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Effects of Interleukin-18 on Cardiac Fibroblast Function and Gene Expression

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Abstract

Fibroblasts are the primary cell type responsible for synthesis and remodeling of the extracellular matrix in the heart. A number of factors including growth factors, hormones and mechanical forces have been identified that modulate the production of extracellular matrix by cardiac fibroblasts. Inflammatory mediators including proinflammatory cytokines and chemokines also impact fibrosis of the heart. Recent studies have illustrated that interleukin-18 promotes a pro-fibrotic response in cardiac fibroblasts; however the effects of this cytokine on other aspects of fibroblast function have not been examined. While fibroblasts have long been known for their role in production and remodeling of the extracellular matrix, other functions of these cells are only now beginning to be appreciated. We hypothesize that exposure to interleukin-18 will stimulate other aspects of fibroblast behavior important in myocardial remodeling including proliferation, migration and collagen reorganization. Fibroblasts were isolated from adult male rat hearts and bioassays performed to determine the effects of interleukin-18 on fibroblast function. Treatment of fibroblasts with interleukin-18 (1–100 ng/ml) resulted in increased production of extracellular matrix components and remodeling or contraction of three-dimensional collagen scaffolds by these cells. Furthermore, exposure to interleukin-18 stimulated fibroblast migration and proliferation. Treatment of heart fibroblasts with interleukin-18 resulted in the rapid activation of the c-Jun N-terminal kinase (JNK) and Phosphoinositide 3-kinase (PI3-kinase) pathways. Studies with pharmacological inhibitors illustrated that activation of these pathways is critical to interleukin-18 mediated alterations in fibroblast function. These studies illustrate that interleukin-18 plays a role in modulation of cardiac fibroblast function and may be an important component of the inflammation- fibrosis cascade during pathological myocardial remodeling.

Keywords

Fibroblast; Collagen; Interleukin 18; Migration; Fibrosis

1. Introduction

A number of cardiovascular diseases including atherosclerosis, myocardial infarction and hypertension, are accompanied by changes in the extracellular matrix (ECM). The ECM

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provides a stress-tolerant network that is essential for cardiac development and function; however, alterations in the accumulation or organization of the ECM are thought to contribute to myocardial dysfunction [1–6]. For instance, hypertension-induced myocardial remodeling is accompanied by cardiomyocyte hypertrophy as well as synthesis and deposition of excessive ECM components or fibrosis. Interstitial fibrosis alters the mechanical properties of the ventricular wall and contributes to altered relaxation of the myocardium. Fibroblasts are the cells primarily responsible for production and remodeling of the ECM components of the heart [7–9]. A number of factors have been identified that can modulate cardiac fibroblast activity including growth factors like transforming growth factor- β , hormones, particularly the renin-angiotensin-aldosterone system, and mechanical forces. Recently, attention has focused on the relationship between inflammation and cardiac fibrosis [10–12]. Several cytokines have been shown to affect cardiac fibroblast gene expression [13–15]; however, many questions remain regarding the contributions of these to cardiac fibrosis and heart function.

Interleukin 18 (IL-18) is a pro-inflammatory cytokine of the IL-1 family that has been correlated to a number of disease processes including atherogenesis [16–17]. A direct correlation between IL-18 levels and severity of myocardial dysfunction has been observed [18–19]. Neutralization of IL-18 significantly reduces myocardial damage following ischemia/ reperfusion [18]. Administration of IL-18 to normal animals results in increased left ventricular mass and a substantial increase in myocardial collagen content [20–21]. In vitro studies have illustrated that IL-18 stimulates the expression of the ECM component fibronectin by cardiac fibroblasts further implicating this cytokine in the promotion of myocardial fibrosis [22].

While fibroblasts have long been recognized as the major cell type that produces ECM in the heart, it has become increasingly clear that these cells have other important functions [7]. Little is known about the effects of IL-18 on other aspects of cardiac fibroblast function besides ECM expression. The present studies were carried out to determine whether IL-18 affects not only expression of ECM components by cardiac fibroblasts, but other functions of these cells including proliferation, migration and remodeling of the ECM. The present studies illustrate that exposure of heart fibroblasts to IL-18 stimulated the expression of ECM components including interstitial collagens and periostin. In addition, treatment with IL-18 promoted collagen remodeling, migration and proliferation of heart fibroblasts. Exposure of cardiac fibroblasts to IL-18 resulted in the rapid activation of JNK and Akt signaling pathways. Experiments with pharmacological inhibitors illustrated that both of these pathways play important roles in IL-18 induced collagen remodeling and migration by heart fibroblasts. These studies illustrate an important role for IL-18 in promoting not only expression of ECM components by cardiac fibroblasts, but other activities of these cells as well.

2. Materials and Methods

2.1 Isolation of cardiac fibroblasts

Eight week old adult male Sprague Dawley rats (200–250 grams body weight) were purchased from Harlan and housed in an AAALAC-approved animal facility. All animals were provided food and water *ad libitum*. All experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and were approved by the University of South Carolina Institutional Animal Use and Care Committee (IACUC). Animals were euthanized, hearts removed and rinsed in sterile, physiological saline. Extracardiac tissue was discarded, heart tissues were minced in sterile saline and fibroblasts were isolated by digestion of the minced tissue with Liberase 3 (Roche Applies Science;

Indianapolis, IN) as described previously [23–24]. Cells collected from digested tissue were plated into T75 flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% neonatal calf serum, 5% fetal bovine serum and antibiotics (hereafter called normal fibroblast medium). Fibroblasts were purified by their rapid attachment to tissue culture plastic. Cells were passaged at approximately 80% confluency following detachment with a 0.25% trypsin/ 0.1% ethylenediaminetetraacetic acid (trypsin/EDTA) solution. Fibroblasts were used prior to passage four.

2.2 Western blots

The effect of IL-18 on the expression of ECM components and their receptors in isolated cardiac fibroblasts was determined by western blot analysis. Fibroblasts were cultured as above in normal fibroblast medium. Twenty-four hours prior to experimental treatment, medium was removed and replaced with low serum DMEM (1.5% fetal bovine serum) containing antibiotics. After 24 hours of culture in low serum medium, fibroblasts were treated with varying doses of IL-18 (0, 1, 10 and 100 ng/ml) in low serum medium for an additional 24 hours. The doses of IL-18 used in the present studies were chosen based upon those used in previous research with isolated fibroblasts and other cell types [22,25–26]. Conditioned medium was removed for analysis of secreted proteins (interstitial collagens and periostin). Cells were subsequently rinsed with sterile saline and total protein was extracted in lysis buffer containing 10mM Tris-HCl (pH 7.4), 1% sodium dodecyl sulfate, 1 mM sodium orthovanadate and protease inhibitors. Cell lysate samples were used for the analysis of integrins by Western blots. Total protein content of the media and cell lysate samples was determined using the BCA Assay (Pierce; Rockford, IL). Equal amounts of protein were separated on 4–20% gradient polyacrylamide gels (Pierce; Rockford, IL). Proteins were transferred onto nitrocellulose membranes, which were stained with Fast Green to verify efficient transfer of proteins. Membranes containing conditioned medium proteins were incubated in primary antisera (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) against ECM components including collagen type I, collagen type III and periostin. Signals from these antisera were normalized to the Fast Green stained membrane.

Membranes containing cellular proteins were incubated with primary antisera against cell surface receptors of the integrin family including $\beta 1$, $\alpha 1$ and $\alpha 2$ integrins, the major collagen-binding integrins in cardiac fibroblasts (Chemicon; Billerica, MA) and against membrane type 1-matrix metalloprotease (MT1-MMP). For normalization purposes, these blots were also probed with antibodies against glyceraldehyde 3-phosphate dehydrogenase (Chemicon; Billerica, MA). Western blots were developed with SuperSignal reagent (Pierce; Rockford, IL) and exposed to x-ray film. Relative protein expression was determined by image analysis of x-ray films using an Alpha Innotech gel analysis system.

2.3 Reverse transcriptase polymerase chain reaction

The expression of mRNAs encoding ECM components was assayed by semiquantitative reverse transcriptase- polymerase chain reaction (RT-PCR). Adult cardiac fibroblasts were cultured as described above in normal fibroblast medium. Twenty-four hours prior to treatment, medium was removed, cells rinsed in sterile saline and culture continued in low serum medium (DMEM plus 1.5% fetal bovine serum). Fibroblasts were subsequently treated for 24 hours with IL-18 (0, 1, 10 and 100 ng/ml). Following treatment with IL-18, cells were rinse with phosphate-buffered saline and extracted in TRIzol Reagent (Invitrogen; Carlsbad, CA). RNA was precipitated, resuspended in nuclease-free water and quantified spectrophotometrically. cDNA was produced from 2 μ g of RNA using the iScript cDNA kit (BioRad; Hercules, CA). Semiquantitative PCR was carried out using primers specific for markers of fibrosis including collagen type I, collagen type III and periostin. The relative expression of mRNA encoding extracellular matrix components was normalized to the

acidic ribosomal phosphoprotein PO [27]. Preliminary experiments were conducted to determine the appropriate amplification cycle number for each of the mRNAs of interest.

2.4 Collagen gel contraction

The three-dimensional collagen gel system has been extensively used to examine interactions between cardiac fibroblasts and the collagenous ECM [28–30]. Fibroblasts were cultured as described above in normal fibroblast medium. Twenty-four hours prior to experimental treatment, medium was removed and replaced with low serum DMEM (1.5% fetal bovine serum). After 24 hours in low serum medium, fibroblasts were detached from culture plates by incubation in trypsin/EDTA. Cells were rinsed, pelleted by centrifugation and resuspended in low serum medium. An equal volume of cells was combined with a 1.25 mg/ml collagen solution to yield a final concentration of 100,000 cells per milliliter. One milliliter of the collagen:cell mixture was added to wells of 24-well plates that had been precoated with bovine serum albumin to prevent collagen gels from attaching to the culture plastic. Collagen gels were allowed to polymerize one hour at 37°C. Following polymerization, one milliliter of low serum medium was gently added to each well of the plate and collagen gels dislodged from the well to allow the gels to float in the medium. Interleukin-18 was added at varying concentrations (0, 1, 10, 100 ng/ml) and culture was continued for 48 hours. Collagen gels were photographed and the relative contraction of the collagen gel was determined by measuring the top surface of the gels at 48 hours relative to their initial size. Collagen gels were performed in triplicate in each of at least four independent experiments. In experiments including inhibitors to specific signaling pathways, pharmacological inhibitors were added 1 hour prior to the addition of IL-18. Doses of pharmacological inhibitors used in the present studies were based upon previous use of these in isolated fibroblasts.

2.5 Fibroblast migration assay

The relative migratory ability of fibroblasts was determined using a scratch assay as previously described [31]. Fibroblasts were plated into wells of 6-well plates precoated with collagen type I (10 µg/ml). After 24 hours of culture, normal fibroblast medium was replaced with DMEM containing 1.5% fetal bovine serum. After 24 hours of culture in low serum medium, a scratch was gently created through the confluent cells with a pipet tip. Interleukin-18 was added at varying concentrations (0, 1, 10, 100 ng/ml) and culture was continued for 24 hours. The area devoid of cells was photographed at the time the scratch was created and 24 hours later. The relative migration of fibroblasts into the denuded area was measured using ImageJ (National Institutes of Health; Bethesda, MD). Migration experiments were performed in triplicate wells with each of at least three independent experiments. In experiments including inhibitors to specific signaling pathways, pharmacological inhibitors were added 1 hour prior to the addition of IL-18.

2.6 Fibroblast proliferation

The proliferation of isolated cardiac fibroblasts was assayed by BrdU incorporation. For analysis of proliferation, cells were cultured on glass coverslips that were precoated with collagen type I. Culture was continued for 24 hours in the presence of normal fibroblast medium. Medium was removed and culture continued in the presence of low serum (1.5% fetal bovine serum) DMEM for 24 hours. Subsequently, culture was continued for 24 hours in the presence of varying concentrations of IL-18 (0, 1, 10, 100 ng/ml) and BrdU (Roche Applied Science; Indianapolis, IN). Following culture, coverslips were rinsed with phosphate-buffered saline and fixed in absolute ethanol containing 10 mM glycine (pH 2.0) for 30 minutes at –20°C. Cells were rinsed and BrdU incorporation detected by immunocytochemical staining with anti-BrdU serum and fluorescein-conjugated secondary antibodies (Roche Applied Sciences; Indianapolis, IN). Cells were simultaneously stained

with propidium iodide to detect all nuclei. Cells were examined and the percentage of BrdU-positive fibroblasts determined using a Nikon E600 microscope equipped for epifluorescence. Proliferation assays were performed on duplicate coverslips from each of at least four independent experiments. In experiments utilizing pharmacological inhibitors to specific signaling pathways, inhibitors were added 1 hour prior to the addition of IL-18.

2.7 Analysis of signal transduction pathways

The effects of IL-18 on the activation of specific signaling proteins was assayed by Western blot analyses in cardiac fibroblasts. Cells were cultured as described above in normal fibroblast medium. Fibroblasts were rinsed and culture continued in low serum medium for 24 hours. Cells were rinsed and culture continued in low serum medium. Fibroblasts were treated for 15, 30, 60 and 120 minutes with IL-18 (100 ng/ml). Following treatment, fibroblasts were rinsed with phosphate-buffered saline and subsequently extracted in boiling lysis buffer containing 10 mM Tris Base (pH 7.4), 1% sodium dodecyl sulfate, 1 mM sodium orthovanadate and protease inhibitors. Western blots were performed as described above with primary antisera specific for phosphotyrosine and total Erk 1/2, JNK kinase and Akt (Cell Signaling Technology, Inc; Danvers, MA). Western blots for phosphorylated proteins are presented relative to the total protein.

2.8 Statistical analysis

Results are shown $\pm$ S.E.M. Statistical significance was determined by ANOVA with Dunnett's post-hoc test ($p < 0.05$). Analyses were performed using GraphPad Prism Software.

3. Results

3.1 Expression of ECM components

Several recent studies have illustrated that IL-18 stimulates fibrosis of the heart and other organs [20–22,32–33]. Experiments were carried out to determine the effects of IL-18 on the expression of ECM components and ECM-modifying enzymes by adult cardiac fibroblasts. Semiquantitative RT-PCR was performed with primers for ECM components and RNA from adult cardiac fibroblasts treated for 24 hours with varying doses of IL-18. These studies indicated an increase in collagen $\alpha 1(I)$, collagen $\alpha 1(III)$ and periostin mRNA expression in a dose-dependent manner following 24 hours treatment with IL-18 (Figure 1a, 1b and 1c). The increase in the expression of ECM components was significant at the highest doses of IL-18 used (10 and 100 ng/ml for collagen $\alpha 1(III)$ and periostin and 100 ng/ml for collagen $\alpha 1(I)$). Conditioned medium was collected from treated cells and collagen type I, collagen type III and periostin protein expression assayed by Western blot analysis (Figure 1). Accumulation of these proteins in the conditioned medium essentially paralleled the respective mRNA expression (Figure 1d, 1e and 1f). Collagen type I and type III protein in the conditioned medium increased in a stepwise manner with increasing doses of IL-18; however, this was significant only at the highest dose used (Figure 1d and 1e).

Accumulation of ECM components is regulated not only by the expression and secretion of the ECM proteins, but also by the expression of enzymes that degrade these proteins. The matrix metalloproteases (MMP) are the major family of enzymes that degrade ECM components. Conditioned medium was subjected to gelatin zymography to assay the relative levels of MMP-2, a major matrix-degrading enzyme secreted by cardiac fibroblasts [34]. The level of the 68 kDa (active) form of MMP-2 in the medium was increased in a dose-dependent manner by treatment of the fibroblasts with IL-18 and this was significant at the highest dose of IL-18 (Figure 2A). MMPs are secreted as inactive zymogens that require cleavage of a prodomain to become activated. As membrane type 1- matrix metalloprotease (MT1-MMP) is capable of activating MMP-2, we also assayed the expression of this

enzyme following treatment of cardiac fibroblasts with IL-18 (Figure 2B). Western blot analysis illustrated that MT1-MMP expression was significantly increased following 24 hours treatment of fibroblasts with 10 and 100 ng/ml IL-18.

3.2 Collagen gel contraction

The 3-dimensional collagen gel model is widely used to assay the ability of fibroblasts to interact with and remodel the collagen matrix. A number of biochemical factors promote contraction of collagen gels by fibroblasts including transforming growth factor – β [35–36], insulin-like growth factor [24], platelet-derived growth factor [37] and angiotensin II [38]. Experiments were conducted to determine the effects of IL-18 on collagen gel contraction by fibroblasts isolated from adult male rat hearts. These experiments illustrated that addition of IL-18 at the higher doses (10 and 100 ng/ml) to the culture medium consistently and significantly enhanced contraction of 3-dimensional collagen gels (Figure 3 – note that smaller bars indicate greater collagen gel contraction). Addition of the lowest dose (1 ng/ml) of IL-18 had no significant effect on collagen gel contraction by adult heart fibroblasts. Despite enhanced collagen gel contraction, treatment of cardiac fibroblasts with IL-18 did not appear to promote the conversion of fibroblasts to myofibroblasts as indicated by the expression of α -smooth muscle actin (data not shown).

3.3 Migration assay

Several studies have illustrated a role for IL-18 in immune and inflammatory cell migration; however, the effects of this cytokine on cardiac fibroblast migration have not been examined [39–40]. A scratch (wound healing) assay was performed to determine whether IL-18 modulates cardiac fibroblast migration. These studies illustrated that, at the highest dose used in the present study (100 ng/ml), IL-18 slightly but significantly, enhanced the ability of fibroblasts to migrate into the denuded area of the culture dish (Figure 4).

3.4 Fibroblast proliferation

Cardiac fibrosis is characterized by an increase in the expression and accumulation of ECM components including collagens and other glycoproteins. In general, this is accompanied by proliferation of the interstitial fibroblasts. Experiments were performed *in vitro* to determine the effects of IL-18 on cardiac fibroblast proliferation. Similar to collagen gel contraction and migration assays described above, the highest dose of IL-18 used in the present study promoted a consistent and significant increase in fibroblast proliferation as measured by BrdU incorporation (Figure 5).

3.5 Integrin expression

ECM receptors of the integrin family are intimately involved in cardiac function [41–42] and mediate collagen gel contraction by heart fibroblasts [38,43]. The effect of IL-18 on the expression of the major collagen-binding integrins was examined in the present studies. The α 1 β 1 and α 2 β 1 heterodimers are two of the major collagen-binding integrins in heart fibroblasts. Western blot analyses illustrated that treatment of adult cardiac fibroblasts with IL-18 for 24 hours resulted in an approximately 2-fold increase in the expression of β 1 and α 2 integrins (Figure 6a and 6c, respectively). Interleukin-18 had no significant effect on the expression of α 1 integrin in the present studies (Figure 6b).

3.6 Activation of signal transduction pathways

Previous studies have indicated that IL-18 activates several different signaling pathways including Erk 1/ 2, JNK, PI3 kinase and NF- κ B in various cell types. Experiments were conducted to analyze the activation of signaling components by IL-18 in adult cardiac fibroblasts. Since significant alterations were largely seen in the 24 hour assays with only

the highest dose of IL-18 (100 ng/ml), that dose was chosen for the signal transduction experiments. The present studies illustrated that IL-18 had little effect on Erk 1/2 activation at the times assayed (not shown). In contrast, the activation of JNK and Akt (a marker of the PI3 kinase pathway) were significantly increased (as indicated by phosphorylation) within 30 minutes of treatment with 100 ng/ml of IL-18 (Figures 7a and 7b). Phosphorylation of these proteins continued to be increased relative to controls for at least 120 minutes of treatment. The antibodies used for Akt in the present studies did not distinguish between the individual Akt isoforms.

Recent studies have begun to elucidate the signal transduction pathways involved in specific cellular processes regulated by IL-18. Activation of Akt and PI3 kinase is essential for enhanced fibronectin expression following exposure of cardiac fibroblasts to IL-18 [22]. Induction of monocyte chemoattractant protein-1 expression by synovial fibroblasts involves activation of PI3 kinase and JNK [44]. As indicated above, treatment of adult cardiac fibroblasts in the present studies resulted in the rapid activation of JNK and PI3 kinase pathways. As previous studies have evaluated the roles of specific signaling pathways in ECM expression, the present experiments were conducted with pharmacological inhibitors to JNK and PI3 kinase to evaluate their roles in IL-18-induced collagen gel contraction and migration. The JNK inhibitor, SP600125 (10 μ M), had little effect on basal or IL-18-stimulated collagen contraction (Figure 8a). Inhibition of JNK appeared to have a slight inhibitory effect on basal migration of cardiac fibroblasts; however, this was not significant (Figure 8b). SP600125 did partially attenuate IL-18 induced migration (Figure 8b). Treatment of fibroblasts with the Akt inhibitor, LY294002 (20 μ M), almost completely prevented collagen gel contraction (Figure 8a) and migration by cardiac fibroblasts (Figure 8b). At the doses used here, the inhibitors did not appear to have cytotoxic effects on the cardiac fibroblasts.

Discussion

Tissue damage and stress are typically accompanied by a robust inflammatory response that includes recruitment of inflammatory cells to the damaged tissue and secretion of diverse biochemical factors including cytokines and growth factors. Although the inflammatory response is necessary for defense against foreign microbes, it has been suggested that this response is not required for tissue repair and that it plays a detrimental role by promoting tissue fibrosis [45–47]. A number of pro-inflammatory factors have been shown to promote fibrosis including several interleukins (IL-1 α , IL-6 and IL-17, for instance), chemokines of the CCL and CXCL families, growth factors including transforming growth factor- β and other biochemical factors. Several recent studies have illustrated that IL-18 also promotes fibrosis in the heart [20–22] and other organs [33,48–49]. In the heart, interstitial fibroblasts produce the bulk of the ECM components including collagens type I and III. Recent studies have shown that exposure of isolated cardiac fibroblasts to IL-18 stimulates ECM production and have begun to dissect the underlying mechanisms of this response [21,22]. Data presented herein also illustrate that exposure of cardiac fibroblasts to IL-18 results in enhanced expression of ECM components including collagen type I, collagen type III and periostin. It has been increasingly realized that fibroblasts are versatile cells that perform a number of functions in the heart in addition to ECM production [7,9,50]. The present studies were designed to examine the effects of IL-18 on additional functions of cardiac fibroblasts and illustrate that this cytokine promotes collagen remodeling, migration and proliferation by these cells.

Based upon protein structure, receptor family and overall function, IL-18, formerly called interferon gamma inducing factor, is a member of the IL-1 cytokine family [51–52 for reviews]. Originally, the primary function of IL-18 was described as promoting the T-cell

helper type 1 response (Th1) by inducing interferon gamma production in T cells and Natural Killer cells. Over the past decade, it has become clear that IL-18 is expressed by a number of cell types and has more widespread functions than induction of interferon gamma. These functions include regulation of gene expression including those for angiogenic factors like vascular endothelial growth factors [44], modulation of proliferation of various cell types including immune and nonimmune cells [53–55], and regulation of cell migration and metastasis [25,56–57]. In the cardiovascular system, increased levels of IL-18 have been identified in atherosclerotic plaques and a role for this cytokine has been suggested in plaque progression [16–17]. Elevated levels of IL-18 are also seen following myocardial infarction, heart failure and stroke [19,58–59]. That this cytokine plays a fundamental role in ischemia-induced myocardial remodeling was illustrated in studies utilizing antibodies to neutralize IL-18 [18]. In a mouse model of ischemia/reperfusion, administration of IL-18 antibodies resulted in reduced tissue damage and infarct size. Interleukin-18 plays an important role in myocardial hypertrophy as indicated by treatment of isolated cardiomyocytes [60] and mice with this cytokine [61]. Further evidence supporting the pro-hypertrophic role of IL-18 was seen by reduced myocardial hypertrophy following pressure overload in mice lacking IL-18 relative to that seen in wildtype mice [62]. These studies and others illustrate the diverse roles that IL-18 plays in tissue maintenance, remodeling and repair; however, much less is known about the effects of this cytokine on cardiac fibroblasts.

As mentioned above, IL-18 likely plays an important role in myocardial fibrosis through direct actions on the cardiac fibroblasts. Treatment of isolated fibroblasts with IL-18 results in increased expression of ECM components including fibronectin [22], interstitial collagens and periostin (present studies). This is consistent with previous studies illustrating a pro-fibrotic effect of IL-18 in other organs (33, 48–49). Interestingly, a recent study showed that IL-18 treatment of isolated dermal fibroblasts resulted in diminished expression of interstitial collagen mRNA and protein (63). Whether IL-18 elicits an anti-fibrotic or pro-fibrotic effect likely depends upon the cell type, cell phenotype and microenvironment. This is further suggested by contradictions in the effects of IL-18 on other cellular functions. Myocardial fibrosis is accompanied by an increased density of fibroblasts, either through proliferation of local fibroblasts or recruitment/ differentiation of exogenous cells. Experiments presented here illustrate that exposure of cardiac fibroblasts to IL-18 results in moderate, but significant increases in proliferation and migration. This is in agreement with studies illustrating that IL-18 stimulates vascular smooth muscle cell proliferation and neointima expansion [26,64–65]. Similarly, IL-18 enhances smooth muscle cell migration [26,66] as well as migration/ metastasis of gastric cancer and melanoma cells [25,67]. While these effects of IL-18 appear to be relatively widespread, several studies have indicated that IL-18 inhibits migration of some cell types including B Cells [68]. The diverse effects of IL-18 on cells may also be in part due to the levels of IL-18 to which the cells are exposed. While the concentrations of IL-18 used in the present study have been considered “physiological” levels (56), the actual levels of IL-18 in tissue are difficult to ascertain. Recent studies have illustrated normal levels of IL-18 in serum to be in the picogram per milliliter range; however, following myocardial ischemia, serum levels can increase into the nanogram per milliliter range (69). Thus, the concentrations of IL-18 used in the present study and other in vitro studies likely represent pathological levels.

Previous studies from our lab and others have illustrated that remodeling of collagen gels and migration of cells on collagen substrata are mediated at least in part by collagen-binding integrins (70–71). The fact that $\alpha 2$ and $\beta 1$ integrin expression are increased by IL-18 treatment suggests that up-regulation of the $\alpha 2\beta 1$ integrin complex is involved in IL-18-induced fibroblast migration and collagen remodeling. This is in agreement with previous studies that illustrated increased alpha M integrin (CD11b) and migration in leukocytes

following treatment with IL-18 (72). Numerous studies have also illustrated that MMPs are essential to migration in and remodeling of the ECM. Increased expression of MT1-MMP and activation of MMP-2 would again be supportive of enhanced migration of cardiac fibroblasts following exposure to IL-18. Combined these data suggest that IL-18 induces the expression of specific integrins and MMPs, which ultimately enhance the ability of cardiac fibroblasts to migrate in and remodel the collagenous ECM.

Significant interest has been placed recently on elucidating the signaling mechanisms whereby IL-18 mediates cellular function. Interleukin-18 acts via the IL-18 receptor, a heterodimer consisting of the ligand-binding α subunit and the β subunit, which is essential for signal transduction. Binding of IL-18 to the α subunit leads to recruitment of the β subunit and initiation of diverse signaling cascades. As discussed above, activation of specific signaling pathways is likely dependent on the cell type and microenvironment. Increased expression of fibronectin by heart fibroblasts is dependent upon activation of PI3 kinase, Akt and NF- κ B [22]. In the present studies, we also show that treatment of adult heart fibroblasts resulted in the activation of the PI3 kinase pathway (as indicated by Akt phosphorylation). Furthermore, treatment of fibroblasts with the PI3 kinase inhibitor, LY294002, substantially impaired basal and IL-18 induced collagen gel contraction and migration by these cells. Several prior studies have illustrated similar effects of PI3 kinase inhibition on collagen remodeling and contraction [73–75]. In contrast, the JNK inhibitor, SP600125, had little effect on collagen gel contraction; however, treatment of cells with SP600125 attenuated IL-18-induced fibroblast migration. This is in agreement with previous studies that showed a role for JNK in fibroblast migration stimulated by phorbol 12-myristate 13-acetate (PMA) [76]. These studies suggest that activation of specific signaling pathways by IL-18 may be necessary for particular downstream cellular events.

Interleukin-18 is beginning to be appreciated as an important modulator of myocardial remodeling. The present studies provide further evidence that this cytokine impacts not only ECM production by cardiac fibroblasts but other cellular processes important in myocardial fibroblast function including interactions with and remodeling of the ECM. Further studies will be critical to understanding the role of IL-18 in modulation of the interstitial fibroblast in vivo.

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Figure 1.

This illustrates the effects of IL-18 on expression of ECM components by fibroblasts isolated from adult rat hearts. Semiquantitative RT-PCR analyses of collagen α 1(I) (Figure 1a), collagen α 1(III) (Figure 1b) and periostin (Figure 1c) are graphically shown. Quantification of PCR products (examples are shown in the insets) with primers specific for collagen α 1(I), collagen α 1(III) and periostin was normalized to acidic ribosomal phosphoprotein P0. Data are presented relative to control, untreated cells. Also shown is graphic analysis of collagen I (Figure 1d), collagen III (Figure 1e) and periostin (Figure 1f) protein determined by Western blot assays of conditioned medium from fibroblasts treated for 24 hours with varying doses of IL-18. (insets show representative Western blots with the

approximate molecular weights of immune-positive proteins indicated) Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc test of IL-18 treated samples to control untreated samples.

Figure 2.

This graphically illustrates quantification of active matrix metalloprotease -2 (MMP-2) in conditioned medium of untreated fibroblasts and fibroblasts treated for 24 hours with varying doses of IL-18 (Figure 2A). The inset illustrates a representative zymograph indicating the 68 kD (active) and 72 kD (pro or latent) forms of MMP-2. Also shown is analysis of MT1-MMP protein expression (Figure 2B) in fibroblasts treated for 24 hours with varying concentrations of IL-18. The inset shows a representative Western blot with the MT1-MMP antisera, which recognized a major protein of approximately 60 kilodaltons. Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc comparison of IL-18 treated samples to control untreated samples.

Figure 3.

This graphically illustrates the effect of IL-18 on collagen gel remodeling and contraction by adult heart fibroblasts. Note that smaller bars are indicative of enhanced collagen gel contraction. The inset shows representative collagen gels treated with varying doses of IL-18. Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc test of IL-18 treated samples to control untreated samples.

Figure 4.

These photomicrographs (Figure 4a) show examples of the scratch migration assay at the time of the initial scratch through confluent untreated fibroblasts (0 Hr) and 24 hours after the scratch was created (24 Hr). The dotted lines indicate the edges of the scratch in each photomicrograph. Quantification of the effects of IL-18 on the ability of cardiac fibroblasts to repopulate the denuded area of the culture dish is shown in Figure 4b. Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc comparison of IL-18 treated samples to control untreated samples.

Figure 5.

This graphically illustrates the effects of IL-18 on proliferation of isolated cardiac fibroblasts. Fibroblast proliferation was determined by BrdU incorporation. BrdU was subsequently detected immunocytochemically and the percentage of BrdU-positive cells determined. Data are presented as the percentage of BrdU-positive cells in IL-18 treated cultures relative to that in untreated control cultures. Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc test comparison of IL-18 treated samples to control untreated samples.

Figure 6.

The expression of $\beta 1$, $\alpha 1$ and $\alpha 2$ integrins was evaluated by Western blot analyses and are presented graphically (Figures 6a, 6b and 6c, respectively). The insets show representative Western blots for the respective integrin with the approximate molecular weight of immune-positive proteins indicated. Data with integrin antibodies was normalized to signals obtained with glyceraldehyde 3-phosphate dehydrogenase. Data are presented as the integrin:glyceraldehyde 3-phosphate dehydrogenase ratio in IL-18 treated cells compared to that in control untreated cells. Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc test of IL-18 treated samples to control untreated samples.

Figure 7.

These show quantitative analysis of JNK (Figure 7A) and Akt (Figure 7B) activation following treatment of adult heart fibroblasts for varying times with 100 ng/ml IL-18. The insets show Western blots with antisera to the phosphorylated and total JNK (approximately 50 kilodaltons) and Akt (approximately 60 kilodaltons) proteins. Data are presented graphically as the ratio of phosphorylated JNK or Akt to total JNK or Akt levels in the IL-18 treated samples relative to that in the untreated samples. The dark bars represent IL-18-treated samples while the lighter bars represent the untreated controls at corresponding timepoints. Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's

posthoc comparison of IL-18 treated samples to corresponding control untreated samples at each timepoint.

8a

8b

Figure 8.

These show collagen gel contraction (Figure 8A) and migration (Figure 8B) assays performed with pharmacological inhibitors to JNK (SP600125) and PI3 Kinase (LY294002). Statistical significance (* $p < 0.05$) was determined by ANOVA with Dunnett's posthoc comparison of samples treated with pharmacological inhibitors versus control untreated samples.