000 × g for 15 min at 4°C. Protein concentrations were determined using a Bio-Rad protein assay.

Cells were lysed in RNAzol™, and total RNA was quantitated by absorbance at 260 nm. 20 μg of RNA was separated on a 1% agarose, 1:1 formaldehyde gel and blotted onto nitrocellulose membrane. The hybridization was carried out using 32P-labeled MMP-2 and GAPDH probes. GAPDH was used as a control to normalize RNA loading. The hybridization buffer and conditions used in our experiments were: 2× Denhardt’s, 50% formamide, 100 μg/ml herring sperm DNA, and 0.25% SDS at 42°C incubation for 12 h. MMP-2 mRNA levels were also quantitated by densitometry of the MMP-2 signals and normalized to the GAPDH levels.

To study the functions of CTGF, we stably transfected a CTGF over-expression construct into VSMC. MMP-2 activity was first measured in the conditioned media using standard zymography. We observed that in the CTGF over-expression VSMC samples there is an approximate 3-fold increase in MMP-2 activity compared with untransfected VSMC media (Fig. 1). We further found that this increase was due to an increase of MMP-2 pro-enzyme in the media (Fig. 2). Because MMP-2 is secreted as pro-enzyme before being activated, the MMP-2 pro-enzyme was used as competitor and was incubated with the nuclear extracts before the addition of labeled probe. Bound and free DNA were resolved by electrophoresis through a regular polyacrylamide gel and then scanned using the Molecular Dynamics PhosphorImager system.

Statistics—All densitometry data are presented as the mean ± the standard error about the mean, and all experiments were performed at least three times.

RESULTS
MMP-2 Expression Is Increased in CTGF Over-expression VSMC—To study the functions of CTGF, we stably transfected a CTGF over-expression construct into VSMC. MMP-2 activity was first measured in the conditioned media using standard zymography. We observed that in the CTGF over-expression VSMC media there is an approximate 3-fold increase in MMP-2 activity compared with untransfected VSMC media (Fig. 1). We further found that this increase was due to an increase of MMP-2 pro-enzyme in the media (Fig. 2). Because MMP-2 is secreted as pro-enzyme before being activated, the MMP-2 pro-enzyme was detected in conditioned VSMC culture. Cells were growth-arrested for 48 h before the supernatant was collected for the assay. The clear bands migrate at 72 kDa and are consistent with the pro-form of MMP-2. For each lane, 25 μl of the conditioned medium was used. There are no apparent differences between the two controls (No Transfection and Vector Only). Increased MMP-2 levels are seen in CTGF over-expression and CTGF-treated cells, but these increases can be inhibited by anti-CTGF antibody and by cycloheximide as well as partially by an AP-3 antisense oligo.

Northern Analysis—Cells were lysed in RNAzol™, and total RNA was isolated according to the vendor’s instructions. RNA samples were quantitated by absorbance at 260 nm. 20 μg of RNA was separated on a 1% agarose, 1:1 formaldehyde gel and blotted onto nitrocellulose membrane. The hybridization was carried out using 32P-labeled MMP-2 and GAPDH probes. GAPDH was used as a control to normalize RNA loading. The hybridization buffer and conditions used in our experiments were: 2× Denhardt’s, 50% formamide, 100 μg/ml herring sperm DNA, and 0.25% SDS at 42°C incubation for 12 h. MMP-2 mRNA levels were also quantitated by densitometry of the MMP-2 signals and normalized to the GAPDH levels.

Delivery of Antisense Oligonucleotide in Vitro—Purified ODNs were introduced into VSMC cells using a cationic liposome reagent, Lipofectin (Invitrogen). Briefly, the cells were incubated at 37°C until they reached confluency. For each transfection, ODNs were mixed with Lipofectin reagent in serum-free medium and overlaid onto the cells 1–2 hours before the addition of labeled probe. Bound and free DNA were resolved by electrophoresis through a regular polyacrylamide gel and then scanned using the Molecular Dynamics PhosphorImager system.

MMP-2 Expression Is Increased in CTGF Over-expression VSMC—To study the functions of CTGF, we stably transfected a CTGF over-expression construct into VSMC. MMP-2 activity was first measured in the conditioned media using standard zymography. We observed that in the CTGF over-expression VSMC media there is an approximate 3-fold increase in MMP-2 activity compared with untransfected VSMC media (Fig. 1). We further found that this increase was due to an increase of MMP-2 pro-enzyme in the media (Fig. 2). Because MMP-2 is secreted as pro-enzyme before being activated, the MMP-2 pro-enzyme was detected in Western analysis by using a spe-
cific anti-pro-MMP-2 monoclonal antibody (Fig. 2). The levels of increase of MMP-2 proteins in both CTGF over-expression cells (Fig. 2A) and media (Fig. 2B) over the controls are more than 3-fold, comparable with that found in MMP-2 activity. There are no apparent differences in protein and activity levels between the untransfected and the vector transfected cells, and we used both as controls.

We further examined the mRNA level of MMP-2 and found that CTGF over-expression increases MMP-2 mRNA over the untransfected and vector controls on an average of 3.5-fold (Fig. 3), which is also consistent with the increase in MMP-2 protein level. Vector transfection alone does not change the level of MMP-2 mRNA (Fig. 3).

**Anti-CTGF Antibody Blocks the Induction of MMP-2 by CTGF**—To confirm that the increased MMP-2 level is the result of over-expression of CTGF, we used a recombinant CTGF to treat normal VSMC and found that by adding an exogenous recombinant CTGF (25 ng/ml) to the cultured VSMC for 6 h, the MMP-2 mRNA, antigen, and activity were increased to levels comparable with those in CTGF over-expression cells (Figs. 1–3). Furthermore, the increase of MMP-2 mRNA, protein, and activity levels by exogenous addition can be blocked by a specific anti-CTGF antibody (Figs. 1–3). We also tested whether the increase of MMP-2 expression by CTGF requires de novo protein synthesis and found that this induction can be inhibited by pretreatment with cycloheximide for 30 min (Figs. 1–3). However, we also observed that the basal MMP-2 expression in normal cells does not show a marked decrease by this antibody (not shown), which may be because a different mechanism regulates constitutive MMP-2 expression.

**Tissue Inhibitor of Metalloproteinase-2 Is Not Affected by CTGF**—TIMPs are endogenous antagonists for their corresponding MMPs and in many cases are regulated by similar inducers and repressors (1, 3). We also explored the possible effects of CTGF on TIMP-2 expression and examined its expression at the mRNA level in the CTGF over-expression and CTGF protein-treated cells. Our data show that there are no significant changes between the CTGF over-expression cells and the control cells (Fig. 4).

**Transcription Factor AP-2 Mediates the Up-regulation of MMP-2 by CTGF**—The transcription regulation of MMP-2 has not been completely defined, and there are only a few transcription factors reportedly involved. One of them is the transcription factor AP-2, which has been shown specifically to bind to the regulatory region and activate MMP-2 gene expression (16). In this study, we investigated whether AP-2 mediates CTGF action in up-regulating MMP-2 expression. Our results show that in both CTGF over-expression and exogenous CTGF-treated cells, AP-2 mRNA levels were about 2-fold higher than in normal and vector control cells (Fig. 5). To confirm that the increased AP-2 level was directly responsible for the increase of MMP-2 levels, we used an antisense oligo against the AP-2 transcription to treat the CTGF-transfected cells and found that this antisense oligo was effective and could block about 75% of the increase in MMP-2 mRNA, antigen, and activity levels (Figs. 1–3), whereas a control oligo did not have much effect. However, the use of AP-2 antisense oligo did not completely block the increase of MMP-2, suggesting that other

![Fig. 2. A and B, Western analyses of MMP-2 protein in total cell extract (A) and in conditioned medium (B) demonstrating a visible increase of MMP-2 protein in CTGF over-expression cells and in the conditioned media. Total proteins were extracted from growth-arrested VSMC or precipitated from the conditioned media and probed with a MMP-2 specific monoclonal antibody. C and D, graphs represent the average densitometric data ± S.E. for A and B from three experiments. There are significant increases (more than 3-fold) of MMP-2 in both CTGF over-expression cells in total cell extract and in the conditioned media versus vector control. The inhibitory effects of anti-CTGF antibody, AP-1 antisense oligo, and cycloheximide are evident from the protein levels.](http://www.jbc.org/)

VSMC or precipitated from the conditioned media and probed with a MMP-2 specific monoclonal antibody. C and D, graphs represent the average densitometric data ± S.E. for A and B from three experiments. There are significant increases (more than 3-fold) of MMP-2 in both CTGF over-expression cells in total cell extract and in the conditioned media versus vector control. The inhibitory effects of anti-CTGF antibody, AP-1 antisense oligo, and cycloheximide are evident from the protein levels.
factors may also be involved in CTGF-regulated MMP-2 expression. We further examined the binding activities on the AP-2 element in the MMP-2 promoter region and found that there is an enhanced binding to this site by nuclear extract from CTGF over-expression cells compared with the extract from normal control cells. We used a labeled synthetic DNA fragment that contains the consensus AP-2 binding sequence (5'-CCCCACCCGTCGCTCAGT-3') and performed a gel mobility shift assay to determine the DNA-protein complex formation for cells over-expressing CTGF and for the normal control. As shown in Fig. 6, two DNA-protein complexes were detected, and there were marked increases in shifted bands in CTGF over-expressing and exogenous CTGF-treated cells. In addition, the complexes could be competed away by an excess of unlabeled AP-2 fragment (Fig. 6) but were not affected by an unlabeled non-specific fragment (GATA fragment). These findings confirm that the increased binding to AP-2 site is specific in CTGF over-expression and CTGF-treated cells.

DISCUSSION

CTGF has recently been identified as a novel mitogen for endothelial, smooth muscle, and fibroblast cells (12). It also is involved in the regulation of ECM protein production and thus in wound repair and fibrosis. CTGF is strongly induced by TGF-β1 and may serve as its downstream effector (12). Oemar and Luscher (17) have found that CTGF is rarely detectable in normal blood vessels but is overexpressed in atherosclerotic lesions. The overexpression was found mainly in VSMC in the lesions, indicating that it may play a role in the development and progression of atherosclerosis. We previously found that VSMC proliferation and migration were stimulated by CTGF over-expression (14), and in this study, we observed that CTGF increases the expression of MMP-2 in VSMC culture; this increase is not accompanied by a corresponding increase of TIMP-2. We further determined that the elevated MMP-2 activity is the result of an increased level of MMP-2 mRNA. In searching a number of possible transcription factors that might mediate the transcription of MMP-2, we determined that AP-2 is involved in CTGF-induced MMP-2 expression.

The expanding family of MMPs now comprises 25 members of the zinc-dependent metallopeptidases. The pathophysiological importance of these MMPs has been determined gradually, based on the growing list of their functions, which range from degrading basement membrane constituents such as fibronectin, laminin, collagen, and elastin (1, 18) to interacting with a number of nonmatrix proteins such as tumor necrosis factor-β, fibroblast growth factor receptor-1, and angiogenic factors (19, 20). This wide variety of activity reflects the multiple functions of MMPs in regulating biological and pathological processes such as cell migration, differentiation, and atherogenesis.

The involvement of MMP-2 in the development of atheroscler-
rosis and restenosis, which are characterized by lesion formation after arterial injury, has been reported in several studies (7, 21, 22). Increased MMP-2 activity was found in and around atherosclerotic lesions. During lesion development in experimental balloon injury, MMP-2 plays a key role in degrading basement membrane and promoting the migration of VSMC from media to intima (7). Mechanistic studies by Koshikawa et al. (23) have shown that the cleavage of laminin-5 by MMP-2 exposes cryptic promigratory sites on laminin-5, which in turn triggers cell motility. In light of our previous and current results, it is conceivable that the CTGF-initiated pro-migratory effect is at least partially mediated by MMP-2 activity.

In contrast to functional characterization, the regulation of MMP-2 transcription is inadequately delineated; there are only a few transcription factors that have been identified so far as binding to the MMP-2 promoter region. Qin and et al. (16) have shown that binding to the MMP-2 promoter area by transcription factors Sp1, Sp3, and AP-2 leads to constitutive expression in astrogliaoma cells. However, growth factors, inflammatory cytokines commonly involved in VSMC proliferation, i.e. platelet-derived growth factor and interleukin-1, fail to up-regulate MMP-2 expression (24), suggesting that a different regulatory mechanism governs MMP-2 induction. TGF-β was shown to induce the transcription of MMP-2 during embryogenesis, but the role this regulation plays in atherogenesis remains unclear (25). In another report, Benbow and Brinckerhoff (26) have shown that AP-1 activity is needed but that AP-2 is critical for tissue-specific MMP-2 expression. AP-1 alone does not regulate MMP-2 transcription, and there is an essential interaction with other cis-acting sequences in the promoter and with other transcription factors that bind to these sequences. We found in this study that the AP-2 level is increased in CTGF over-expression cells, which contributes significantly to the increase of mRNA level of MMP-2 because an AP-2 antisense oligo blocks most of the increase of MMP-2. Other factors may also be at work in CTGF-stimulated MMP-2 expression because blocking AP-2 alone does not completely inhibiting the induction of MMP-2.

Transcription factor AP-2 has also been found in the regulation of a number of other genes, including biglycan and vascular endothelial growth factor, both of which were shown to be involved in various stages of atherosclerotic development (27, 28). Previous studies have shown that the mechanisms for AP-2 induction may involve three major pathways: activation of the retinoic acid receptor (29), cAMP-dependent protein kinase A (30), and protein kinase C activation (31). Further
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studies are needed to help elucidate which pathway is critical in the CTGF induction of AP-2 and the subsequent increase of MMP-2.

Biglycan is a small leucine-rich proteoglycan that is involved in several pathophysiological processes including atherogenesis through its interactions with other ECM molecules and TGF-β (28). Its synthesis is controlled by TGF-β, and biglycan and TGF-β are thought to form a negative feedback loop regulating TGF-β activity (32–34). Because CTGF is also closely controlled by TGF-β, it should be very interesting to investigate whether the modulation of biglycan expression by TGF-β is via CTGF and AP-2 and, if so, whether the TGF-β-biglycan loop should include CTGF and AP-2 as well.

In general, in the current study we have demonstrated an increased level of MMP-2 regulated by CTGF and underscored the importance of understanding the complex interactions of gene activities under the direct and indirect control of TGF-β. Because TGF-β plays a pivotal role as an endogenous growth regulatory factor in the pathogenesis of cardiovascular disease, e.g. progressive neointimal thickening (restenosis) after angioplasty (35) and atherosclerosis, a better understanding of its mechanisms will surely advance future plans to block targeted molecules to treat and prevent the development of cardiovascular disease.

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