CITED2-mediated Regulation of MMP-1 and MMP-13 in Human Chondrocytes under Flow Shear*

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CITED2 (CBP/p300-interacting transactivator with ED-rich tail 2) is a member of the Cited family of nuclear regulators, previously known as mrg1 (melanocyte-specific gene-related gene 1). CITED2 is inducible by varying stimuli including lipopolysaccharide, hypoxia, and cytokines such as interleukin 9 and interferon γ. Using the immortalized human chondrocyte cell line, C-28/I2, we investigated whether CITED2 could be responsive to mechanical stimuli, and if so, whether CITED2 could mediate shear-driven regulation of matrix metalloproteinase (MMP) genes. The C-28/I2 cells were cultured under flow shear at 1–20 dyn/cm², and the role of CITED2 in regulation of MMPs was examined using the plasmids encoding sense and antisense CITED2 DNA sequences. The results showed that flow shear at 5 dyn/cm² increased CITED2 mRNA and protein levels and down-regulated MMP-1 and MMP-13 mRNA and protein levels as well as enzyme activities. Consistent with the coordinated expression patterns of CITED2 and MMPs, overexpression of CITED2 repressed MMP-1 and MMP-13 mRNA levels and activities, whereas antisense CITED2 plasmids prevented the shear-induced down-regulation of MMP expression. Interleukin-1β induced the formation of p300-Ets-1 complexes without affecting expression of CITED2. Transforming growth factor-β as well as flow shear at 5 dyn/cm² stimulated not only the expression of CITED2 but also the association of CITED2 with p300 by dissociating Ets-1 from p300. These results indicate that CITED2 plays a major role in shear-induced down-regulation of MMP-1 and MMP-13 via a transforming growth-factor-β-dependent pathway.

Physical stimuli at appropriate intensities are essential for growth and maintenance of bone and joint tissues (1–3). In vivo studies demonstrate that mechanical loading facilitates the strengthening of bone, and flow-induced shear in articular cartilage stimulates a repair response (4, 5). Chondrocytes in cartilage experience a variety of stresses, strains, and pressure that result from normal activities of daily living. Determining how shear stress alters chondrocyte metabolism is fundamental to understanding how to limit matrix destruction and stimulate cartilage repair and regeneration (31). Matrix metalloproteinases (MMPs)¹ are a family of collagen-degrading proteinases whose expression and activities are altered by mechanical stimuli in various cell types (6–8). In inflammatory joint diseases such as rheumatoid arthritis and osteoarthritis, MMPs are considered pivotal proteinases of cartilage degradation. Because IL-1 and tumor necrosis factor-α are known to stimulate the expression and activities of MMPs, cytokine antagonists and receptor-blocking antibodies have been studied as potential agents for blocking cartilage destruction in joint diseases (9–11). Our recent study using a human synovial cell line showed that gentle mechanical shear with intensity at a few dyn/cm² had anti-inflammatory effects and reduced the expression and activities of many MMPs including MMP-1 and MMP-13 (7). The molecular mechanism underlying anti-inflammatory responses to flow shear, especially down-regulation of MMPs, has not been identified.

Many MMPs possess transcription-factor binding motifs such as AP1, AP2, NFκB, PEA3, Sp1, and STAT in the 5′-flanking regulatory DNA sequences (12–14). Transcription factors such as Ets-1, NFκB, and Stat2 not only bind DNA directly but also interact with CREB-binding protein (CBP) and p300 (15). CBP and p300 are transcriptional co-activators, and they play a critical role in many cellular functions through interactions with TFII B and RNA polymerase II as well as regulatory transcription factors (16). Recently, a new family of transcriptional co-regulators, the CITED (CBP/p300-interacting transactivator with ED-rich tail) family, was discovered. These factors interact with the first cys-teine-histidine-rich (CH1) region of CBP/p300 (17) and appear to be key transcriptional modulators in embryogenesis, inflammation, and stress responses (18–21). Recent studies have shown that CITED2 is involved in regulation of stress-responsive genes such as HIF1 and LIM (21). To our knowledge, however, the role of CITED in mechanical stress and regulation of MMPs has not been investigated.

MMP-1 and MMP-13 are collagenases whose expression is elevated in inflamed joints (22) and are associated with regions of collagen destruction in osteoarthritic chondrocytes (21, 23). Thus, we investigated whether CITED2 could be induced by flow shear in chondrocytes and, if so, whether CITED2 could mediate the down-regulation of MMP expression and activities in response to flow shear. As a reproducible model, we used the C-28/I2 chondrocyte cell line and analyzed MMP-1 and MMP-13 mRNA levels and enzyme activities after application of flow shear with intensity up to 20 dyn/cm². Our results using the sense and antisense CITED2 plasmids, overexpressed in the C-28/I2 cells prior to application of flow shear, support the

¹ The abbreviations used are: MMP, matrix metalloproteinase; IL, interleukin; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; TGF-β, transforming growth factor-β; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
critical role of CITED2 in flow shear-induced down-regulation of MMP-1 and MMP-13. Ets-1 is responsive to IL-1 and known to activate the transcription of many MMPs (24, 25), and TGF-β leads to suppression of Ets-1 through its dissociation from CBP/p300 (26). In this report, we have identified a mechanism whereby IL-1β and inflammatory flow shear at 20 dyn/cm² facilitate association of Ets-1 with p300 and TGF-β and anti-inflammatory flow shear at 5 dyn/cm² stimulate formation of p300-CITED2 complexes.

EXPERIMENTAL PROCEDURES

Cell Culture—The immortalized human chondrocyte cell line, C-28/I2, was used for this study (27, 28). The use of human cell cultures was approved by the Indiana University-Purdue University Indianapolis Institutional Committee. Cells were plated on a glass slide coated with 2 μg/ml type I collagen and grown to reach ~70% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. After a mild starvation in medium containing 1% serum for 18 h, the cells were exposed to 1-h flow shear at intensity of <20 dyn/cm² in a Streamer Gold flow device (Flexcell International). In some experiments, the cells were incubated with recombinant human IL-1β (Calbiochem) or TGF-β (Calbiochem) at concentrations of 5–100 ng/ml for 0–6 h.

Transfection of Sense and Antisense CITED2 Plasmids—Expression vectors containing the sense or antisense CITED2 DNA sequences were constructed in pcDNA3.1 to examine the function of CITED2 (Fig. 1). Sequences from the coding region of human CITED2 DNA was amplified by PCR using the primers containing EcoRI and BamHI sites, and the amplified fragments were subcloned into the pcDNA3.1 vector. The sense CITED2 plasmid (pcDNA3.1-CITED2S) was used to overexpress CITED2, and the antisense CITED2 plasmid (pcDNA3.1-CITED2AS) was designed to block translation of CITED2 mRNA. The plasmids were transfected in chondrocytes at a concentration of 0.1 or 1 μg of DNA/2 cm². The collected agarose beads were washed four times with an ice-cold lysis buffer, and the precipitated proteins were eluted by boiling the proteins for 2 h. Fluorescent intensity, a measure of MMP activity, was determined by boiling the beads for 5 min in the SDS sample buffer. Fractions of the supernatant were analyzed by SDS-PAGE and immunoblotting using antibodies against p300, CITED2, and Ets-1 (Santa Cruz Biotechnology). The expected molecular sizes of p300 and Ets-1 were 300 and 54 kDa, respectively.

MMP Activity Assay—MMP activities were determined using fluorophore-labeled substrates specific for MMP-1 or MMP-13 and collagenases or gelatinases (Molecular Probes) (30). The proteins isolated from the culture medium were incubated with the fluorescent substrates in a reaction buffer consisting of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl₂, and 0.2 mM sodium azide at room temperature for 2 h. Fluorescent intensity, a measure of MMP activity, was determined by a Fluoromax-2 spectrofluorometer ( Instruments S. A., Inc.). An absorption/emission wavelength was set to 382/441 nm (MMP-1), 325/393 nm (MMP-13), and 495/515 nm (collagenases and gelatinases). The culture medium itself exhibited basal MMP activity that was >5% of that in the medium incubated with control cells.

RESULTS

MMP-1 and MMP-13 mRNA, Protein Levels, and Enzyme Activities Were Altered by Flow Shear—The C-28/I2 cells expressed constitutive levels of MMP-1 and MMP-13 mRNA that could be detected by the sensitive RT-PCR technique under the...
Overexpression of Antisense CITED2—43%, respectively.

enzyme activity by 43 and 66% and MMP-13 activity by 23 and

CITED2 expression occurs at the same level of fluid sheer, 5 dyn/cm², (Fig. 3). These data indicate that the induction of CITED2 did not alter either mRNA or protein level relative to controls 2.0-fold, respectively. However, flow shear at 10 or 20 dyn/cm² increased CITED2 mRNA and protein levels by 1.9- and 5, 10, and 20 dyn/cm² for 1 h, MMP-1 and MMP-13 mRNA, enzyme activities. In response to flow shear at 0 (control), 1, 2, 5, 10, and 20 dyn/cm² for 1 h. Actin was used as loading control for Western blots.

**Increased Expression of CITED2 in Response to Flow Shear at 5 dyn/cm²**—CITED2 is a transcriptional activator known to be inducible by many biological stresses, but no study on the responses to mechanical stress has been conducted previously. Thus, we wished to determine whether CITED2 could be expressed in chondrocytes in response to flow shear. The C-28/I2 cells expressed detectable levels of CITED2 mRNA and proteins as shown in Fig. 3. Application of flow shear at 5 dyn/cm² for 1 h increased CITED2 mRNA and protein levels by 1.9- and 2.0-fold, respectively. However, flow shear at 10 or 20 dyn/cm² did not alter either mRNA or protein level relative to controls (Fig. 3). These data indicate that the induction of CITED2 expression occurs at the same level of fluid shear, 5 dyn/cm², that causes down-regulation of MMP expression.

**Suppression of the Levels of MMP-1 and MMP-13 mRNA, Protein, and Enzyme Activities by Overexpression of CITED2**—To examine the role of CITED2 in regulation of MMP-1 and MMP-13, we overexpressed CITED2 in C-28/I2 cells. Transfection of pcDNA3.1-CITED2S elevated the mRNA protein levels of CITED2, and the increases were dependent on the dosage of plasmids used for transfection. A 10-fold increase in the amount of plasmid DNA raised transfection efficiency by 3.3-fold (Fig. 4, A and B). The MMP-1 and MMP-13 mRNA levels and enzyme activities were reduced by overexpression of CITED2 (Fig. 4, A and C). Compared with the cells transfected with the empty pcDNA3.1 vector, transfection of the sense CITED2 plasmids at 0.1 and 1 μg/2 × 10⁶ cells reduced MMP-1 enzyme activity by 43 and 66% and MMP-13 activity by 23 and 43%, respectively.

**Loss of Shear-induced Repression of MMP-1 Expression after Overexpression of Antisense CITED2**—To determine whether flow shear-induced CITED2 was indeed capable of suppressing MMP-1 expression, we transfected antisense CITED2 in the C-28/I2 cells for 72 h followed by application of flow shear at 5 dyn/cm² for 1 h. The antisense plasmid reduced the level of CITED2 protein by 81% in control cells and 80% in shear-treated cells at 5 dyn/cm² (Fig. 5A). In the cells transfected with the empty vector alone, the expression of CITED2 was not altered either in the presence or absence of flow shear (Fig. 5A). However, the level of MMP-1 mRNA was increased by overexpression of antisense CITED2 in either the absence or presence of flow shear (Fig. 5B).

**Suppression of IL-1β-stimulated MMP-1 and MMP-13 mRNA Levels and Enzyme Activities by Flow Shear**—IL-1β is known to up-regulate the expression and activities of many members of the MMP family in connective tissues. In response to IL-1β, we observed that the constitutive mRNA levels of
MMP-1 and MMP-13 were further elevated. Consistent with the expected anti-catabolic effects of flow shear at 5 dyn/cm², the IL-1β-stimulated mRNA levels were significantly reduced by flow shear. In the real-time PCR assay, the MMP-1 mRNA levels were 4.3-fold (IL-1β), 0.4-fold (shear at 5 dyn/cm²), and 1.9-fold (IL-1β and shear at 5 dyn/cm²) of the level in control cells (Fig. 6A). Similarly, MMP-1, MMP-13, gelatinases, and collagenases enzyme activities were all up-regulated by IL-1β, whereas the IL-1β-driven increases in MMP activities were suppressed by flow shear (Fig. 6B). The average percentages of reduction in MMP activities were 45% (with IL-1β) and 24% (without IL-1β).

**CITED2 Expression in C-28/I2 Chondrocytes Was Unaltered by IL-1β but Activated by TGF-β**—We wished to determine whether the anti-catabolic effects of CITED2 were via modulation of IL-1β expression or TGF-β expression in C-28/I2 chondrocytes. After a 1-h incubation with IL-1β at a concentration of 5, 25, 50, and 100 ng/ml, the level of CITED2 mRNA was unchanged (Fig. 7A). Furthermore, the level of CITED2 mRNA was not affected by incubation with 25 ng/ml IL-1β for up to 4 h (Fig. 7B). On the other hand, a 1-h incubation with TGF-β at a concentration of 5, 25, 50, and 100 ng/ml elevated the mRNA level of CITED2 (Fig. 7C). The elevated level of CITED2 mRNA after a 30-min incubation with 25 ng/ml TGF-β was maintained at least for up to 4 h (Fig. 7D). The mRNA level of MMP-1 was undetectable in the presence of TGF-β (data not shown).

**Formation of p300-Ets-1 Complexes by IL-1β and Inflammatory Shear at 20 dyn/cm² and Formation of p300-CITED2 Complexes by TGF-β and Anti-inflammatory Shear at 5 dyn/cm²**—Because CITED2 and Ets-1 are known to interact with p300, we determined whether IL-1β or TGF-β, which have opposite effects on MMP expression, could modulate the interactions with this cofactor. The C-28/I2 cells were incubated with 25 ng/ml IL-1β or 25 ng/ml TGF-β for 1 h. Nuclear extracts were prepared and incubated with the antibody specific for p300 to immunoprecipitate p300 protein complexes. Western blotting identified equal amounts of p300 in control and treated extracts (Fig. 8). In control cells, no p300-Ets-1 or p300-CITED2 complex was detectable. In contrast, p300-Ets-1 complexes were detected in the extracts from IL-1β-treated cells and cells under flow shear at 20 dyn/cm² and p300-CITED2 complexes were identified in the TGF-β-treated cells and cells under flow shear at 5 dyn/cm². These results suggest that CITED2 mediates TGF-β-dependent effects on gene transcription via protein-protein interactions with p300.

**DISCUSSION**

In this study, we provide evidence that CITED2 is a shear-responsive transcriptional mediator in chondrocytes and that it plays a critical role in reducing expression and activity of MMP-1 and MMP-13 at intensities of flow shear that are physiologically relevant (31). We used the immortalized human chondrocyte cell line, C-28/I2, which has served as a reproducible model for examining intracellular signaling and gene transcriptional mechanisms relevant to chondrocyte functions (28, 32–34). Our results clearly show that CITED2 mRNA and protein levels are elevated most effectively by shear stress at 5 dyn/cm² and that this shear intensity down-regulates expression and activities of MMP-1 and MMP-13. A positive correlation between the MMP protein level and the activity level indicates that expression of tissue inhibitors of metalloproteinase-1 (TIMPs) may contribute to the anti-catabolic effects of shear stress on chondrocytes.

**Fig. 3.** CITED2 expression under flow shear at 0 (control), 5, 10, and 20 dyn/cm² for 1 h. A, CITED2 mRNA level. GAPDH was used as control for RT-PCR. B, CITED2 protein level. Actin was used as loading control for Western blots.

**Fig. 4.** Effects of CITED2 overexpression on mRNA expression and activity of MMP-1 and MMP-13. The sense plasmid (pcDNA3.1-CITED2S) was transfected at a concentration of 0.1 and 1 μg of DNA/2 × 10⁵ cells. A, mRNA levels of MMP-1, MMP2, and CITED2. B, CITED2 protein levels after transfection of sense plasmids. C, reduced MMP-1 and MMP-13 enzyme activities in response to overexpression of CITED2. The asterisk indicates statistical difference from the plasmid control (vector alone) at p < 0.05.
ases, a natural inhibitor of MMPs, does not overturn the shear-induced regulation of MMP-1 and MMP-13. Overexpression of CITED2 results in the reduction of expression of MMP-1 and MMP-13, and transfection of antisense CITED2 suppresses the shear-induced down-regulation of MMPs. Because association of CITED2 with p300 is affected by TGF-β but not by IL-1β, the CITED2-mediated mechanical responses leading to down-regulation of MMP-1 and MMP-13 expression appear to be regulated by the TGF-β signaling pathway.

The observed anti-inflammatory effects of mechanical stimuli are consistent with previous studies in various cell types, although excessive mechanical loads elevate expression of MMPs in chondrocytes (35). In peridontal ligament cells, 6–10% of mechanical strain was shown to reduce IL-1β-induced expression of proinflammatory cytokines such as IL-6 and IL-8 (8). Expression of MMP-1 was decreased by 1–4% of strain in vascular smooth muscle cells and by 6% of strain in fibrochondrocytes (36, 37). Using synovial cells, we have shown previously that mechanical stimuli such as small strain at ~1% and flow shear at ~2 dyn/cm² are effective in reducing expression and activities of MMPs induced by IL-1β and tumor necrosis factor-α (30).

Elucidation of the molecular mechanism underlying the CITED2-mediated down-regulation of MMP-1 and MMP-13 re-
quires investigation of the interactions of CITED2 with p300/CBP and many transcription factors that control MMP promoter activities by interacting with p300/CBP, such as c-Fos, Ets-1, NFκB, and SMAD family members (16, 25, 26). In our previous studies using human synovial cells, the mRNA expression of c-Fos and Ets-1 was responsive to mechanical stimuli and the response was dependent on shear intensity (7). In computational simulations where the expression of MMPs in response to mechanical stimuli was modeled using multiple transcription-factor binding motifs, the PEA3 binding motif, which is recognized by Ets-1, was identified as a stimulatory factor (12). In the same model, the AP-2 binding motif was predicted as an inhibitory factor that is known to be activated by CITED2 (19). Taken together, these experimental and computational results suggest that CITED2 could orchestrate the expression of MMPs by regulating interactions between p300 and other transcription factors that either down-regulate (AP-2) or up-regulate (Ets-1 and NFκB) MMP gene expression. The multifunctional p300/CBP has been shown to play a major role in SMAD-dependent TGF-β stimulation of the type I collagen promoter (38). Our studies suggest that CITED2 provides an alternative mechanism of TGF-β-induced metabolic responses.

The observed link of the CITED2-mediated reduction in MMP expression to the TGF-β pathway is consistent with the known role of TGF-β in down-regulation of MMP-1 expression (28). TGF-β leads to dissociation of Ets-1 from the p300/CBP complexes (26), and TGF-β-null mice develop uncontrolled inflammation and joint destruction (18). The observation that the anti-catabolic effects of CITED2 were not directly linked to the IL-1β signaling pathway was unexpected, because a previous study in mouse D10 T cells reported that CITED2 was inducible by IL-1α. Differential responses to IL-1 in T cells and chondrocytes may result from cell-type dependent signaling pathways. We used IL1-β (29), because this is the dominant IL-1 signal in human tissues and it plays a central role in the inflammation and connective tissue destruction observed in rheumatoid arthritis and osteoarthritis. The observed quick increase in MMP-1 expression in chondrocytes after 1 h of the IL-1β treatment is consistent with the responses to IL-1 in synoviocytes (30). IL-1β suppresses the expression of the type II collagen gene in C28/I2 chondrocytes (39). However, because CITED2 down-regulates and IL-1β up-regulates MMP expression in chondrocytes, CITED2 could be interfering with the actions of IL-1-induced transcription factors on MMP promoters. Consistent with our observation in C28/I2 chondrocytes, Xu et al. (40) reported that cyclic tensile strain, which generated an anti-inflammatory signal in chondrocytes, was not mediated through IL-1 receptors but instead was an antagonist of IL-1 actions (40).

In conclusion, we have identified CITED2 as the critical repressor of MMP-1 and MMP-13 gene expression and associated proteolytic activities using flow shear as a probe. Further elucidation of CITED2-mediated mechanotransduction of chondrocytes would contribute to controlling catabolic responses in rheumatoid arthritis and osteoarthritis.
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