Combined effects of interleukin-1α and transforming growth factor-β1 on modulation of human cardiac fibroblast function

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Abstract

During cardiac remodeling, cardiac fibroblasts (CF) are influenced by increased levels of interleukin-1α (IL-1α) and transforming growth factor-β1 (TGFβ1). The present study investigated the interaction between these two important cytokines on function of human CF and their differentiation to myofibroblasts (CMF). CF were isolated from human atrial appendage and exposed to IL-1α and/or TGFβ1 (both 0.1 ng/ml). mRNA expression levels of selected genes were determined after 6-24 h by real-time RT-PCR, while protein levels were analyzed at 24-48 h by ELISA or Western blot. Activation of canonical signaling pathways (NFκB, Smad3, p38 MAPK) was determined by Western blotting. Differentiation to CMF was examined by collagen gel contraction assays. Exposure of CF to IL-1α alone enhanced levels of IL-6, IL-8, matrix metalloproteinase-3 (MMP3) and collagen III (COL3A1), but reduced the CMF markers α-smooth muscle actin (αSMA) and connective tissue growth factor (CTGF/CCN2). By contrast, TGFβ1 alone had minor effects on IL-6, IL-8 and MMP3 levels, but significantly increased levels of the CMF markers αSMA, CTGF, COL1A1 and COL3A1. Co-stimulation with both IL-1α and TGFβ1 increased MMP3 expression synergistically. Furthermore, while TGFβ1 had no effect on IL-1α-induced IL-6 or IL-8 levels, co-stimulation inhibited the TGFβ1-induced increase in αSMA and blocked the gel contraction caused by TGFβ1. Combining IL-1α and TGFβ1 had no apparent effect on their canonical signaling pathways. In conclusion, IL-1α and TGFβ1 act synergistically to stimulate MMP3 expression in CF. Moreover, IL-1α has a dominant inhibitory effect on the phenotypic switch of CF to CMF induced by TGFβ1.

Keywords

Cardiac fibroblasts, Cytokines, Extracellular matrix, Fibrosis, Inflammation, Remodeling

Abbreviations

αSMA, alpha-smooth muscle actin; CF, Cardiac fibroblast; CMF, cardiac myofibroblast; COL1A1, collagen I; COL3A1, Collagen III; CTGF, connective tissue growth factor; ECM, extracellular matrix; IL, Interleukin; MMP, matrix metalloproteinase; TGFβ1, transforming growth factor β1; TNFα, tumor necrosis factor α

1. Introduction

Cardiac fibroblasts (CF) are abundant in the heart and are important in maintaining the cardiac extracellular matrix (Brown et al., 2005; Porter and Turner, 2009; Souders et al., 2009). During cardiac structural remodeling, CF function is influenced by changes in electromechanical and biochemical signals. The remodeling heart is characterized by increased levels of various pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNFα) (Nicoletti and Michel, 1999; Porter and Turner, 2009; Souders et al., 2009). CF actively contribute to this inflammatory milieu by secreting various cytokines and chemokines, which attract and activate immune cells (Sinfield et al., 2013; Turner et al., 2011; Turner et al., 2009; Turner et al., 2010). On the other hand, elevated levels of anti-inflammatory factors such as transforming growth factor β1 (TGFβ1), derived from both infiltrating immune cells and myocardial cells, reduce inflammation and lead to differentiation of CF to myofibroblasts (CMF) (Leask, 2010; Lijnen et al., 2000; van den Borne et al., 2010). This phenotypic switch of CF to CMF is characterized by increased expression of alpha-smooth muscle actin (αSMA) and elevated production of extracellular matrix (ECM) proteins such as type I collagen. Excess ECM synthesis and reduced degradation leads to ECM accumulation, myocardial fibrosis and cardiac dysfunction. The interplay between the pro- and anti-inflammatory signals in the heart and their effect on CF is incompletely understood.

In isolated human CF, IL-1α strongly stimulated the expression of the pro-inflammatory cytokines IL-1β, TNFα and IL-6, implicating that CF are important in mediating the inflammatory response after myocardial infarction (Turner et al., 2009). Furthermore, IL-1α enhanced the expression of several CXC chemokines and adhesion molecules important in neutrophil infiltration (Turner et al., 2011). Moreover, IL-1α induced metalloproteinases MMP1, MMP3, MMP9 and MMP10 (Sinfield et al., 2013; Turner et al., 2010); indicating that increased levels of this cytokine have important consequences for ECM degradation. Increased MMP expression and activity was also observed in rat CF stimulated by IL-1β (Brown et al., 2007) or TNFα (Siwik et al., 2000). The latter study also showed decreased CF collagen synthesis upon IL-1β or TNFα treatment, and suggests that these cytokines may be contributing to ventricular dilation (Siwik et al., 2000). Studies with isolated CF show that TGFβ1 induces differentiation of CF to CMF (Swaney et al., 2005), and stimulates collagen production (Butt et al., 1995; Chua et al., 1991; Eghbali et al., 1991; Lijnen et al., 2000; Swaney et al., 2005). In addition, TGFβ1 enhances in vitro collagen gel contraction by CF (Burgess et al., 1994; Drobic et al., 2007; Lijnen et al., 2003).

Although it is clear that TGFβ1 and IL-1α have opposing effects on some aspects of CF biology, such as collagen synthesis, surprisingly few studies have been performed to unravel the effect of combinations of cytokines and growth factors on CF function (van Nieuwenhoven and Turner, 2013). The aim of the present study was therefore to determine the interaction between IL-1α and TGFβ1 on the function of human CF. The effect of co-stimulation with these two cytokines was investigated by

analyzing the expression and secretion of IL-6, IL-8 and MMP3 which are known target genes of IL-1α. In addition, the differentiation of CF to CMF was determined by measuring αSMA expression and incorporation into stress fibers. Moreover, expression levels of collagen I (COL1A1), COL3A1 and connective tissue growth factor (CTGF/CCN2) were determined as markers for TGFβ1 target gene expression. Collagen gel contraction assays were used to assess the contractile behavior of CF. Finally, the effects of co-stimulation of IL-1α and TGFβ1 on canonical downstream signaling pathways were investigated.

2. Results

2.1 TGFβ1 does not influence IL-1α-induced IL-6 and IL-8 expression

Real-time RT-PCR revealed that exposure of human CF to 0.1 ng/ml IL-1α for 6 h increased IL-6 mRNA levels by 14-fold (Fig 1A), and this was maintained for at least 24 h (Fig 1B). IL-6 mRNA levels were only slightly elevated (2-fold) in response to 6 h treatment with 0.1 ng/ml TGFβ1 (Fig 1A), but this returned to basal levels within 24 h (Fig 1B). Exposure of cells to both IL-1α and TGFβ1 in combination appeared to elicit a comparable response to that of IL-1α alone. ELISA analysis of conditioned medium revealed similar results (Fig 1C). IL-1α increased IL-6 protein secretion by 12-fold over a 24 h period, whereas TGFβ1 increased IL-6 secretion by 2-fold. IL-6 secretion was even higher in response to both IL-1α and TGFβ1 in combination (18-fold), although this was not statistically significant. Generally comparable results were observed when the proinflammatory chemokine IL-8 (CXCL8) was studied (Fig 1D, E), although IL-1α induced IL-8 more potently than it did IL-6, and TGFβ1 alone did not appear to modulate IL-8 levels.

2.2 IL-1α inhibits TGFβ1-induced αSMA expression

αSMA is encoded by the ACTA2 gene. RT-PCR analysis revealed that neither TGFβ1 nor IL-1α, either alone or in combination, had any effect on ACTA2 mRNA levels after 6 h (Fig 2A). After 24 h however, TGFβ1 had elicited a 1.7-fold elevation in ACTA2 levels, whereas in stark contrast IL-1α had reduced ACTA2 mRNA levels by 44% (Fig 2B). These opposing effects of IL-1α and TGFβ1 on ACTA2 mRNA levels were neutralized when cells were exposed to both cytokines together, resulting in ACTA2 mRNA levels similar to those in untreated control cells (Fig 2B).

Measurement of αSMA protein levels by Western blotting revealed that TGFβ1 upregulated αSMA protein expression by 4.5-fold after 48 h, whereas IL-1α had no discernible inhibitory effect (Fig 2C). When cells were treated with both cytokines together, TGFβ1-induced αSMA levels were tempered to 2.3-fold, approximately 50% less than observed in response to TGFβ1 alone (Fig 2C), indicating that IL-1α was opposing the effects of TGFβ1.

Immunocytochemical analysis of αSMA protein expression revealed that 48 h TGFβ1 treatment potently increased expression and organization of αSMA fibers and led to larger cells, consistent with the myofibroblast phenotype (Fig 2D). IL-1α alone also appeared to induce a morphological change in the cells, with more smaller spindle-shaped cells being evident (Fig. 2D). When cells were exposed to both cytokines in combination, αSMA expression and cell morphology were similar to control untreated cells (Fig. 2D).

2.3 Effect of IL-1α on TGFβ1-induced collagen I, collagen III and CTGF expression

TGFβ1, IL-1α or their combination did not induce changes in mRNA expression levels of COL1A1 and COL3A1 after 6h (Fig 3A,C). After 24h however, TGFβ1 had stimulated a 45% increase in expression of COL1A1 mRNA (Fig 3B), while IL-1α still had no effect (Fig 3B). TGFβ1-induced COL1A1 mRNA expression remained unaffected by co-incubation with IL-1α (Fig 3B). COL3A1 mRNA levels were elevated by 47% by TGFβ1 after 24 h (Fig 3D). Surprisingly, IL-1α treatment more strongly induced COL3A1 mRNA expression by 2-fold after 24 h. Combining TGFβ1 and IL-1α showed a similar effect to IL-1α alone.

An upregulation of CTGF mRNA was apparent after 6 h (Fig 4A) and 24 h (Fig 4B) exposure to TGFβ1, although this did not reach statistical significance. In contrast, IL-1α reduced CTGF mRNA levels by 29% after 6 h (Fig 4A) and by 48% after 24 h (Fig 4B). This inhibitory effect of IL-1α on CTGF mRNA expression was fully prevented by the presence of TGFβ1 (Fig 4A,B). Similar results were obtained when protein levels of CTGF were analyzed by Western blotting, although the induction by TGFβ1 was much greater than that observed at the mRNA level (Fig 4C).

2.4 Synergistic effect of TGFβ1 and IL-1α on MMP3 expression

IL-1α stimulated MMP3 mRNA expression after 6 h (Fig 5A) and 24 h (Fig 5B), and increased MMP3 protein secretion measured after 24 h (Fig 5C). Although TGFβ1 had no discernible effect on MMP3 mRNA levels at 6 or 24 h (Fig 5A, B), it did stimulate a small (37%) increase in MMP3 secretion (Fig 5C). When the two cytokines were added together, MMP3 mRNA levels (Fig 5A, B) and protein secretion (Fig 5C) were increased further, suggesting that IL-1α and TGFβ1 were acting synergistically to increase MMP3 mRNA and protein levels.

2.5 IL-1α inhibits TGFβ1-induced collagen-gel contraction

The ability of fibroblasts to contract collagen gels is a marker of their differentiation to the myofibroblast phenotype. The effects of TGFβ1 and IL-1α on this process were investigated using

collagen gel contraction assays. TGFβ1 (0.1 ng/ml, 24 h) stimulated a visible reduction in collagen gel size (Fig 6A), with a concomitant 15% reduction in collagen gel weight (Fig 6B), confirming the ability of TGFβ1 to induce the myofibroblast phenotype. In marked contrast, IL-1α increased collagen gel size and weight compared with the untreated control (Fig 6A, B), suggesting reduced myofibroblast activity. When cells were treated simultaneously with both cytokines, the results were similar to those observed with IL-1α alone, indicating that IL-1α was dominant over TGFβ1 for regulating myofibroblast activity (Fig 6A, B).

2.6 Signaling pathways activated by TGFβ1 and IL-1α

The ability of IL-1α and TGFβ1 to regulate gene and protein expression and activity is regulated by complex networks of intracellular signaling pathways. To investigate whether cross talk between IL-1- and TGFβ-stimulated pathways may underlie some of the effects we had observed on CF function, we investigated the influence of these cytokines on activation of canonical pathways for TGFβ (i.e. Smad and p38 MAPK) and IL-1 (NFκB and p38 MAPK) by Western blotting with phospho-specific antibodies (Fig 7).

Exposure of CF to TGFβ1 stimulated robust Smad3 phosphorylation that increased over a 60 min period. In contrast, IL-1α did not modulate Smad3 signaling either alone or together with TGFβ1. Although TGFβ1 has been shown to activate p38 MAPK in adult rat CF (Voloshenyuk et al., 2011), we did not observe TGFβ1-induced p38 activation in human CF at 0.1 ng/ml concentrations (Fig 7), or indeed at concentrations up to 10 ng/ml (data not shown). However, IL-1α did strongly stimulate p38 phosphorylation over the 10-60 min period, and this was not affected by co-incubation with TGFβ1. Similarly, IL-1α stimulated activation of the NFκB pathway (measured by proteasome-mediated degradation of IκB-α), but TGFβ1 had no effect either alone or in combination with IL-1 α (Fig 7). Thus, we found no evidence of cross talk between the IL-1 and TGFβ signaling pathways in human CF that could have contributed to the interaction of these two cytokines on CF function.

3. Discussion

CF orchestrate cardiac remodeling as the key source of ECM molecules, including ECM proteins, ECM-degrading proteases, matricellular proteins and autocrine/paracrine factors. IL-1α and TGFβ1 levels are increased in cardiac remodeling and both influence CF function. The major findings of the present study are that IL-1α clearly inhibits TGFβ1-induced CF to CMF differentiation, and that IL-1α and TGFβ1 also act synergistically to induce MMP3 expression. The interaction between IL-1α and TGFβ1 at the gene expression level is depicted in Fig 8.

We used sub-maximal concentrations of IL-1α and TGFβ1 (0.1 ng/ml) in our study in order to provide optimal conditions for observing potential interactions between these two cytokines, and to

mimic the transition between the inflammatory and fibrotic stages of post-MI healing (van Nieuwenhoven and Turner, 2013). Even at these relatively low concentrations, typical CF responses were observed. For example, phosphorylation of p38 MAPK was enhanced by IL-1α as shown earlier (Turner et al., 2009), while TGFβ1 induced sustained Smad-phosphorylation (>60 min), as previously described (Cucoranu et al., 2005). Prolonged IL-1α exposure induced the typical target genes IL-6 and IL-8 (Turner et al., 2011; Turner et al., 2009), while TGFβ1 enhanced αSMA, COL1A1, COL3A1 and CTGF levels (Cucoranu et al., 2005). Surprisingly, IL-1α also induced COL3A1 mRNA expression by 2 fold. In cardiac disease states, increased collagen III has been described in early myocardial remodeling, while collagen I accumulation is observed at a later stage (Graham et al., 2008). Possibly, the inflammatory cytokine IL-1α plays a role in this early synthesis of collagen III, while TGFβ1 will be more important at the late stages of myocardial remodeling.

The induction of CTGF mRNA levels by TGFβ1 was only modest, which might be due to the high basal level of CTGF expression in these cells or our use of a relatively low concentration of TGFβ1 to stimulate the cells. However, the cumulative CTGF protein level as determined by western blotting was clearly enhanced 6-fold by TGFβ1 treatment. Moreover, TGFβ1 induced a small but statistically significant enhancement of collagen gel contraction, as was described previously (Drobic et al., 2007). Together these results show that CF were responsive to the low concentrations of IL-1α and TGFβ1 used in the present study. Some differences were observed in the scale of changes in levels of mRNA and protein of the genes studied. This is most likely caused by differences in start of synthesis (protein translation follows mRNA expression), time of harvesting cells and turnover rates. However, the direction of response was generally comparable.

Co-stimulation of CF with IL-1α and TGFβ1 revealed that TGFβ1 had no inhibitory effect on the IL-1α-induced increase of IL-6 and IL-8. Surprisingly, the MMP3 induction observed with IL-1α treatment was further increased in the cells co-stimulated with both factors. The synergistic effect on MMP3 was observed both at the cellular mRNA level and secreted protein level in conditioned media. This finding is in sharp contrast to a previous study that showed that TGFβ1 inhibited IL-1β-induced MMP activity and migration (Brown et al., 2007). In the latter study by Brown and colleagues, TGFβ1 strongly inhibited IL-1β-induced MMP2, MMP3, and MMP9 in adult rat CF. We can only speculate on the discrepancy between our current findings and the previous study by Brown and colleagues; it might be related to the difference in species of the CF or to the use of different concentrations of IL-1 and TGFβ1. Synergistic upregulation of MMP9 by combinations of IL-1α and either platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) was observed earlier in dermal fibroblasts (Bond et al., 1998). In combination with these earlier studies, the present study supports the idea that complex interactions between various cytokines and growth factors will ultimately determine the nature and quantity of MMP synthesis and release by CF.

While TGFβ1 did not inhibit several IL-1α-induced effects in CF (e.g. IL-6, IL-8 expression) the opposite was clearly observed. IL-1α inhibited several features of TGFβ1-induced CF-CMF

differentiation. The hallmark of CMF differentiation, αSMA expression, was inhibited by IL-1α at both mRNA and protein levels. Although IL-1α decreased basal expression of CTGF, it was unable to counteract the TGFβ1-induced increase in CTGF levels. Finally, the contractile ability of cells (an indicator of CMF phenotype), studied by collagen gel contraction assays, was strongly inhibited by IL-1α. This is the first study showing the inhibition of CMF differentiation by IL-1α. Earlier studies using co-cultures of keratinocytes and dermal fibroblasts showed that TGFβ1-induced αSMA expression was suppressed by IL-1α (Shephard et al., 2004). Moreover, blocking the IL-1 receptor potentiated αSMA expression in the dermal fibroblasts, indicating an inhibitory effect of IL-1 on TGFβ1-induced myofibroblast differentiation (Shephard et al., 2004). In human pulmonary fibroblasts, TGFβ1-induced αSMA expression was inhibited by TNFα, indicating that other pro-inflammatory cytokines show similar inhibitory effects on myofibroblast differentiation (Liu et al., 2009).

The dominant inhibitory effect of IL-1α on CMF differentiation is likely to be important during cardiac remodeling. Inflammation plays a role in various types of cardiac remodeling and might be acute, such as after myocardial infarction, or chronic, such as in hypertensive disease. If IL-1α is abundant, our data suggest that the phenotypic switch from CF to CMF would be prevented even in the presence of TGFβ1. The combined presence of both factors at temporal stages during the remodeling process would synergistically increase MMP3 expression and secretion from CF, which would increase ECM turnover. Finally, differentiation to myofibroblasts would most likely occur when inflammation and IL-1α levels decrease and TGFβ1 signaling prevails (van Nieuwenhoven and Turner, 2013).

In conclusion the present study shows IL-1α and TGFβ1 each have robust effects on CF function and phenotype. IL-1α has a dominant inhibitory effect on the phenotypic switch of CF to CMF induced by TGFβ1. In combination, these two cytokines have a synergistic stimulatory effect on MMP3 secretion. Taken together, our data suggest that many functions of CF that are of key importance to myocardial remodeling can be influenced by both proinflammatory and profibrotic stimuli. The net effect of these opposing stimuli on CF function, and hence remodeling, is complex and dependent on relative concentrations of different bioactive molecules at different stages of the remodeling process.

4. Experimental procedures

4.1 Materials

All cell culture media and solutions were purchased from Invitrogen, except fetal bovine serum (FBS) that was from BioSera. TGFβ1 was from R&D Systems and IL-1α was from Invitrogen. Goat polyclonal anti-CTGF antibody was from Santa Cruz Biotechnology and mouse monoclonal anti-αSMA antibody was from Sigma. Signaling antibodies for phospho-Smad3, phospho-p38 MAPK and IκB-α were from Cell Signaling Technology.

4.2 Cardiac fibroblast isolation and culture

Right atrial appendage biopsies were obtained from patients undergoing coronary artery bypass surgery after informed patient consent and approval of the local ethical committee of the Leeds General Infirmary. Cardiac fibroblasts were isolated and cultured as described previously (Turner et al., 2003). Experiments were performed on cells of passage number 3-6. Cells were cultured in 6-well plates and serum-starved for 24 h before exposure to IL-1α and/or TGFβ1 (both 0.1 ng/ml). Effects on gene expression were determined at 6 and 24 h and protein levels were assessed after 24 or 48 h. To determine the effect of IL-1α and TGFβ1 on signaling pathways, cells were stimulated for 10-60 min before preparing whole cell homogenates.

4.3 Collagen gel contraction

The collagen gel contraction assay was performed in 24-well plates. Wells were coated with bovine serum albumin (Sigma) at 37°C for 1 hour. Collagen gels containing cells were prepared by mixing ice-cold type I rat tail collagen solution (Gibco) with ice-cold 2x concentrated DMEM (Sigma), adjusting pH to 7.4 with NaOH, and then immediately mixing with freshly trypsinized cells. The final mix contained 1 mg/ml collagen I, 10^5 cells/ml and 0.4% FBS in 1x DMEM. After mixing, 0.5 ml of the cell solution was added to each well of the coated 24-well plates and gels were allowed to solidify at 37°C for 1 hour. Following solidification, 0.5 ml DMEM was added to the gels, and the cells were exposed to IL-1α and/or TGFβ1 (0.1 ng/ml). Gels were photographed and weighed 24 h later to assess their relative contraction.

4.4 qRT-PCR

Cellular RNA was extracted from cells using the Aurum RNA isolation kit (BioRad). After cDNA synthesis (Promega Reverse Transcription System), real-time RT-PCR was performed using an

Applied Biosystems ABI-7500 Real-Time PCR System and intron-spanning primers and Taqman probes for human IL-6 (Hs00174131_m1), IL-8 (Hs99999034_m1), ACTA2 (Hs00426835_g1), COL1A1 (Hs00164004_m1), COL3A1 (Hs00943809_m1), CTGF (Hs00170014_m1) and MMP3 (Hs00233962_m1) (Applied Biosystems). Data are presented as percentage of GAPDH expression using the formula $2^{-\Delta CT}$ x 100.

4.5 ELISAs

Conditioned media were collected, centrifuged to remove any residual cells and stored at -40^{o}C for analysis. ELISAs were performed according to the manufacturer's instructions (R&D Systems).

4.6 Western blotting

Whole cell homogenates were prepared by scraping cell layers directly into SDS-PAGE sample buffer containing protease and phosphatase inhibitors, and Western blotting performed as described previously (Turner et al., 2001). Prior to running the gels, the protein concentration of cell homogenates were determined and loading volumes adjusted to ensure equal amounts of proteins (30 μg) were loaded. Additionally, Western blotting of β-actin was performed to further confirm equal protein loading and blotting.

4.7 Immunocytochemistry

Serum-starved cells cultured in 8-well LabTek chamber slides were treated with 0.1 ng/ml TGFβ1 and/or IL-1α for 48 h before fixing in 4% paraformaldehyde and permeabilizing with 1% Triton X-100. Immunocytochemistry was performed using 1:200 diluted anti-αSMA monoclonal antibody, 1:200 diluted FITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) and 4',6'-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories) to stain nuclei. Images were obtained under equivalent optical conditions using a Zeiss LSM-510 confocal microscope.

4.8 Statistical analyses

Results are mean ± SEM where n represents the number of experiments on cells from different patients. Data were analyzed as ratios using repeated measures one-way ANOVA test followed by Newman-Keuls post hoc test (GraphPad Prism software, www.graphpad.com). $P<0.05$ was considered statistically significant.

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Figure Legends

Fig. 1. Effect of TGFβ1 and IL-1α on IL-6 and IL-8 expression. Cells were stimulated with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 6 h **(A,D)** or 24 h **(B, E)** before collecting RNA and measuring IL-6 **(A, B)** or IL-8 **(D, E)** mRNA levels by RT-PCR (n=6). **(C)** IL-6 protein levels in conditioned media were analyzed by ELISA after 24 h (n=6). ***P<0.001, **P<0.01, NS not significant for effect of treatment vs. control (Ctrl), or comparisons as indicated.

Fig. 2. Effect of TGFβ1 and IL-1α on αSMA mRNA and protein expression. Cells were stimulated with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 6 h **(A)** or 24 h **(B)** before collecting RNA and measuring ACTA2 mRNA levels by RT-PCR (n=6). **(C)** αSMA protein levels were analyzed by Western blotting after 48 h (n=6). ***P<0.001, *P<0.05, NS not significant for effect of treatment vs. control (Ctrl), or comparisons as indicated. **(D)** Cells were treated with 0.1 ng/ml TGFβ1 and/or IL-1α for 48 h before performing immunocytochemistry with anti-αSMA antibody (green). Cell nuclei were stained with DAPI (blue). Scale bar = 100 μm. Representative images shown.

Fig. 3. Effect of TGFβ1 and IL-1α on COL1A1 and COL3A1 mRNA expression. Cells were stimulated with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 6 h **(A,C)** or 24 h **(B, D)** before collecting RNA and measuring COL1A1 **(A, B)** or COL3A1 **(C, D)** mRNA levels by RT-PCR (n=6). **P<0.01, *P<0.05, NS not significant for effect of treatment vs. control (Ctrl), or comparisons as indicated.

Fig. 4. Effect of TGFβ1 and IL-1α on CTGF mRNA and protein expression.
Cells were stimulated with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 6 h **(A)** or 24 h **(B)** before collecting RNA and measuring CTGF mRNA levels by RT-PCR (n=6). **(C)** CTGF protein levels were analyzed by Western blotting after 48 h (n=3). ***P<0.001, **P<0.01, *P<0.05, NS not significant for effect of treatment vs. control (Ctrl), or comparisons as indicated.

Fig. 5. Effect of TGFβ1 and IL-1α on MMP3 mRNA and protein secretion. Cells were stimulated with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 6 h **(A)** or 24 h **(B)** before collecting RNA and measuring MMP3 mRNA levels by RT-PCR (n=6). **(C)** MMP3 protein levels in conditioned media were analyzed by ELISA after 24 h (n=6). ***P<0.001, **P<0.01, *P<0.05, NS not significant for effect of treatment vs. control (Ctrl), or comparisons as indicated.

Fig. 6. Effect of TGFβ1 and IL-1α on collagen gel contraction. Collagen gels containing cardiac fibroblasts were allowed to solidify for 1 h before stimulating with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 24 h. **(A)** Representative images of contracted gels in wells of culture plate. **(B)** Mean gel

weights from n=6 separate experiments. ***P<0.001, *P<0.05, NS not significant for effect of treatment vs. control (Ctrl), or comparisons as indicated.

Fig. 7. Effect of TGFβ1 and IL-1α on Smad3, p38 and NFκB signaling. Cells were stimulated with 0.1 ng/ml TGFβ1 and/or 0.1 ng/ml IL-1α for 10-60 min and phosphorylation of signaling molecules analyzed by Western blotting. β-actin expression was monitored to confirm equal protein loading and blotting. Representative images from two separate experiments are shown.

Fig. 8. Summary of effects of TGFβ1 and IL-1α on human CF. Representation of interaction between TGFβ1 and IL-1α on expression of proinflammatory molecules (IL-6, IL-8), ECM molecules (MMP3, CTGF, COL1, COL3) and myofibroblast markers (αSMA, CTGF). IL-1α stimulated IL-6, IL-8 and COL3, and this was not modulated by TGFβ1. IL-1α potently increased MMP-3 gene expression, and this was increased further by TGFβ1. TGFβ1 elevated COL1 and COL3 mRNA levels and the myofibroblast markers αSMA and CTGF (and collagen gel contraction); latter effects opposed by IL-1.

Figure 1

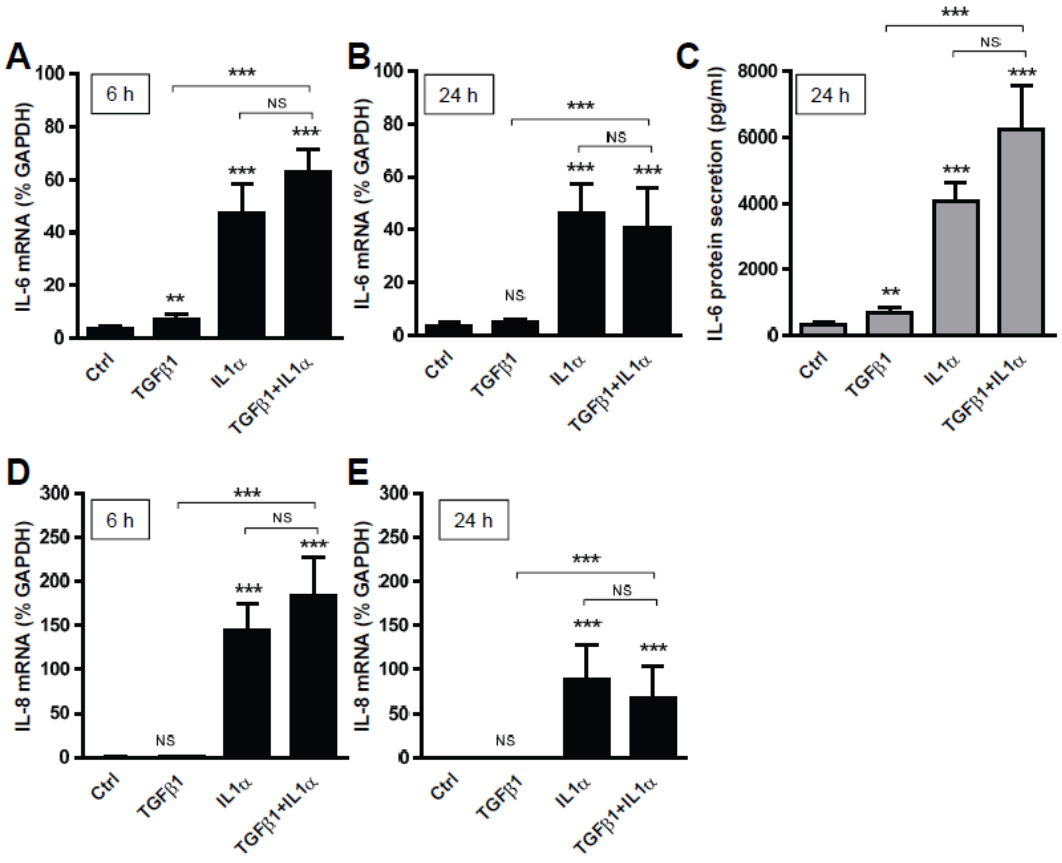
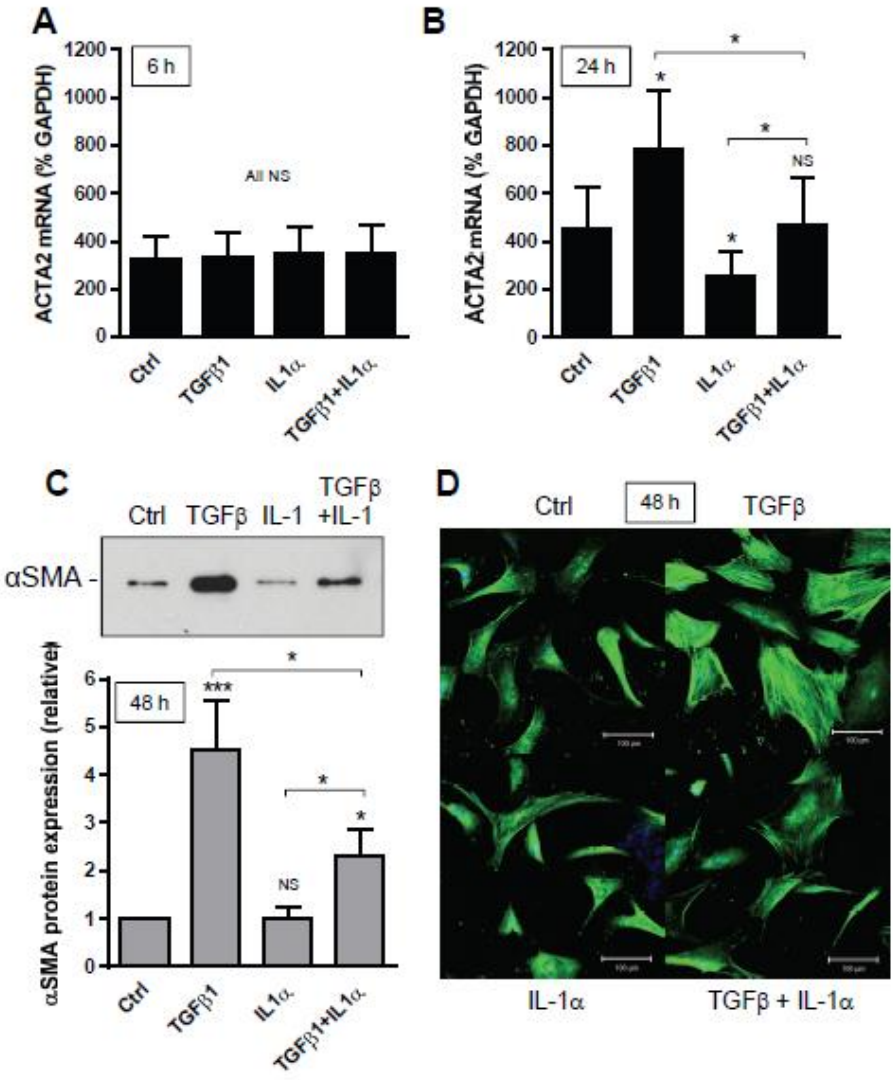


Figure 2



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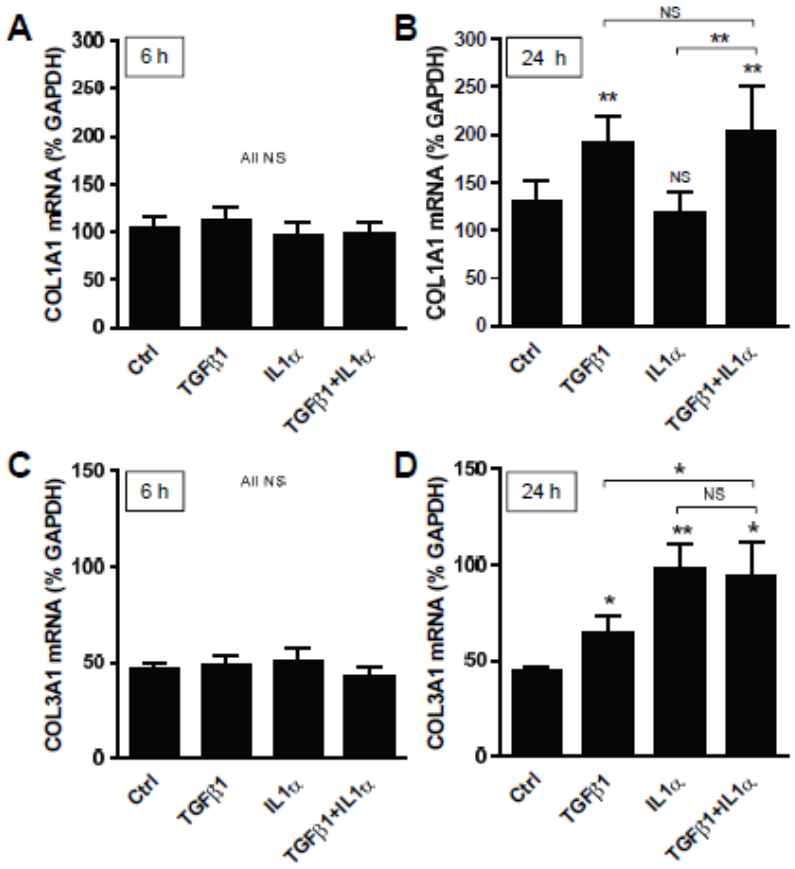

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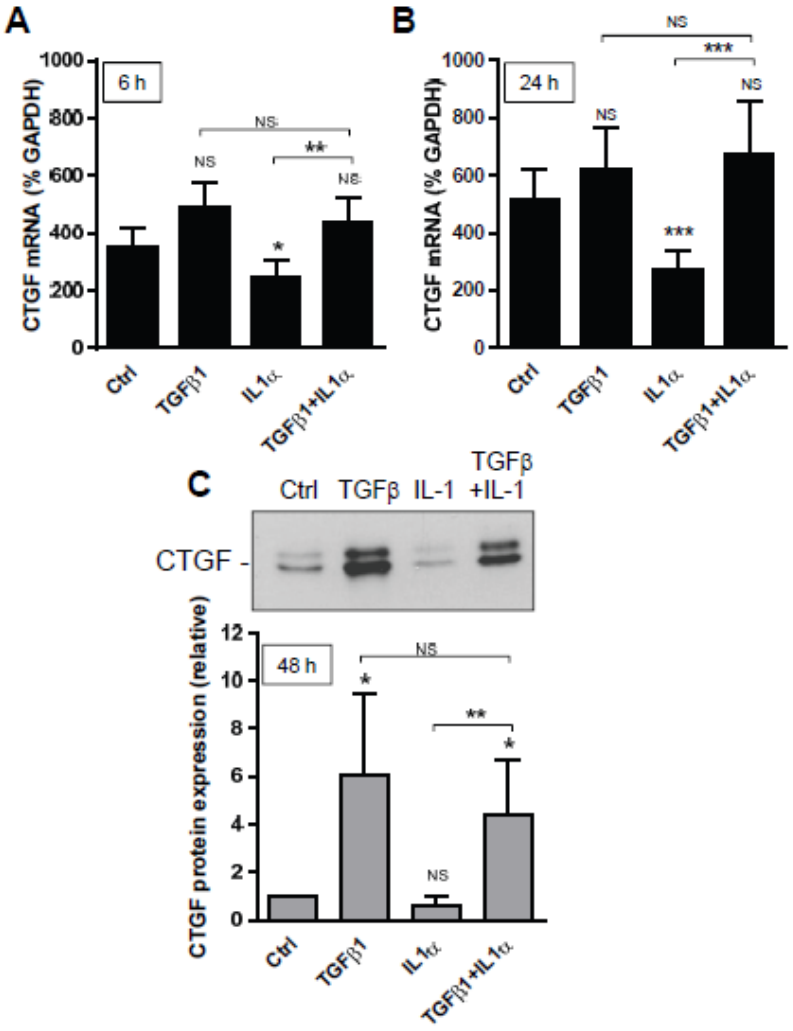


Figure 5

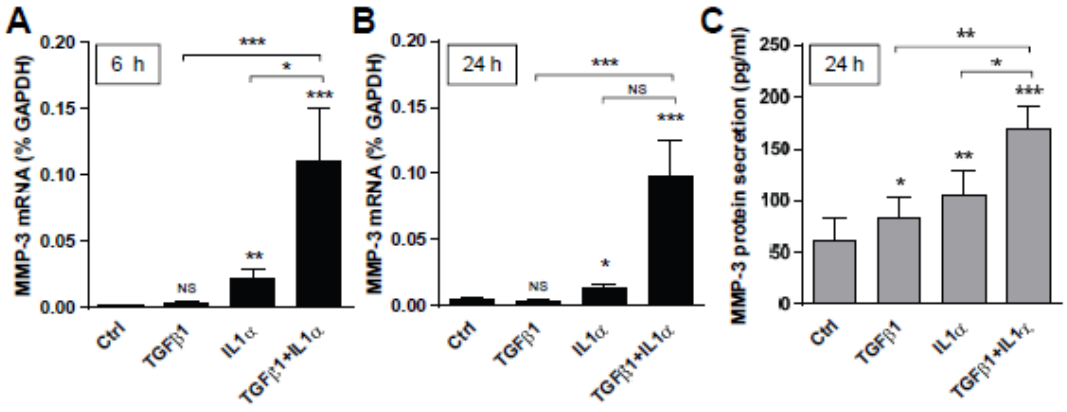


Figure 6

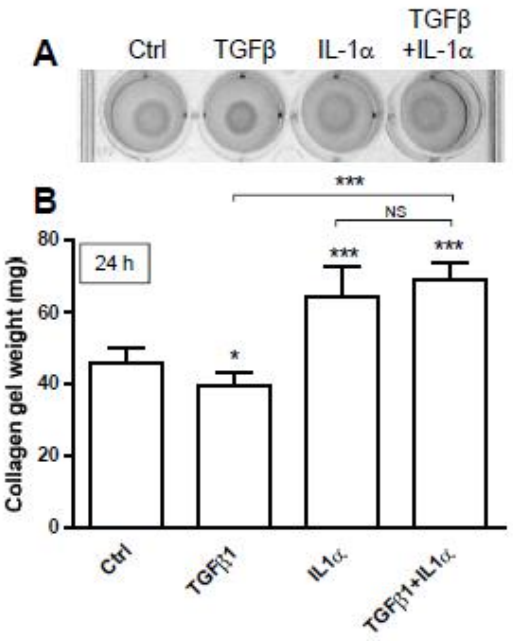


Figure 7

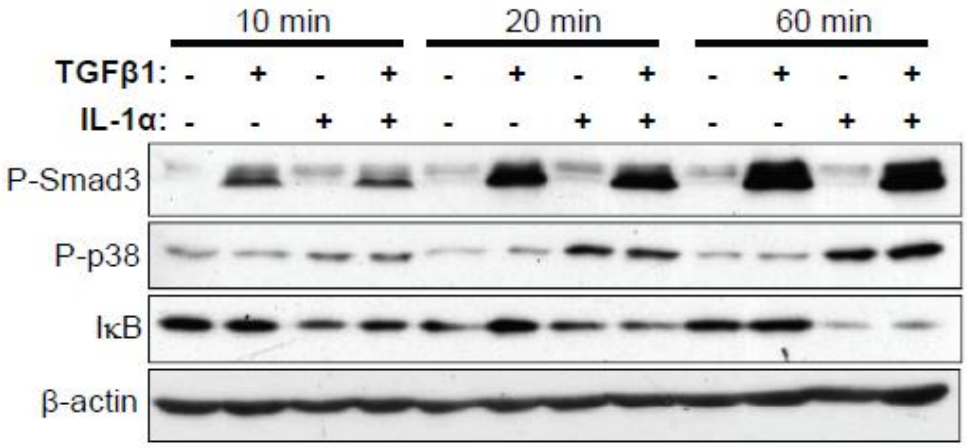
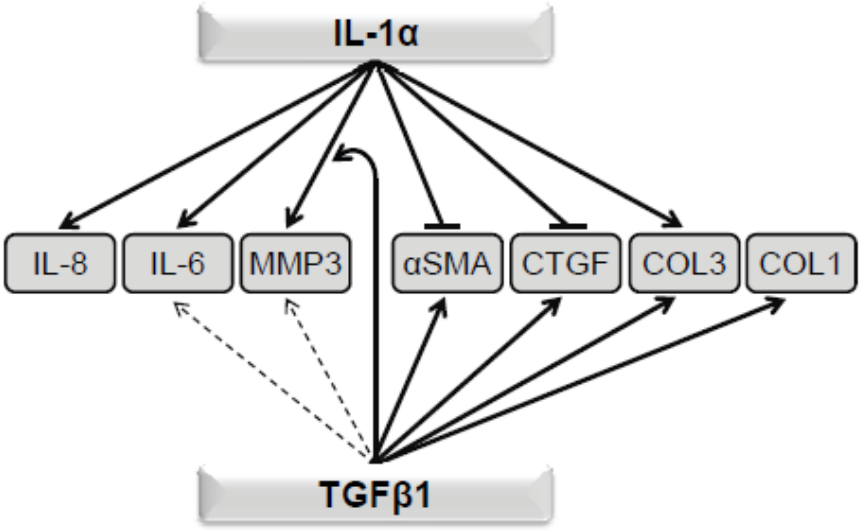


Figure 8



Highlights

- Combined effects of IL-1α and TGFβ1 on cardiac fibroblast (CF) function were studied
- IL-1α exposure alone enhanced IL-6, IL-8, MMP3 and COL3A1 expression, but reduced αSMA and CTGF expression
- TGFβ1 exposure alone significantly increased expression of αSMA, CTGF, COL1A1 and COL3A1
- Co-stimulation of CF with both IL-1α and TGFβ1 synergistically increased MMP3 expression
- IL-1α has a dominant inhibitory effect on the phenotypic switch of CF to myofibroblasts induced by TGFβ1