Interleukin-1β inhibits collagen synthesis and promotes its decomposition in cultured cardiac fibroblasts

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Abstract: The present study aimed to investigate the effects of interleukin-1β (IL-1β) at different doses on collagen synthesis and decomposition in cultured cardiac fibroblasts from neonatal Sprague-Dawley rat. Cardiac fibroblasts were treated with IL-1β (0.01, 0.1, 1, 10, 100 ng/mL) for 24 h. Cell DNA synthesis was measured by 3H-thymidine (3H-TdR) incorporation and collagen synthesis was measured by 3H-proline (3H-Pro) incorporation. Matrix metalloproteinase (MMP) activity was measured by gelatinase zymography. MMP-2, MMP-9 protein expressions were measured by Western blot. mRNA expressions of MMP-2 and MMP-9 were detected by reverse transcription-polymerase chain reaction (RT-PCR). Compared with that in the control group, the incorporation of 3H-TdR and 3H-Pro decreased in high-dose IL-1β groups (≥0.1 ng/mL) but not in low-dose IL-1β group (0.01 ng/mL). IL-1β significantly increased MMP-2 and MMP-9 activities. IL-1β (0.01-100 ng/mL) also dose-dependently increased the protein and mRNA expressions of MMP-2 and MMP-9 (P<0.05, P<0.01), respectively. These results suggest that IL-1β decreases collagen synthesis and MMP activities through transcriptional and posttranslational processes via degrading collagen in a dose-dependent way. Elevation of IL-1β is possibly involved in the process of ventricular remodeling after myocardial infarction, and the concentration of IL-1β is possibly a major factor which affects the extent of ventricular remodeling.

Key words: interleukin-1β; fibroblasts; collagen; matrix metalloproteinases

Ventricular remodeling in the process of post-myocardial infarction (MI) is the major mechanism of heart failure. It contributes to ventricular dilation and dysfunction, patients’ disability and death. Ventricular remodeling is a complex
dynamic process including cardiomyocyte hypertrophy, apoptosis, necrosis and extracellular matrix (ECM) remodeling, especially dynamic equilibrium of synthesis and decomposition of collagen, the major composition of ECM\(^{[9]}\). Collagen is mainly synthesized by cardiac fibroblasts (CFs) and decomposed by extracellular matrix metalloproteinases (MMPs). MMPs, a family of endopeptidases, have been proved upregulated during left ventricular (LV) remodeling following MI in rats and play a significant role in the development of ECM remodeling\(^{[22]}\). MMP-2 and MMP-9 are the two important components of the family, and well-known for its ability to degrade gelatins and type IV collagen in basement membranes\(^{[31]}\). Increasing lines of evidence showed that MI results in the migration of macrophages, monocytes, and neutrophils into the infarct zone, and this initiates the inflammatory response and augmentation of pro-inflammatory cytokines which play an important role in the process of post-MI remodeling. Since the concentration of interleukin-1\(\beta\) (IL-1\(\beta\)) increased gradually accompanied by an enlargement of the size of MI, the incidence of ventricular remodeling was high and the extent was severe with the large size of infarct, we hypothesize that different concentrations of IL-1\(\beta\) have different effects on collagen synthesis and decomposition. So we used IL-1\(\beta\), one of the pro-inflammatory cytokines at different concentrations to the CFs of neonatal Sprague-Dawley (SD) rat and observed the different effects of IL-1\(\beta\) on collagen synthesis and MMP activity.

1 MATERIALS AND METHODS

1.1 Reagents and antibodies

Dulbecco’s modified Eagle’s medium (DMEM), IL-1\(\beta\) and 0.25% pancreatin was purchased from Sigma. Bradford assay was purchased from Bio-Rad. \(^3\)H-thymidine (\(^3\)H-TdR) and \(^3\)H-proline (\(^3\)H-Pro) were purchased from HTA Co., Ltd. Rabbit polyclonal anti-goat-vimentin, rabbit polyclonal anti-goat-fibronectin and rabbit polyclonal anti-goat-\(\alpha\)-actin antibodies were purchased from Boster Tech. Ltd., China. Rabbit polyclonal anti-goat-MMP-2 and anti-goat-MMP-9 antibodies were purchased from Santa Cruz Biotechnology. The primers of MMP-2, MMP-9 and GAPDH were synthesized by Shanghai GeneCore BioTechnologies Co., Ltd. TRIzol and \(AMV\) reverse transcriptase were purchased from TaKaRa Bio. Inc.

1.2 Rat CFs culture and treatment

Hearts were removed aseptically from neonatal (2-3 days old) SD rats. The atria were removed and the ventricles were placed in cold PBS buffer and finely minced with scissors in PBS buffer to form a slurry. The minced tissue was digested with 0.25% pancreatin for 5 min at 37 °C. The supernatant was removed and then digested 8 times. The supernatant was collected and transferred to a 15 mL conical tube containing 2 mL of fetal bovine serum (FBS) to inactivate the enzymes, centrifuged for 10 min at 1 000 r/min, cell supernatant was removed, and cells were resuspened with DMEM containing 20% bovine serum albumin (BSA). Four milliliters of cell suspension was added to the original flask and cultured for 1.5 h, then the medium was changed gently. The expressions of fibronectin and vimentin were determined by immunocytochemistry, and the absence of \(\alpha\)-actin was determined by immunofluorescence. Cells from the second to fourth passages were used for all the experiments and serum-starved for 24 h before use. The cells were treated with IL-1\(\beta\) (0.01, 0.1, 1, 10, 100 ng/mL) for 24 h to measure cell DNA synthesis, collagen synthesis, gelatinase activity, MMP-2, MMP-9 mRNA and protein expressions.

1.3 \(^3\)H-TdR incorporation

After treatment with IL-1\(\beta\) for 20 h, 37 kBq \(^3\)H-TdR and 50 \(\mu\)g/mL ascorbate were added to every well and cultured for 4 h. The cells were then washed three times with distilled water, lysed with 1 mol/L NaOH. After that the cells were harvested onto glass fiber filters and dried. Radioactivity was quantified as count per minute (CPM) value by using liquid scintillation counter (Beckman LS6500, Fullerton, USA).

1.4 \(^3\)H-Pro incorporation

After treatment with IL-1\(\beta\) for 20 h, 37 kBq \(^3\)H-Pro and 50 \(\mu\)g/mL ascorbate were added to every well and cultured for 4 h. Medium was then removed, and the cells were lysed with 0.25% pancreatin and harvested onto glass fiber filters, dried. Radioactivity was quantified as CPM value by using liquid scintillation counter.

1.5 In-gel zymography

MMP activity in the conditioned medium of fibroblasts was measured as described previously\(^{[27]}\). After treatment with IL-1\(\beta\) for 24 h, the conditioned medium was collected, centrifuged for 10 min at 12 000 r/min to remove cells and debris, and lyophilized. The pellet was resuspended in water, and protein content was determined using Bradford assay. Samples (500 ng protein) were loaded under non-reducing condition onto 4% stacking and 8% separating SDS-polyacrylamide gel polymerized with 1 mg/mL gelatin and electrophoresed at 15-20 mA. Then the gels were washed in 2.5% Triton X-100 for 30 min at 4 °C twice followed
by 1 h of wash by distilled water. The gels were then incubated overnight at 37 °C in gelatinase substrate buffer (containing 50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L CaCl₂, and 200 mmol/L NaCl), stained with Coomassie blue R-250, and then destained using 10% acetic acid and 40% methanol solution. Clear and digested regions representing MMP activity were quantified as gray value measured by ImageJ, and molecular weights were estimated using prestained molecular weight markers.

1.6 Western blot analysis
The protein expressions of MMP-2 and MMP-9 in medium were studied using Western blot analysis. Protein content was determined using Bradford assay. Equal amounts of protein (30 µg) were loaded onto 4% stacking and 12% separating SDS-polyacrylamide gel and electrophoresed at 100-200 mV. Then proteins were transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were then probed with polyclonal anti-MMP-2 (1:2 000) and anti-MMP-9 (1:1 000) antibodies. The membranes were then incubated with appropriate secondary antibody and quantified as gray value measured by ImageJ. All protein expressions were normalized to GAPDH amounts.

1.7 RT-PCR analysis
Total RNA from cultured neonatal cardiac fibroblasts was extracted according to the TRIzol reagent protocol. The expressions of MMP-2 and MMP-9 mRNAs were determined by RT-PCR using primers (Table 1) at a final concentration of 0.25 µmol/L. Thirty-five cycles were performed as follows: 94 °C 5 min, 94 °C 45 s, 55 °C 1 min, 72 °C 90 s for MMP-2, and 94 °C 5 min, 94 °C 45 s, 56 °C 1 min, 72 °C 90 s for MMP-9. The adequacy of the different PCR products was verified by 1% agarose gel electrophoresis and quantified as gray value measured by ImageJ. All mRNA expressions were normalized to GAPDH amounts.

1.8 Statistical analysis
All data were expressed as mean±SD. Statistical analysis was performed using one-way ANOVA and a post hoc SNK test. P<0.05 was considered to be statistically significant.

2 RESULTS

2.1 Effects of IL-1β on DNA and collagen synthesis in rat CFs
Low-dose (0.01 ng/mL) of IL-1β had no effects on DNA synthesis and collagen synthesis in CFs as reflected by 3H-TdR (11 091.44±489.21 in control) and 3H-Pro incorporation (14 179.11±255.52 in control). However, ≥0.1 ng/mL IL-1β significantly reduced DNA synthesis (Fig.1A; 0.1 ng/mL: 8 647.48±468.26; P<0.05; 1 ng/mL: 8 025.65±273.45; 10 ng/mL: 7 123.75±417.74; 100 ng/mL: 7 038.54±253.37; P<0.01) and collagen synthesis (Fig.1B; 0.1 ng/mL: 11 279.50±920.32; 1 ng/mL: 10 501.50±397.24; 10 ng/mL: 10 263.71±639.44; 100 ng/mL: 9 045.36±630.48; P<0.01) in a dose-dependent manner.

2.2 Effects of IL-1β on MMP activity
MMP activity in the conditioned medium was measured by in-gel zymography. IL-1β increased MMP-2 and MMP-9 activities (Fig.2A). MMP-2 activity increased (vs 119.48±6.62 in control) in a dose-dependent manner (Fig.2B; 0.01 ng/mL: 139.07±9.43; P<0.05; 0.1 ng/mL: 156.58±9.25; 1 ng/mL: 163.33±7.90; 10 ng/mL: 172.77±11.15; 100 ng/mL: 180.95±9.69; P<0.01) and MMP-9 activity also significantly increased (vs 88.43±10.25 in control) in conditioned medium after exposure of CFs to IL-1β for 24 h (Fig.2C; 0.01 ng/mL: 105.35±6.60; 0.1 ng/mL: 110.03±9.26; 1 ng/mL: 112.79±10.66; P<0.05; 10 ng/mL: 122.37±5.90; 100 ng/mL: 132.95±11.09; P<0.01). But the effect of IL-1β on MMP-9 activity was not significantly different among 0.01, 0.1 and 1 ng/mL IL-1β.

2.3 Effects of IL-1β on MMP-2 and MMP-9 protein expressions
MMP activity in the conditioned medium was measured by Western blot. IL-1β
Fig. 1. Effects of IL-1β on DNA and collagen synthesis in rat cardiac fibroblasts. Rat cardiac fibroblasts were treated with different doses of IL-1β for 24 h in the presence of 37 kBq ³H-TdR and ³H-Pro during last 4 h. Incorporation of ³H-TdR (A) and ³H-Pro (B) was measured as count per minute (CPM) value. Data were shown as mean±SD of CPM from 6 experiments. *P<0.05, **P<0.01 vs control.

Fig. 2. Effects of IL-1β on MMP activity in rat cardiac fibroblasts. Rat cardiac fibroblasts were treated with different doses of IL-1β for 24 h.

MMP-2 and MMP-9 activities were measured as gray value. A: In-gel zymography result. B: Quantification of MMP-2 activity. C: Quantification of MMP-9 activity. Data were shown as mean±SD of gray value from 3 experiments. *P<0.05, **P<0.01 vs control.

dose-dependently increased MMP protein expression (Fig. 3). IL-1β stimulated MMP-2 protein expression (fold change vs 1.00±0.05 in control; 0.01 ng/mL: 1.35±0.05; 0.1 ng/mL: 1.56±0.10; P<0.05; 1 ng/mL: 2.15±0.32; 10 ng/mL: 2.34±0.19; 100 ng/mL: 2.41±0.26; P<0.01) and MMP-9 protein expression (fold change vs 1.00±0.05 in control; 0.01 ng/mL: 1.38±0.20; P<0.05; 0.1 ng/mL: 2.37±0.21; 1 ng/mL: 2.56±0.31; 10 ng/mL: 2.60±0.19; 100 ng/mL: 2.67±0.15; P<0.01), respectively.

2.4 Effects of IL-1β on MMP-2 and MMP-9 mRNA expressions

MMP-2 and MMP-9 mRNA expressions were measured by RT-PCR. IL-1β dose-dependently increased MMP mRNA expressions (Fig. 4). IL-1β stimulated MMP-2 mRNA expression (fold change vs 1.00±0.04 in control; 0.01 ng/mL: 1.81±0.16; P<0.05; 0.1 ng/mL: 2.17±0.19;
Fig. 3. IL-1β stimulated MMP protein expressions in the conditioned medium of rat cardiac fibroblasts. Rat cardiac fibroblasts were treated with different doses of IL-1β for 24 h and the protein expressions of MMP-2 (A, B) and MMP-9 (A, C) were analyzed by Western blot. The experiment was repeated three times with similar results. Data were shown as fold change of gray value as compared with control. *P<0.05, **P<0.01 vs control.

Fig. 4. Effects of IL-1β on MMP mRNA expressions in rat cardiac fibroblasts. Rat cardiac fibroblasts were treated with different doses of IL-1β for 24 h and mRNA expressions of MMP-2 (A and B) and MMP-9 (C and D) were analyzed by RT-PCR. The experiment was repeated three times with similar results. Data were shown as fold change of gray value as compared with control. *P<0.05, **P<0.01 vs control.
1 ng/mL: 2.18±0.04; 10 ng/mL: 2.27±0.06; 100 ng/mL: 2.31±0.07; \( P<0.01 \) and MMP-9 mRNA expression (fold change was 1.00±0.04 in control; 0.01 ng/mL: 1.36±0.13; \( P<0.05 \); 0.1 ng/mL: 1.49±0.08; 1 ng/mL: 1.50±0.11; 10 ng/mL: 1.51±0.24; 100 ng/mL: 1.52±0.31; \( P<0.01 \), respectively.

3 DISCUSSION

Post-MI ventricular remodeling includes cardiomyocyte hypertrophy and myocardial fibrosis. Myocardial fibrosis is the most important process of ventricular remodeling, which involves alteration in the ECM including collagen synthesis decrease, degradation and denaturation. Those changes promote the ventricular dilation and rupture\(^4\). IL-1\( \beta \), tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and IL-6 are pro-inflammatory cytokines that have been found to be elevated in the serum of patients who underwent MI\(^5,6\). This initiates pro-inflammatory cytokines which may play an important role in the process of post-MI remodeling. Previous study showed that IL-1\( \beta \) (4 ng/mL), TNF-\( \alpha \) (100 ng/mL) decreased collagen synthesis without change in cell number or total protein synthesis, increased total MMP activity, causing specific increases in the bands corresponding to MMP-13, MMP-2, and MMP-9 and the expressions of proMMP-2 and proMMP-3 mRNA\(^7\). But the incidence and extent of ventricular remodeling were accompanied with infarct size depending on the number of cardiomyocyte necrosis and detriment. The elevation of IL-1\( \beta \) was also accompanied with the enlargement of MI, but the relationship between ventricular remodeling and different concentrations of IL-1\( \beta \) was not clear.

Collagen is the major composition of ECM, so the balance between the synthesis and degradation of collagen is the major determinant of ECM integrity. Collagen is mainly synthesized by CFs and decomposed by extracellular MMPs. This study demonstrated that interfering with high-dose of IL-1\( \beta \) (≥0.1 ng/mL), the decrease in collagen synthesis as reflected by \(^{3}H\)-Pro incorporation occurred accompanied with the change in cell number in a dose-dependent manner as reflected by \(^{3}H\)-TdR incorporation and it was different from the previous study by Siwik et al. which showed the cell number and total protein were not changed\(^8\). The difference may be caused by the methods to calculate the cell number and DNA synthesis could have derangement with protein synthesis. Higher dose of IL-1\( \beta \) could decrease more cell and collagen synthesis, indicating that the decrease in cell number may be the major cause of collagen synthesis decrease and the different concentrations of IL-1\( \beta \) after MI were may be the major influencing factor of collagen synthesis in CFs.

Collagen degradation is regulated by the balance between MMPs and their inhibitors called TIMPs in ECM. MMPs are a class of the zinc-dependent enzymes that have a high specificity for components of ECM and secreted in a pro-enzyme form and require proteolytic cleavage for activation\(^9\). MMPs selectively degrade extracellular proteins such as the fibrillar collagens. According to the different substrates, MMPs can be divided into collagenases (MMP-1, MMP-8, and MMP-13), stromelysins (MMP-3 and MMP-10), and gelatinases (MMP-2 and MMP-9)\(^9\). MMP-2 and MMP-9 are the important compositions of MMP family which can degrade the denatured collagen fibril and elastin. MMP-9 contributes to cardiac rupture and MMP-9\(^{-/-}\) mice could partially protect against rupture after acute MI. It may be related to complete degradation of collagen fibers depending on a concerted action of interstitial collagenases and gelatinases\(^{10}\). MMP activity is regulated by both transcriptional and posttranslational mechanisms. We founded that IL-1\( \beta \) increased MMP-2 and MMP-9 protein expressions as measured by Western blot and MMP activity as measured by in-gel zymography in a dose-dependent manner, indicating that IL-1\( \beta \) may increase MMP activity partly due to protein expression through posttranslational mechanism. It also dose-dependently increased mRNA expressions of MMP-2 and MMP-9, suggesting that IL-1\( \beta \) also can influence MMPs through transcriptional way. So up-regulation of mRNA expressions of MMP-2 and MMP-9 by IL-1\( \beta \) was partly contributed to protein expressions of MMP-2 and MMP-9, followed with increasing activity of MMPs and the different concentrations of IL-1\( \beta \) after MI were also the major influencing factor in collagen decomposition in CFs just like its synthesis. So in the process of MI, the decrease in infarct size maybe not only preserve the function of heart and degrade the inflammation response but also reduce the incidence and extent of ventricular remodeling.

The data presented here indicate that IL-1\( \beta \) regulates the MMP activities through different mechanisms in neonatal SD rat CFs and the effects were in a dose-dependent manner. It is important to note that the present study deals specifically with the effect of IL-1\( \beta \) on rat CFs. Though IL-1\( \beta \) had been found to be elevated in the serum of patients who underwent MI, it was not clear whether the mechanisms of IL-1\( \beta \) in human CFs were the same as that in rat CFs. Further, the pathway of those effects should be studied in future.
REFERENCES


