Hypoxia-stimulated cardiac fibroblast production of IL-6 promotes myocardial fibrosis via the TGF- β 1 signaling pathway

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Interleukin-6 (IL-6) is a multifunctional cytokine produced by several cell types that has a role in fibrosis. Fibroblasts (FBs) maintain this underlying pathogenic change through regulation of IL-6 production; however, its potential functional role in regulating surrounding cellular structural changes during ischemic myocardial remodeling remains unexplored. Here, we generated FBs, cardiomyocytes (CMs), and blood vascular endothelial cells (ECs) from the ventricles of neonatal rats. IL-6 was then overexpressed in FBs and the cells were treated with IL-6 receptor inhibitor (IL6RI), TGF- β 1 receptor inhibitor (T β RI), or MMP2/MMP9 inhibitor (MMPI) using monoculture or coculture models under hypoxic conditions. The results indicate that overexpression of IL-6 is sufficient to induce myofibroblastic proliferation, differentiation, and fibrosis, probably via increased TGF- β 1-mediated MMP2/MMP3 signaling. The use of IL6RI, T β RI, or MMPI diminished these effects. In addition, IL-6 activated the apoptosis-associated factors Caspase3 and Smad3, and decreased the expression of anti-apoptotic factor Bcl2, resulting in apoptosis of CMs under hypoxic coculture: IL6RI or T β RI inhibited these effects. Unexpectedly, IL-6-overexpressing FBs significantly increased the angiogenesis of ECs, which involved significant increases in the expression of proangiogenic growth factors. Treatment of FBs with IL6RI or T β RI in coculture with ECs reduced the levels of secreted proangiogenic growth factors, and the angiogenesis of ECs was significantly downregulated. Thus, IL-6 functions in ischemic myocardial remodeling through multifunctional reprogramming of hypoxia-associated FBs towards fibrosis via upregulation of the TGF- β 1 signaling pathway.

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Myocardial remodeling following myocardial infarction (MI) is emerging as a key cause of chronic infarct mortality. Ischemia/reperfusion injury causes cardiomyocyte (CM) apoptosis and ventricular remodeling, leading to a dilated heart. Hypoxia is one of the causes of ischemia damage, and cardiac fibroblasts (FBs), the most prevalent cell type in the ischemic heart, have a key role in adverse myocardial remodeling. Ischemia-associated FBs have a critical influence on myocardial remodeling progression and fibrosis.^{1–3} Myocardial remodeling is the product of interactions between FBs and adjacent cells, such as CMs, and blood vascular endothelial cells (ECs), although the exact mechanisms remain poorly understood.^{4,5}

Pleiotropic effects of cardiac FBs are mediated through differentiation to a myofibroblast phenotype and secretion of pro-inflammatory cytokines (eg, IL-6, TGF β , TNF α , and IL-1).⁶ IL-6 is a classic pro-inflammatory cytokine with pleiotropic effects in the acute-phase response in MI,⁷ and drives chronic inflammation, autoimmunity, EC dysfunction, and fibrogenesis.⁸ Recent evidence has suggested that IL-6 increases continuously during the early stage after induction of MI,⁹ and may be a powerful predictor of acute coronary syndrome.¹⁰ A comprehensive understanding of the biological roles of IL-6 in myocardial ischemia leading to inflammatory response and disease-related ventricular remodeling would help to find a solution to chronic heart failure. Other studies

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indicate that IL-6 may have important roles in EC dysfunction and fibrogenesis in systemic sclerosis, and clinical trials are currently being designed to explore whether tocilizumab, a monoclonal antibody directed against the IL-6 receptor, is of therapeutic benefit to patients with this disease.⁸ Recently, heart aging and toxicosis were observed to be associated with increased expression of IL-6 from cardiac FBs, contributing to CMs' senescence and the differentiation of FBs to myofibroblasts, which could be alleviated by blocking the TGF β 1-mediated pathway.^{11,12}

However, the effects of IL-6-mediated signals on progression of myocardial fibrosis and alteration of cardiac cell components have not been investigated in an experimental model of hypoxia-induced myocardial injury. Therefore, we examined the effects of IL-6-mediated signals using three different cells (FBs, CMs, and blood vascular ECs) to investigate their multiple effects on myocardial fibrosis, apoptosis, and angiogenesis during left ventricular remodeling induced by continuous hypoxia in rats.

MATERIALS AND METHODS

An expanded Materials and Methods section containing details regarding the culture and purification of CMs, ECs, and FBs, cell treatments and group, analysis of cell proliferation and apoptosis, assays of endothelial colony forming units, tube formation, and angiogenesis, quantitative real-time reverse transcription–polymerase chain reaction (PCR), immunoblotting, immunocytofluorescence, and statistics is available in the online-only data supplement.

Culture and Purification of CMs, ECs, and FBs

Primary cultures of neonatal ventricular CMs, ECs, and FBs were prepared from the ventricles of 1–3-day-old Wistar strain rats. These cells were purified by magnetic-activated cell sorting.

Cell Treatments and Groups

As described in Supplementary Figure S1, the purified FBs were divided into two parts: monoculture conditions and coculture with myocardial cells or ECs. In the monoculture conditions, FBs were cultured for analysis of the effects of IL-6 on their proliferation and differentiation under normoxic condition (NO, serving as the control group) with recombinant IL-6 (NO+IL-6) or IL-6 receptor inhibitor (IL6RI) (NO+IL6RI), and hypoxic culture (HO) with recombinant IL-6 (HO+IL-6) or IL6RI (HO+IL6RI). These FBs were also cultured under hypoxia without (CON) or with IL-6 (IL-6), IL-6 plus IL6RI (IL-6+IL6RI), IL6RI (IL6RI), IL-6 plus TGF β 1 receptor inhibitor (T β RI, IL-6+T β RI), and T β RI to analyze their conversion and fibrosis. MMP inhibitors were also used to support the expression changes.

In the coculture experiments, FBs were analyzed by hypoxic coculture for the effects of FB-mediated IL-6 signals on CMs apoptosis and ECs' angiogenesis. Coculture was performed in the absence (CON) or presence of IL-6, IL-6

plus IL6RI (IL-6+IL6RI), IL6RI (IL6RI), IL-6 plus T β RI (IL-6+T β RI), or T β RI (T β RI).

Cell Proliferation and Apoptosis Analysis

FBs proliferation were assessed by fluorescence staining for proliferation marker Ki67. Cells were analyzed by a fluorescence-activated cells sorter (BD), western blot, and immunofluorescence. Apoptotic cell death under hypoxic conditions was evaluated through annexin V staining and the TUNEL assay.

Angiogenesis Assay

The effects on angiogenesis of paracrine secretion by ECs under hypoxia were studied using ECs cocultured with FBs in the presence or absence of IL-6 overexpression from three individual experiments. The ECs were lysed with 2 $\times$ cell lysis buffer (RayBiotech, Norcross, GA, USA) and quantified using an Angiogenesis Protein Array kit (QAR-ANG-100, RayBiotech).

Assay of Colony Forming Units, Tube Formation, and Angiogenesis of ECs

ECs colonies were counted visually under an inverted microscope. Tube formation was observed through a reverted and phase-contrast photomicroscope. Angiogenesis of ECs was detected using immunofluorescence with factor VIII-positive-staining.

Reverse Transcriptase PCR and Immunoblotting

Cells were harvested and pulverized to extract RNA or protein for quantitative real-time reverse transcription–PCR and immunoblotting.

Immunocytofluorescence

Cells were fixed in 4% paraformaldehyde solution, and then stained using immunocytofluorescence.

RESULTS

IL-6 is Mainly Produced by Cardiac FBs in Response to Hypoxia

We first evaluated the cellular source of IL-6 in ischemia-induced cardiac fibrosis. CMs, ECs, or FBs derived from the hearts of neonatal rat were cultured under hypoxic conditions for 12 h. To normalize the expression levels of IL-6 in these different cell populations, levels in normoxic culture was served as controls. Quantitative real-time reverse transcription–PCR revealed that the IL-6 expression was primarily present in cardiac FBs, especially during hypoxia (hypoxia, 8.7fold over CMs, 7.0fold over ECs, all $P < 0.001$, Figure 1a). After normalization of IL-6 expression between normoxia and hypoxia, the change in IL-6 expression during hypoxia was still significantly higher in FBs than in CMs and ECs (Figure 1b). No significant difference was seen between CMs and ECs. Western blotting also confirmed that the highest level of IL-6 protein expression was in the FBs under HO (Figure 1c). Furthermore, immunofluorescence revealed

Figure 1 IL-6 expression in cardiac fibroblasts, cardiomyocytes, and blood vascular endothelial cells. **(a)** Quantitative analysis of the mRNA expression levels of IL-6 in cardiac fibroblast, cardiomyocyte, and blood vascular endothelial cells, 12 h post normoxic/hypoxic culture. **(b)** The mRNA expression level of IL-6 during hypoxic culture relative to normoxia. $P < 0.05$: *vs Cardiac fibroblast under the same culture condition, [†]vs normoxic culture ($n = 10$, per group). **(c)** Western blot analysis of the protein level of IL-6 in these three cells 12 h after normoxic culture or hypoxic culture. Cells were subjected to western blotting analysis using an antibody directed against IL-6. GAPDH was used as internal control. IL-6 was strongly expressed in cardiac fibroblasts, but very weakly in cardiomyocytes and blood vascular endothelial cells. **(d)** Representative immunofluorescence staining of IL-6 (green) and specific cell marker proteins (red), including vimentin, myosin heavy chain, and factor VIII. Nuclei are DAPI stained (blue). Compared with cardiac fibroblasts, IL-6 staining in cardiomyocytes and blood vascular endothelial cells was significantly weaker. IL-6 was expressed simultaneously in the nucleus and cell cytoplasm of fibroblasts, but there appears to be exclusively nuclear/perinuclear distribution of IL-6 in cardiomyocytes and endothelial cells compared with fibroblasts during both hypoxic culture and normoxic culture. Scale bars: 50 μm .

significantly greater IL-6 levels in the vimentin-positive FBs than in MHC-positive CMs and factor VIII-positive ECs under HO (Figure 1d). Thus, endogenous IL-6 is mainly produced by cardiac FBs in response to hypoxia.

IL-6 Stimulated FB Proliferation and Myofibroblastic Differentiation

To test the hypothesis that IL-6 increases the proliferation and differentiation of cardiac FBs, cells were incubated with medium containing no IL-6/IL6RI, IL-6, or IL6RI for 72 h under hypoxic conditions. Normoxic cultivation with medium containing no IL-6/IL6RI, IL-6, or IL6RI was also performed to clarify these effects under hypoxia. Cell proliferation was assessed using Ki67 expression, as assessed by fluorescence-activated cells sorter, western blotting, and immunofluorescence. As shown in Figure 2a, dynamic analysis of cell proliferation and staining with Ki67 was assessed by fluorescence-activated cells sorter at each time point after different treatments, which revealed that IL-6

stimulated FB proliferation kinetically, and IL6RI abolished this kinetic stimulation. Compared with normoxia, hypoxia enhanced the increase in IL-6 expression in FBs and increased cell proliferation. This promotion was also observed in the IL-6 overexpressing FBs. Under hypoxic conditions, IL-6 overexpression strongly promoted FBs' proliferation compared with cells cultured with IL-6 under normoxic culture and those cells without adding IL-6 or IL6RI under hypoxia. IL6RI canceled this increase in proliferation, which was more pronounced in the HO group (the HO+IL6RI group). Western blotting revealed that the level of Ki67 was consistently higher in the HO+IL-6 group compared with the NO+IL-6 group, higher in the HO group compared with the NO group, and that this change trend correlated well with the expression of IL-6 (Figure 2b). These results showed that the cellular density under hypoxic conditions was higher in the HO+IL-6 group than in the HO group, and was the lowest in the HO+IL6RI group (Figure 2c). Immunofluorescence showed significantly more positively stained cells in the group

with IL-6 overexpression than in the group without IL-6 overexpression, and showed more positively stained cells under hypoxia than under normoxia, with the lowest number of stained cells in the HO+IL6RI group (Figure 2c). To

evaluate if IL-6 expression promotes myofibroblastic differentiation, FBs 72 h after IL-6 intervention were subjected to western blotting analysis using a panel of myofibroblastic markers (Figure 2b). The results showed that IL-6 was

Figure 2 IL-6 overexpression induces cardiac-associated fibroblast proliferation and differentiation. **(a)** Dynamic analysis of cell proliferation evaluated by FACS in the individual groups at 0 h, 24 h, 48 h, and 72 h after different treatments. $P < 0.05$: *vs at 0 h post treatment, †vs 24 h post treatment, ‡vs 48 h post treatment, §vs the normoxia, ‖vs the NO/HO group under the same culture conditions, ¶vs the IL-6 group ($n = 10$, each group). **(b)** Equal protein loading was assessed by immunoblotting for proliferation marker protein, Ki67, and myofibroblast marker proteins, including SMA, fibronectin, and vimentin at 72 h after treatment. Successful overexpression of the IL-6 protein was verified by western blotting analysis in the NO+IL-6 group and the HO+IL-6 group. Note that IL-6 overexpression strongly induced the expression of myofibroblast markers, such as α -SMA, fibronectin, and vimentin in these two groups, although its significant decreases in the levels of these proteins were observed in the NO+IL6RI group and the HO+IL6RI group. **(c)** Fluorescence microscope images of the cells double stained with DAPI and Ki67, showing the greatest positive staining in the HO+IL-6 group, the second highest in the HO group, and the lowest in the HO+IL6RI group. **(d)** Immunocytofluorescence images of the cells positively double stained with DAPI and myofibroblast characteristic marker SMA (red) at 72 h post treatment. Nuclei are stained blue with DAPI. The density of SMA-positive staining was the greatest in the HO+IL-6 group, and the smallest in the HO+IL6RI group. Scale bars: 50 μ m.

Figure 2 Continued.

sufficient to induce increased protein expression of α -SMA, fibronectin, and vimentin in the IL-6 overexpressing FBs, and that IL6RI significantly reduced the expression levels of these proteins. Hypoxia promoted these changes. Immunofluorescence further confirmed that the number of cells positively stained with α -SMA was the greatest in the HO+IL-6 group, followed by the NO+IL-6 group, with the smallest number in the HO+IL6RI group (Figure 2d).

IL-6 Overexpression Induces the Activation of TGF- β 1/MMP2/MMP9 Signaling under Hypoxia

Several lines of evidence indicate that activated FBs increase their expression and secretion of TGF- β 1, thereby promoting myocardial fibrosis.^{11,12} Therefore, we next tested if IL-6 overexpression upregulates the expression of TGF- β 1-related signaling molecules under hypoxic conditions. Consistent with this hypothesis, Figures 3a and d show that the mRNA and protein levels of TGF- β 1 were significantly upregulated by IL-6 overexpressing and downregulated by inhibition of its receptor inhibitor by IL6RI. Immunofluorescence showed that TGF- β 1 was mainly observed in the cytoplasm,

particularly in the perinuclear region (Figure 3e). When treated with IL-6, the TGF- β 1 staining intensity was markedly increased, although IL6RI markedly suppressed the staining intensity. The inhibitory effect was observed whether IL6RI was combined with IL-6 or not. T β RI did not affect the expression of IL-6. All these data indicated that IL-6 overexpression indeed activated TGF- β 1 signaling.

Next, we observed the effects of IL-6 on the expression of two matrix metalloproteinases (MMPs): MMP2 and MMP9 under hypoxia. As shown in Figures 3b–d, IL-6 overexpression increased the mRNA, and protein expression of MMP2 and MMP9 after 72 h under HO compared with the control. These changes were canceled by treatment with IL6RI or T β RI, and there was no significant difference in the expression of MMP2 and MMP9 between FBs receiving IL6RI or T β RI combined with IL-6 and FBs receiving IL6RI or T β RI only.

IL-6-TGF- β 1/MMP2/MMP9 Signaling Promotes Conversion and Fibrosis of FBs under Hypoxia

To examine the potential effect of IL-6 on myofibroblast conversion and fibrosis, FBs were treated with IL-6, with or

Figure 3 IL-6 overexpression induced TGF- β 1 signaling under hypoxia. (a), (b), and (c), qRT-PCR analysis of mRNA expression of TGF- β 1, MMP2, and MMP9 revealing that these three genes were significantly increased in IL-6 overexpressing FBs, compared with control FBs. Conversely, the IL-6 receptor inhibitor IL6RI significantly reduced the mRNA and protein expression of TGF- β 1, MMP2, and MMP9. $P < 0.05$: *vs the CON fibroblast, † vs IL-6 overexpressing fibroblast ($n = 10$, each group). (d) Cells were subjected to immunoblot analysis using antibodies directed against TGF- β 1, MMP2, and MMP9. Note that IL-6 increased the expression of these three proteins, and IL6RI decreased their expression, when compared with control FBs. (e) After 72 h of hypoxic culture, the cells were fixed and stained with TGF- β 1 (cytoplasm, red) and DAPI (nuclei, blue). Note that IL-6- overexpressing FBs displayed an increased number of TGF- β 1-positive staining FBs compared with the control cells. IL6RI significantly decreased the numbers of these TGF- β 1-positive staining fibroblasts. Scale bars: 50 μ m.

without treatment with IL6RI, T β RI, or MMPI. Real-time PCR and immunofluorescence were used to examine the expression levels of α -SMA, a symbol of FBs phenotypic conversion, and collagen I, a marker of fibrosis-related genes. We found that IL-6 remarkably increased the mRNA levels of α -SMA and collagen I. In contrast, the pretreated FBs with

IL6RI, T β RI, or MMPI exhibited a downregulation of α -SMA and collagen I, and no significant difference was seen in the expression of α -SMA and collagen I between FBs receiving IL6RI, T β RI, or MMPI combined with IL-6 and FBs receiving IL6RI, T β RI, or MMPI only (Figure 4a). The immunofluorescence showed the similar change trends in the protein

Figure 4 Overexpression of IL-6 induced FBs differentiation into myofibroblasts and fibrosis under hypoxia. **(a)** Quantitative analysis of α -SMA and collagen I mRNA expression in FBs treated with IL-6 in the presence or absence of the inhibitors of IL-6, TGF- β 1 and MMP2/MMP9. $P < 0.05$: *vs the CON fibroblast, † vs IL-6 overexpressing fibroblast ($n = 10$, each group). **(b)** Representative images of immunofluorescence staining for α -SMA (red) and collagen I (green) in FBs treated as mentioned previously. Nuclei were stained with DAPI (blue). Scale bar: 50 μ m.

expression levels of α -SMA and collagen I. Immunofluorescent staining also revealed increased cell particles that were positively stained for α -SMA and collagen I, which was indicative of myofibroblasts formation and collagen transformation in IL-6-overexpressed cells. In contrast, using inhibitors of IL-6, TGF- β 1, or MMP2/MMP9 significantly reduced the increase in α -SMA and collagen I protein expression in cells treated with IL-6 or without IL-6 (CON) (Figure 4b). It appears that the treatment of FBs with inhibitors of IL-6, TGF- β 1, or MMP2/MMP9 could significantly reduce the expression levels of α -SMA, collagen I mRNA, and protein induced by IL-6.

FBs Overexpressing IL-6 Activate Apoptosis of CMs under Hypoxia

Acute release of IL-6 and TGF- β could regulate the survival or apoptosis of CMs in the infarcted zone;¹³ therefore, we validated that IL-6 could induce not only the apoptosis of CMs, but also the activation of apoptosis-related signaling pathways. As shown in Figures 5a and b, CMs cocultured with FBs under hypoxic conditions revealed 18–24% of apoptosis, as assessed by PI/annexin V staining and TUNEL staining, respectively. Moreover, FBs overexpressing IL-6 resulted in further increased levels of CMs apoptosis. IL6RI or T β RI significantly decreased the cell apoptosis rate. TUNEL labeling showed that the number of TUNEL-positive cells reached the highest level in the CMs cultured with IL-6 overexpressing FBs, which showed signs of degeneration, such as condensed chromatin, typical of apoptosis (arrow, Figure 5c). By contrast, CMs treated with IL6RI or T β RI-treated FBs showed less apoptosis.

Next, we evaluated the apoptotic pathway. Quantitative real-time reverse transcription-PCR and western blotting (Figures 5d–f and h) showed that IL-6 overexpression significantly increased the mRNA and protein expression levels of apoptotic signaling molecules, including Smad3 and caspase3, compared with the control cells; however, IL6RI or T β RI significantly decreased their expression levels. Similar to Smad3 expression, both the representative western blots using an anti-pSmad3 antibody and the bar graph of pSmad3 level showed the greatest protein and phosphorylation levels in the cells treated with IL-6 (Figures 5g and h). Conversely, the influence of IL-6, IL6RI, and T β RI on anti-apoptotic protein

Bcl2 showed the opposite trend: IL-6 overexpression down-regulated Bcl2 expression, and IL6RI and T β RI upregulated its expression. These results suggested that FBs induced apoptosis of CMs under hypoxic conditions via the IL-6-TGF- β 1-mediated Smad3/caspase3/Bcl2 pathway.

FBs Overexpressing IL-6 Promote Angiogenesis of Vascular ECs under Hypoxia

We used a rat angiogenesis array to analyze the expression of 60 angiogenesis-related cytokines from ECs harvested under hypoxia, in the absence or presence of IL-6. The results showed that two isoforms of angiopoietins, Ang-1 and Ang2, and their tyrosine kinase receptors (Tie1, Tie2), Angiogenin, basic fibroblast growth factors (bFGF), follistatin, GM-CSF, GCSF, hepatocyte growth factor (HGF), IL-1, IL-2, IL-6, insulin-like growth factor I, metalloproteinase inhibitor 2 (TIMP-2), MCP-1, MCP-2, MCP-3, MMP-1, MMP2, MMP9, TGF β 1, VEGF, VEGFR2, IL-17, IP-10, RANTES, TNF α , and I-TAC were present in both conditions. Among them, Ang-1, bFGF, GM-CSF, GCSF, IL-1, IL-2, IL-6, HGF, MCP-1, MCP-2, MCP-3, MMP2, MMP9, TGF β 1, Tie2, TNF α , VEGF, and VEGFR2 were expressed abundantly, all at >60 pg in 30 μ g of hypoxia-cultured EC total proteins and 10 pg in 30 μ g of normoxia-cultured EC total proteins. We compared the quantity of these cytokines expressed under both conditions and found that Ang-1, bFGF, IL-6, HGF, MCP-1, MCP-2, MCP-3, MMP2, MMP9, TGF β 1, Tie2, VEGF, and VEGFR2 in ECs were expressed in greater quantities in the presence of IL-6 expression than in the absence of IL-6 expression ($P < 0.01$, Figures 6a and b). However, activin A, AgRP, ANGPTL4, angiostatin, CXCL16, FGF-4, ENA-78, GRO, IL-4, IL-10, IL-12p40, IL-12p70, LIF, PIGF, TGF α , TGF β 3, Tie1, TPO, VEGFR3, VEGF-D, TNF β , Leptin, PDGF-BB, PIGF, PECAM-1, and MCP-4 were expressed neither in the presence or absence of IL-6 overexpression (data not shown). Hypoxia can upregulate TGF- β 1 expression by microvascular pericytes that secrete IL-6, which enhances pericyte angiogenesis.¹⁴ Therefore, based on the results of antibody array, we selected Ang-1, bFGF, HGF, and VEGF for western blotting detection in ECs receiving IL-6 overexpression or not to validate the result of antibody array, and to evaluate whether IL-6 could promote angiogenesis induced by blood vascular ECs. For this purpose, we developed a

Figure 4 Continued.

coculture model. IL-6-overexpressing FBs were cocultured with ECs in the presence or absence of IL6RI or T β RI. Figures 6c–f shows that compared with the control cells, IL-6 overexpression in FBs significantly promoted mRNA of vascular growth factors in ECs, such as Ang-1, bFGF, HGF, and VEGF. IL6RI or T β RI abolished this promotion. These results were further confirmed by representative Western blot analysis of bFGF and VEGF (Figure 6g).

After replating on fibronectin-coated dishes, the ECs cocultured with the IL-6 overexpressing FBs had the greatest number of colony forming units, and the smallest number of colony forming units was seen in the ECs cocultured with the IL6RI-treated FBs or the T β RI-treated FBs (Figures 6i and k). To investigate the angiogenesis of ECs after coculture with FBs, an *in vitro* matrigel tube formation assay was performed. ECs cocultured with the IL-6 overexpressing FBs showed a significant enhancement of tube-forming activity compared with those cocultured with T β RI-treated FBs (Figure 6j). Western blotting and immunofluorescence were performed to analyze the expression of the vascular characteristic marker, factor VIII. The highest expression of factor VIII was observed in the ECs cocultured with the IL-6 overexpressing FBs, followed by that in the CON ECs, and the lowest was observed in the ECs cocultured with the IL6RI-treated FBs or the T β RI-treated FBs (Figures 6g, h and l). The vascular EC density was revealed by staining with anti-factor VIII antibody (red cytoplasmic stain). These results indicate that IL-6 induced angiogenesis in ECs under hypoxic conditions.

DISCUSSION

In this study, we show that FBs enhance the structural alteration of heart cells, termed myocardial fibrosis, displaying fibrosis proliferation, collagen formation, myocardial apoptosis, and angiogenesis in a paracrine manner and in direct coculture *in vitro*. One of the main stimulators secreted by FBs is IL-6. The *in vitro* hypoxic conditioning of myocardial FBs, eg, mimicking the post-MI microenvironment (ischemia), strongly upregulated IL-6 production and further augmented the proliferation and collagen formation of FBs, the apoptosis of CMs, and the angiogenesis of blood vascular ECs. IL-6-stimulated myocardial remodeling was accomplished through activation of TGF- β 1-mediated signaling pathways.

Myocardial apoptosis and fibrosis are common and coexisting pathological features after MI. Clarifying the structural and functional interactions between CMs and FBs is essential to understand the pathophysiological heart.¹⁵ Meléndez *et al*¹⁶ showed that IL-6 infusion, both *in vivo* and *in vitro*, in rats resulted in myocardial remodeling with considerable fibrosis within the myocardium, and the soluble IL-6 receptor in combination with IL-6 was essential to increase the collagen concentration by isolated cardiac FBs. IL-6 also has a role in mediating the phenotypic conversion to myofibroblasts. Our present results confirmed these findings: in comparison with normoxia, hypoxia upregulated IL-6 expression in FBs (Figure 1). The expression of cell proliferation marker Ki67, and the myofibroblast markers α -SMA, fibronectin, and vimentin, were consistent with IL-6 expression. Moreover, IL-6 overexpression and hypoxia further promoted these expression, whereas the IL6RI canceled this promotion and induction (Figure 2). Thus, it can be seen that IL-6 induced by hypoxia triggered cell proliferation and differentiation. Moreover, there was a synergy between hypoxia and overexpression of IL-6 to increase the release of IL-6 and to induce differentiation of fibroblasts into myofibroblasts, which was consistent with the findings of Zhong *et al*,¹⁷ who demonstrated that hypoxia amplifies the effects of adenosine on the release of IL-6 and differentiation of FBs. These data further demonstrated that IL-6 might mediate myocardial fibrosis associated with MI directly. Cardiac FBs contribute to multiple aspects of myocardial function and pathophysiology. However, the pathogenic relevance of cytokine production by these cells under hypoxia, remains unexplored.¹⁸ Our findings are the first to establish that pathological elevations of IL-6 within extensive cardiac fibrosis under hypoxic conditions result in the activation of the TGF- β 1-mediated MMP2/MMP9 signaling pathway. With the use of an *in vitro* cell culture model, we demonstrated that treatment of rat neonatal FBs with recombinant IL-6 stimulated TGF- β 1 expression, and was accompanied by upregulation of MMP2 and MMP9 expression. This was corroborated by a strong decrease of MMP2 and MMP9 expression after addition of an IL6RI or a TGF- β 1 receptor neutralizing antibody to the conditioned medium for the FBs (Figure 3), indicating that the stimulatory effect of FBs conditioned with IL-6 under hypoxic conditions was accomplished through activation of TGF- β 1-mediated MMP2/MMP9 signaling pathways. Cytokines TGF β 1 and

Figure 5 Influence of the IL-6-TGF- β 1 pathway on apoptosis of CMs under hypoxic conditions. (a) and (b) Quantitative analysis of apoptosis of CMs after coculture with IL-6 overexpressing FBs or FBs treated with IL-6 receptor inhibitor (IL6RI) or TGF- β 1 receptor inhibitor (T β RI). FBs were evaluated by apoptosis (annexin V), cell death (propidium iodide (PI)), and TUNEL staining. (c) Typical images showing different apoptosis staining of CMs after hypoxic coculture with IL-6 overexpressing FBs, IL6RI-treated FBs, or T β RI-treated FBs. The nuclei of apoptotic cells were stained green (arrows). The images were revealed as an overlay of fluorescence microscopy and light microscopy. Scale bars: 50 μ m. (d), (e), and (f) Quantitative analysis of the differences in Bcl2, Caspase3, and Smad3 mRNA expression of CMs. (g) The phosphorylation level of Smad3 in CMs after hypoxic coculture with IL-6 overexpressing FBs, IL6RI-treated FBs, or T β RI-treated FBs. All $P < 0.05$: *vs the CON group, [†]vs the IL-6 overexpressing ($n = 10$, each group). (h) Representative immunoblots of Bcl2, Caspase3, pSmad3, and Smad3.

IL-6 are both produced by FBs, and act on FBs to promote inflammatory and fibrotic responses.¹⁹ Yang *et al*²⁰ observed that TGF- β 1 transfection caused overexpression of MMP9

and MMP2 after lung irradiation and led to further radiation-induced lung fibrosis.²⁰ The study of Faturi *et al*²¹ showed that intermittent stretching induces fibrosis via temporal

Figure 5 For caption see page on 847.

Figure 6 IL-6 improved EC angiogenic function under hypoxia. (a) and (b) Comparison of angiogenic factor concentrations (pg/ml) under hypoxic conditions, as detected by antibody array in ECs in the absence or presence of IL-6 overexpression. $P < 0.05$: *vs in the absence of IL-6 overexpression ($n = 3$, each group). (c), (d), (e), and (f) Quantitative analysis of Ang-1 (c), bFGF (d), HGF (e), and VEGF (f) mRNA expression in ECs treated with IL-6 in the presence or absence of the inhibitors of IL-6 or TGF-β1. $P < 0.05$: *vs the CON ECs, †vs IL-6 overexpressing ECs ($n = 10$, each group). (g) Representative western blots of the bFGF, VEGF, factor VIII protein levels in the ECs after coculture with FBs treated with IL-6 in the presence or absence of the inhibitors of IL-6, or TGF-β1. (h), (i), and (j) Quantification of factor VIII expression levels (h), CFU (i), and tube formation (j) of ECs after coculture with the IL-6 overexpressing FBs, the IL6RI-treated FBs, or the TβRI-treated FBs. $P < 0.05$: *vs the CON group, †vs the IL-6 overexpressing ($n = 10$, each group). (k) and (l) Typical images showing the changes in colony formation (arrows) and factor VIII expression of ECs after intervention with IL-6, IL6RI, or TβRI, respectively. Scale bars: 50 μm.

Figure 6 Continued.

modulation of TGF- β 1/myostatin and MMP9 cascades. Islam *et al*²² found that TGF- β 1 induces the development of bladder fibrosis via a Smad2/Smad3-dependent MMP2/MMP9 signal pathway. Thus, like IL-6, TGF- β 1 is a multifunctional cytokine that has different roles in different pathways in the development of fibrotic responses.²³ In the present study, we found that IL-6 remarkably increased the expression levels of α -SMA and collagen I. By contrast, IL6RI, T β RI, or MMP2/MMP9 inhibitor MMP1 significantly downregulated the expression of α -SMA and collagen I (Figure 4). These data suggested that IL-6 has a central role in the phenotypic conversion and collagen formation of myofibroblasts under hypoxia via TGF- β 1-MMP2/MMP9 signaling.

IL-6-TGF- β 1 signals have a critical role in FB proliferation and differentiation. However, their potential roles in myocardial apoptosis are underappreciated. In the current study, we demonstrated that IL-6-overexpressing FBs increased CM apoptosis induced by hypoxia, upregulated

the expression of proapoptotic factors including Smad3 and caspase3, and downregulated expression of anti-apoptotic factor Bcl2. To determine whether TGF- β 1 influences the activation of the apoptotic microenvironment, we added a T β RI to cocultured FBs. The results demonstrated that FBs receiving the T β RI showed significantly less myocardial cell apoptosis, with characteristic reregulation of the expression of apoptosis-related factors: downregulation of Smad3 and caspase3, and upregulation of Bcl2. Moreover, the T β RI could offset the effects of IL-6 when combined with IL-6, resulting in the inhibition of CM apoptosis (Figure 5). Therefore, overexpression of IL-6 is a likely mechanism by which FBs induce TGF- β 1-activation, leading to increased myocardial apoptosis caused by hypoxic stimulation. Our findings were supported by the study of Aguilar *et al*,²⁴ where culturing of cardiac FBs in high-glucose media led to significant increases in TGF- β 1 and Smad2/3, leading to increases in collagen levels. Thus, hypoxic IL-6 overexpressing

FBs create an unfavorable ischemic microenvironment to promote myocardial fibrosis via activation of TGF- β 1-mediated CM apoptosis.

TGF- β 1 is a potent inducer of increased fibrosis and neovessels, which have been implicated in conferring wound-associated angiogenesis.²⁵ Here, we demonstrated that IL-6 overexpression in FBs leads to increased production of TGF- β 1 in blood vascular ECs under hypoxic coculture, which is associated with an increase in the expression of proangiogenic factors, Ang-1, bFGF, HGF, and VEGF, and a significant enhancement of tube-forming activity and vascular EC density: IL6RI and T β RI abolished this promotion. The above cytokines might have a role in angiogenesis. First, the most important effector for this is VEGF.²⁶ Second, Ang-1 regulates sprouting angiogenesis and vascular remodeling.²⁷ Third, VEGF, bFGF, and Ang-1 are specific mitogens of ECs in EC proliferation and migration. Fourth, VEGF and TGF β 1 have been shown to be implicated in lumen formation and vessel stabilization.²⁸ Therefore, increased IL-6 expression in FBs benefits angiogenesis, likely via paracrine effects on ECs. In particular, the release of TGF- β 1 in the vicinity of blood vascular ECs may result in a more hospitable microenvironment, facilitating angiogenesis (Figure 6). Several authors have shown that IL-6 overexpression could lead to a commercial application for therapeutic angiogenesis, because of enhanced neovascularization in ischemic diseases.^{29,30} IL-6 may induce angiogenesis in blood vascular ECs via increased TGF- β 1-mediated endogenous production of angiogenic growth factors. The observed increase in angiogenesis of blood vascular ECs may in turn promote the proliferation of FBs. This assertion is consistent with our previous observations that the coexistence with collateralization and angiogenesis makes a large contribution to generating the ischemic niche that drives survival and neovascularization of blood endothelial progenitor cells.^{31,32} Whether these mechanisms in stem cells promote myocardial fibrosis in infarcted hearts requires further investigation. This may be related to the regulatory feedback mechanism described in Supplementary Figure S2: hypoxia activates IL-6 expression in FBs; promotes TGF- β 1 expression; triggers TGF- β 1-mediated signal pathways by upregulating the expression of MMPs, MMP2 and MMP9, apoptotic factors, Smad3, caspase3, and proangiogenic factors, bFGF and VEGF; and inhibits anti-apoptotic factor Bcl2, which results in enhanced FB proliferation and differentiation, increased myocardial apoptosis, and the promotion of angiogenesis. Upregulated angiogenesis of blood vascular ECs may in turn stimulate fibrosis. By contrast, inhibitors of the IL-6 receptor and TGF- β 1 receptor abolish these effects. Therefore, IL-6 injection may lead to deterioration of myocardial remodeling under hypoxic/ischemic conditions. Future research should use conditioned media from FBs to inhibit the target cells.

In conclusion, our results highlight the critical function of IL-6 as a driver of myocardial fibrosis. This is consistent with its ability to stimulate apoptosis in CMs and angiogenesis in

blood vascular ECs, and with its effects on myocardial remodeling of the ischemic microenvironment.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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