Cardiac Fibroblasts Arrest at the $G_1$/$S$ Restriction Point in Response to Interleukin (IL)-1β

EVIDENCE FOR IL-1β-INDUCED HYPOPHOSPHYRLATION OF THE RETINOBLASTOMA PROTEIN*

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Although responsible for only approximately one-third of the overall myocardial mass, the interstitial fibroblasts of the heart serve a fundamental role in establishing the functional integrity of myocardium and are the major source of myocardial extracellular matrix production. Their importance in clinical medicine is underscored by the observation that fibroblast numbers increase in response to several pathologic circumstances that are associated with an increase in extracellular matrix production, such as long-standing hypertension and myocardial injury/infarction. Up to the present time, however, there has been little information available on either the kinetics of the cardiac fibroblast cell cycle, or the fundamental mechanisms that regulate its entry into and exit from the cell cycle. Previous work from our laboratory examining the effects of interleukin (IL)-1β on myocardial growth and gene expression in culture indicated that cardiac fibroblasts have a diminished capacity to synthesize DNA in response to mitogen in the presence of this cytokine. The mechanism of IL-1β action was not clear, however, and could have resulted from action at several different points in the cell cycle. The investigations described in this report indicate that IL-1β exerts its effect on the fibroblast cell cycle at multiple levels through altering the expression of cardiac fibroblast cyclins, cyclin-dependent kinases, and their inhibitors, which ultimately affect the phosphorylation of the retinoblastoma gene product.

Interleukin-1β (IL-1β) is a pleiotropic 17 $K_β$ hormone-like polypeptide produced by a number of cell types including mononuclear inflammatory cells, epithelial cells, endothelial cells, mesangial cells, smooth muscle cells, and fibroblasts (1). In the heart, expression of IL-1β by myocardial cells has been detected in several clinical circumstances that are characterized by immunologic myocardial injury (e.g., myocarditis, transplant rejection, ischemia, and congestive heart failure) (2–7). IL-1β expression during inflammatory myocardial injury is not unique, however, since myocardial cells respond to other pathologic stresses (such as pressure load) with the production of a number of both known and potentially novel growth substances (8–18) (reviewed in Long (19)). The production of these factors by myocardial cells is noteworthy since several growth factors and cytokines have been implicated in the initiation and regulation of myocardial growth under both developmental and pathologic conditions. In this regard, work from both our laboratory and others has identified the cardiac fibroblast as an important intracardiac source of IL-1β in both the acute response to hypoxia/ischemia as well as in circumstances associated with chronic cardiac dysfunction (20, 21) and IL-1β has been found to alter the growth of myocardial cells in culture (22–24). Specifically, IL-1β increases cardiac myocyte protein content while inhibiting cardiac fibroblast DNA synthesis (22). However, despite the previous findings on myocardial [3H]thy-midine incorporation, little work has been done to localize the effects of IL-1β on myocardial cell cycle control.

In contrast to the cardiac myocyte, whose replicative capacity is limited in the adult heart, the cardiac fibroblast retains the ability to proliferate, and does so in response to many pathologic circumstances. As such, the growth of the heart after birth (both developmental, or “physiologic,” growth as well as abnormal, or “pathologic,” growth) is characterized by hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. This disparity in growth potential is important since the cardiac fibroblast makes up nearly two-thirds of the total myocardial cell numbers, is the major source of extracellular matrix (ECM) production in the heart, and appears to be the major producer of many of the cytokines known to have potent effects on cardiomyocyte growth, fibroblast proliferation, and ECM homeostasis (19). Despite the obvious importance of the cardiac fibroblast in myocardial repair, however, there is little information available on either the kinetics of the fibroblast cell cycle, or the factors that regulate its initiation and progression. In order for the cardiac fibroblast to become a potential target for therapeutic manipulation, the fundamental mechanisms that regulate its entry into (and exit from) the cell cycle as well as the factors responsible for ECM deposition must be identified.

For virtually all mammalian cells with proliferative potential, a general scheme has emerged for the control of cell proliferation in response to mitogen stimulation. This scheme involves the cell cycle-specific stimulation of a binary system of cell regulators consisting of a family of regulatory subunits (the
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EXPERIMENTAL PROCEDURES

Materials—Minimum essential medium with Hanks' salts was obtained from Cell Culture Facility, University of California, San Francisco. Calf serum was obtained from Hyclone Labs (Logan, UT). Bovine serum albumin was obtained from Intergen (Purchase, NY). Propidium iodide (PI), N-hexanoyl-t-sphingosine (C6-ceramide), and N-acetyl-t-sphingosine (C2-ceramide) were obtained from Sigma. [3H]Thymidine (20 Ci/mmol) and [32P]ATP (10 mCi/mmol) were obtained from NEN Life Science Products. Recombinant mouse IL-1β was obtained from Genzyme Corp. (Cambridge, MA). N⁰-Monomethyl-t-arginine, mononacetate salt (l-NMMA) was obtained from Calbiochem. For immunoblotting experiments, fibroblasts were plated in 60-mm dishes (2.5 × 10⁵/dish), grown for 24 h, and then incubated with various stimuli (minimum essential medium, 5% calf serum). Cells were then synchronized by 48 h of serum starvation (minimum essential medium, 0.1% bovine serum albumin) and subsequently treated with calf serum in the presence or absence of IL-1β and the cell cycle kinetics determined by incorporation of [3H]thymidine (1-h pulse with 10 μCi/ml) and flow cytometry as described below.

Sample Preparation for Flow Cytometry—After treatment, cells were trypsinized at the indicated time with 500 ml of trypsin/EDTA (2 mg/ml/0.02%) for 2–5 min, scraped, and pelleted by centrifugation at 500 × g for 5 min. Cell pellets were fixed in 25% ethanol, 15 ml MgCl₂ on ice for 30 min. The fixed cells were pelleted and resuspended in 0.5 ml of calcium- and bicarbonate-free Hanks' solution with Hepes (CBFHH) supplemented with 10 mg/ml RNase A and incubated at 37°C for 30 min. Cellular DNA was stained with 10 mg/ml propidium iodide, and samples were filtered through a 70-mm nylon mesh to remove cell clumps. Samples were used immediately or kept at 4°C until analysis. No differences were seen between fresh and stored samples.

Flow cytometric analysis was done using a FACScan benchtop cytocounter (Becton and Dickinson Immunocytometry System, San Jose, CA) with a standard 15 milliwatt, 488-nm, air-cooled, argon-ion laser and standard filter sets. Single cell populations were gated using forward scatter, an indicator of cell size versus side scatter, an indicator of cell granularity. The FL2 detector measures fluorescent light from PI, which emits a red color at 650-nm wavelength of the FACScan laser, and PI intensity is proportional to the DNA content of the cell. The FL2-PI area versus width plots distinguished true cycling G/M cells from doublets or aggregates of G0/G1 cells by comparison to standardized area versus width plots and were adjusted in all experiments. Using Cell Quest Software (Becton and Dickinson Immunocytometry System), at least 20,000 cells were collected per sample at low flow rate (12 μl/min), and DNA data were analyzed with ModFit software (Verity Software House, Popham, ME).

Immunoblotting—For analysis of cell cycle-specific protein expression, cells treated with serum ± IL-1β were harvested in ice-cold homogenization buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1.0 mM EDTA, 5% calf serum), grown for 24 h, and then incubated with various stimuli (minimum essential medium, 5% calf serum). Cells were then synchronized by 48 h of serum starvation (minimum essential medium, 0.1% bovine serum albumin) and subsequently treated with calf serum in the presence or absence of IL-1β and the cell cycle kinetics determined by incorporation of [3H]thymidine (1-h pulse with 10 μCi/ml) and flow cytometry as described below.

Cell Culture—Primary cultures of neonatal rat cardiac fibroblasts were prepared as described previously (43). For flow analysis and immunoblotting experiments, fibroblasts were plated in 60-mm dishes (2.5 × 10⁵/dish), grown for 24 h, and then incubated with various stimuli (minimum essential medium, 5% calf serum). Cells were then synchronized by 48 h of serum starvation (minimum essential medium, 0.1% bovine serum albumin) and subsequently treated with calf serum in the presence or absence of IL-1β and the cell cycle kinetics determined by incorporation of [3H]thymidine (1-h pulse with 10 μCi/ml) and flow cytometry as described below.

In Vitro Kinase Assays—For the analysis of the cell cycle kinase activity, cells were treated as described previously and harvested in ice-cold homogenization buffer. Lysed cells were preclared for 60 min at 4°C with 20 μl of protein A-agarose and 5 μg of normal rabbit IgG. Precleared lysates were subsequently incubated with 20 μl p125⁰⁰—a-agarose-conjugated beads for 2 h at 4°C and collected by centrifugation (4,000 rpm for 5 min). Beads were washed twice with homogenization buffer and were used for in vitro kinase assay using either GST-Rb fusion protein or histone H1 as a substrate. Kinase reactions were carried out in kinase buffer (20 mM Hepes, pH 7.2, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 25 mM ATP, and 5 μCi of [γ-32P]ATP per reaction) for 15 min at 22°C. After the reaction was terminated by addition of 2× Laemmli buffer, and the proteins were separated by SDS-PAGE, dried, and autoradiographed. The bands corresponding to the phosphorylated GST-Rb and histone H1 were quantified by densitometric analysis and normalized to the control.

Statistics—Results are given as mean ± S.D. Mean values for two groups were compared using Student’s t test, or analysis of variance for
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RESULTS

IL-1β Inhibits Cardiac Fibroblast DNA Synthesis under Both Basal and Mitogen-stimulated Conditions—Previous work in our laboratory suggested that IL-1β had potent dose-dependent effects on cardiac fibroblast DNA synthesis in culture; however, the kinetics of this inhibitory response was unknown (22). Since the determination of the time course of inhibitory effects can provide important clues as to the cell cycle-specific site of action, we first evaluated the time-dependence of the IL-1β effect on mitogen-stimulated cardiac fibroblast proliferation in more detail. As noted in Fig. 1A, the inhibitory effect was seen if IL-1β was added at any point up to 12 h after the addition of mitogen (5% calf serum). If IL-1β was added after this point, however, there was a diminution in its ability to prevent [³H]thymidine incorporation. Similarly, in investigations into the time course of “release” from IL-1β-induced block, the peak of [³H]thymidine incorporation was also delayed approximately 12 h (Fig. 1B). These findings were quite similar to that seen in the response of mink lung cells (Mv1Lu) to transforming growth factor β and suggested that the IL-1β effect may be due to a similar mechanism and site of action (36).

IL-1β Prevents Cardiac Fibroblast Exit from G₀/G₁—Although the time course of IL-1β inhibition defined by [³H]thymidine incorporation suggested a site of action at the G₁/S restriction point, it was critical to define this with certainty. Flow cytometric analysis allows for the measurement of DNA content in isolated cardiac fibroblasts on a per cell basis and is the technique of choice for the determination of cell cycle kinetics. In preliminary experiments, the normal cell cycle distribution of cardiac fibroblasts was determined at various times after mitogen stimulation (range from 3 to 48 h). We found that the highest percent of cells in S phase was at 20 h (data not shown). Using flow cytometric analysis of these cells, we have determined the site of inhibition by IL-1β on cardiac fibroblasts cell cycle progression following their co-treatment with IL-1β and calf serum. As indicated in Fig. 2, IL-1β prevents fibroblast entry into S phase by arresting them at the G₁/S interphase.

IL-1β Effects Are Not Mediated through Prostaglandin, Nitric Oxide, or Sphingomyelin/Ceramide Pathways—As is true for most peptide growth factors/cytokines, the biological action of IL-1β is mediated through specific cell surface receptors. In this regard, the stimulation of IL-1 receptors has been shown to initiate a variety of signaling pathways, including cyclooxygenase/prostaglandins (45), nitric oxide (46), and sphingomyelinase (47–50). In order to understand which of these mechanisms might be involved in the arrest of cardiac fibroblast proliferation seen in our culture system, we performed a series of experiments in which inhibitors of these various pathways were included during the IL-1β treatment. Neither the nitric oxide synthase inhibitor t-NMMA, nor the cyclooxygenase inhibitor indomethacin had any effects on basal levels of DNA synthesis and were incapable of blocking the IL-1β effect. The percentage of cells in S phase for these experiments was 33 ± 2, 19 ± 2, 15.6 ± 1.6, and 16 ± 1.8 for serum, serum/IL-1β, serum/IL-1β/t-NMMA, and serum/IL-1β/indomethacin, respectively (n = 4, p = NS for all inhibitors). In view of the previous suggestion that the production of ceramide from sphingomyelin was associated with an inhibition of proliferation in other cells types (51), additional experiments aimed at understanding the role of the sphingomyelinase pathways were also performed. Using the putative down-stream effectors of sphingomyelin breakdown (C2- and C6-ceramide), we found that the cardiac fibroblasts were relatively refractory to these agents when examined under conditions of serum treatment similar to that used for IL-1β. The percentage S phase in these experiments for serum- and serum/C6-ceramide-treated cells was 30.3 ± 3.1, and 28.20 ± 2.0, respectively (n = 4, p = NS). In light of our previous report suggesting that the growth effect of IL-1β on cardiac myocytes was due to the action of a down-stream tyrosine kinase pathway (22), we attempted to address the effects of tyrosine kinase inhibition (both genestein and tyrphostin) on the IL-1β effect. Unfortunately, we were unable to either confirm or refute the effects of tyrosine kinase inhibition on the cardiac fibroblasts due to the basal effects of this class of inhibitor on cardiac fibroblasts proliferation. The percentage S phase in these experiments for serum, serum/tyrophastin-, serum/IL-1β-, and serum/IL-1β/tyrophastin-treated cells was 37.1 ± 2.5, 19.7 ± 1.4, 21.2 ± 2, and 18.3 ± 1.6, respectively (n = 4).

IL-1β Decreases the Phosphorylation State of Rb in Cardiac Fibroblasts—Studies of cell cycle regulation have identified the pocket protein Rb as a critical checkpoint control protein for the G₁-to-S phase transition (27, 28, 31, 52, 53). To determine...
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whether the phosphorylation status of Rb correlated with the extent of growth inhibition as judged by accumulation of cells in the G0/G1 phase of the cell cycle shown by fluorescein-activated cell sorter analysis, we performed Western blot analysis. As shown in Fig. 3A, in untreated cardiac fibroblasts, Rb was in the hypophosphorylated form (110 kDa), causing it to migrate faster than the hyperphosphorylated form in SDS-PAGE. Investigations into the time course of 5% calf serum treatment indicate that the shift in phosphorylation state of Rb reaches a plateau by 24 h. Cells that remained serum-starved over the 24-h treatment were similar to 0 h cells with Rb remaining in the hypophosphorylated form. As shown in Fig. 3B, cells co-treated with IL-1β showed a dose-dependent decrease in the expression of the higher mobility form of Rb. Using scanning densitometry, the ratio of the fast migrating form (Rb) to the relatively slow migrating forms (pRb) can be determined. When applied to our studies, we found that in calf serum-treated cells, 70 ± 8% of the Rb protein was in the pRb form (Fig. 3C).

IL-1β decreases in vitro GST-Rb phosphorylation. Although the changes in Rb mobility shown in Fig. 3 were likely the result of decrease in the activity of the G1/S cyclin-dependent kinases, it was critical to confirm that this was indeed the case. To assess kinase activity, cardiac fibroblasts were treated with calf serum ± IL-1β for 20 h followed by in vitro kinase reaction using either recombinant GST-Rb or histone H1. The

Figure 3. Calf serum induced Rb phosphorylation is inhibited by IL-1β. A, quiescent cardiac fibroblasts were stimulated with mitogen (5% calf serum) for the indicated times. Total cell protein extracts were prepared and 25 μg subjected to SDS-PAGE. Western blotting was accomplished using an anti-Rb antibody that detects both the hyperphosphorylated (pRb) and the hypophosphorylated (Rb) forms of Rb. B, quiescent cardiac fibroblasts were treated with vehicle (Con), mitogen (5% calf serum (CS)), and calf serum in the presence of increasing concentration of IL-1β (ng/ml). After 24 h, total cell protein extracts were prepared and subjected to SDS-PAGE and Western blotting using the anti-Rb antibody that detects both hyperphosphorylated (pRb) and hypophosphorylated (Rb) forms. C, densitometric analyses of Rb bands was performed and the intensity of the bands indicated by arbitrary units (y axis). Solid black bars indicate percentage hypophosphorylated Rb; gray bars indicate percent hyperphosphorylated form of Rb. Results shown are the mean ± S.D. of three independent experiments.

Of note was the finding that cells treated with IL-1β had a decrease in the overall level of detectable Rb. Similar effects were observed when Mv1Lu cells were treated with transforming growth factor β1 (36) and is of unclear etiology, although it may reflect the relatively short half-life for Rb (54).

Previous studies evaluating the role of the hypophosphorylation of Rb in transforming growth factor β induced G0/G1 growth arrest have shown that SV40 large T-antigen can rescue the cell from the G0/G1 block by bypassing the need for the Rb protein in the G1/S transition (36). To further address the role of Rb in the IL-1β-induced cell cycle arrest, we examined the effect of IL-1β in the SV40 large T-antigen-transformed cardiac fibroblast cell line (TxNMCs) whose [3H]thymidine incorporation had been shown previously to be unaffected by IL-1β (22). In our culture system, IL-1β did not inhibit S phase entry of T-antigen-transformed cardiac fibroblasts (percentage S phase in serum and serum/IL-1β-treated cells 18.3 ± 2.0 and 20.4 ± 2.3, respectively, n = 4, p = NS), confirming the necessity of a functional Rb protein for the antiproliferative effect of IL-1β to be manifest.
versus controls, respectively, in the serum-stimulated cells. Inhibitory effect at the G1/S restriction point with a decrease in protein associates specifically with several of the cyclin:Cdk complexes (55–58). As shown in Fig. 4A, representative autoradiograms of phosphorylated GST-hyperphosphorylated Rb, B, densitometric analysis of kinase activity with values normalized to control. Results shown are the mean ± S.D. of three experiments. *p < 0.05 versus control, †p < 0.05 versus serum.

Fig. 4. IL-1β decreases mitogen-stimulated cyclin-dependent kinase activity. Quiescent cardiac fibroblasts were treated with vehicle (control), mitogen (5% calf serum), and mitogen/IL-1β (5% serum/1 ng/ml IL-1β). After 20 h, cells were harvested, and 100 μg of the indicated protein extract incubated with p135**-conjugated agarose beads. Phosphorylation of GST-hyperphosphorylated Rb substrate was performed as described under “Experimental Procedures.” A, representative autoradiograms of phosphorylated GST-hyperphosphorylated Rb. B, densitometric analysis of kinase activity with values normalized to control. Results shown are the mean ± S.D. of three experiments. *p < 0.05 versus control, †p < 0.05 versus serum.

p135**-conjugated agarose beads were utilized since this protein associates specifically with several of the cyclin:Cdk complexes (55–58). As shown in Fig. 4B, in vitro GST-Rb phosphorylation increases by 4.2 ± 0.7-fold in serum-treated controls versus controls. In contrast, Rb phosphorylation increases only 1.7 ± 0.5-fold in serum/IL-1β-treated cells versus controls. Overall, these results indicate that IL-1β reduces cyclin-dependent kinase activity by 60%. Similar results were obtained using histone H1 as a substrate (data not shown).

IL-1β Inhibits Mitogen-stimulated Fibroblast Cyclin and Cdk Expression—Once we determined that IL-1β exerted its inhibitory effect at the G1/S restriction point with a decrease in cyclin-dependent kinase activity and phosphorylation state of Rb (Fig. 3, B and C), it was important to identify the potential mechanism(s) of this effect. In an effort to clarify this point, we examined the effects of IL-1β on the protein expression of G1/S cyclins (D2, D3, E, and A) and their catalytic subunits, Cdks 2 and 4 protein increase by 1.84 ± 0.1, 1.49 ± 0.03, 6.19 ± 0.4, 4.31 ± 0.80, 2.05 ± 0.17, and 1.90 ± 0.21-fold versus controls, respectively, in the serum-stimulated cells. In contrast, levels of these proteins in cells co-treated with IL-1β were 0.66 ± 0.1, 0.76 ± 0.01, 3.32 ± 0.1, 1.89 ± 0.16, 0.48 ± 0.03, and 1.03 ± 0.02-fold compared with the control, respectively. The decrease in cyclin/Cdk protein was specific for IL-1β treatment since reprobing of these membranes showed an increase in inducible nitric oxide synthase protein compared with both control and serum-treated cells as shown previously (data not shown) (59).

IL-1β Increases p27 and p21 Cyclin Kinase Inhibitor Levels in Cardiac Fibroblasts—The cyclin kinase inhibitors, p21 and p27, have been shown to interact with cyclin D, E, and A subunit as a mechanism of their actions (60–62). For this reason, we determined the extent to which the Cdk inhibitors p21 and p27, contributed to the IL-1β inhibitory activity in cardiac fibroblasts. Serum-starved fibroblasts were treated with 5% calf serum in the presence of increasing concentration of IL-1β. After 20 h, cells were harvested and protein subjected to SDS-PAGE and Western blotting using antibody that recognizes both p21 and p27 proteins. As indicated in Fig. 6A, p27 protein levels are strongly increased by IL-1β even in the presence of mitogen-rich growth medium. In contrast, p21 protein levels increase in both IL-1β- and serum-treated cells. Densitometric analyses of additional investigations into the effect of IL-1β on these CKIs are shown in Fig. 6B, indicating a preferential increase in p27 protein levels in IL-1β treated cells. Confirming that the increase in p27 protein was specific for IL-1β-treated cells, reprobing of the membranes indicate the expected decrease in G1 cyclin expression in the same extracts (but an increase in serum treated cells, data not shown).

**DISCUSSION**

Although the interstitial cells of the heart comprise only approximately one-third of the overall myocardial mass they serve a fundamental role in establishing the functional integrity of the myocardium and are the source of myocardial ECM production. Furthermore, the cardiac fibroblast is also the source of a variety of important growth factors/cytokines that can act via paracrine and autocrine mechanisms to affect both myocyte and fibroblast growth/gene expression, respectively (19). Their importance in clinical medicine is underscored by the observation that fibroblast numbers increase in response to several pathologic circumstances that are associated with an increase in ECM production, such as long standing hypertension and myocardial injury/infarction. The importance of the cardiac interstitium in maintaining overall cardiac health has lead to the term “interstitial heart disease,” indicating that certain disease states may predominantly involve the extracellular space (reviewed in Weber (63)). For example, in hypertensive heart disease, the interstitial compartment undergoes growth that may exceed that of the cardiac myocytes. Rather than cellular hypertrophy, however, interstitial growth takes the form of cellular hyperplasia and an increase in collagens I, III, IV, and fibronectin. This situation ultimately results in hypertrophied hearts, with an increase in both interstitial and myocyte mass (64–67). Similarly, following myocardial infarction, increases in non-myocyte numbers, collagen content, and myocardial stiffness have also been seen (68–71).

Unfortunately, the mechanism(s) underlying the interstitial cell fibroproliferative response to injury are not known with certainty. Recent work from a number of groups investigating the deposition of ECM has suggested that both locally produced and circulating vasoactive peptides (i.e. angiotensin II/aldosterone, norepinephrine, and endothelin-1) play important roles in myocardial remodeling under several pathologic circumstances. Specifically, studies using both the administration of these agonists (or their peptide-specific antagonists) as well as mechanical stretch have shown alterations in fibroproliferative response that support such a cause-effect role (70, 72–78). Additional in vitro work with some of the cytokines produced by the heart in response to injury have indicated that these substances are equally important in ECM homeostasis (76, 79–85).

Previous work in our laboratory with the cytokine IL-1β, one of the factors expressed during myocardial injury, indicate that this peptide has profound effects on both cardiac myocyte and cardiac fibroblast growth and gene expression (99). Although the mechanism(s) of the unique myocyte-specific effects of IL-1β have been explored in detail elsewhere (24), our prelim-
inary findings of an inhibitory effect of IL-1β on cardiac fibroblast DNA synthesis in culture had not been elucidated further. In view of the critical role played by the cardiac fibroblast in the process(es) of myocardial remodeling post injury, however, this remained an important area of research. It was the purpose of the investigations described in the present report to gain additional insight into the growth regulation of the cardiac fibroblast and expand our previous observations in a way that would help to understand the mechanism(s) of the antiproliferative effect of IL-1β. The ultimate goal of research into the mechanisms of fibroblast growth control is in the development of novel therapeutic approaches to instances of myocardial injury, which could target the interstitium of the heart as well as the contractile unit of the heart, the cardiac myocyte.

Entry into the cell cycle upon growth stimulation requires a number of coordination of events from the membrane to the nucleus. As expected, the cardiac fibroblast, like other cell types, enters the cell cycle in response to mitogen-induced signals. They do so by inducing G1/S phase genes, specifically the cyclins and their respective kinase partners, which are required for cell growth and differentiation (86). These protein complexes, which are activated in an ordered fashion, alter the ratio of phosphorylated to dephosphorylated Rb and subsequently the initiation of specific events such as DNA replication and subsequent cell division (reviewed in Morgan (87)).

The major finding of the studies reported here is that IL-1β appears to exert its inhibitory effect on the cardiac fibroblast at the G1/S interphase by preventing the phosphorylation of the retinoblastoma gene product, a key regulator of the G1/S transition in most mammalian cells. More specifically, IL-1β appears to prevent the post-translational modification of Rb in response to mitogen by a dual action on the expression and activity of the cyclins and cyclin-dependent kinases necessary for cell cycle progression as well as that of the G1 cyclin kinase.

**FIG. 5.** IL-1β inhibits mitogen-stimulated fibroblast cyclin and Cdk expression. Quiescent cardiac fibroblasts were treated with vehicle (control), IL-1β (1 ng/ml), serum/IL-1β (5% serum/1 ng/ml IL-1β) and serum (5% calf serum). After 20 h, cells were harvested, and 25 μg of the indicated extract protein subjected to SDS-PAGE and Western blotting using the specific antibodies. A, representative immunoblot analyses. B, densitometric analysis of the levels of protein expression. Values were normalized to control. Results shown are the mean ± S.D. of three independent experiments for each cyclin, and two experiments for each Cdk. *p < 0.01 versus control, **p < 0.05 versus control, †p < 0.01 versus serum treated cells, ††p < 0.05 versus serum treated cells.
overexpression of dominant negative p38/HOG-1 may help in confirming the role for this kinase family in the IL-1β effect. In addition, the importance of the CKIs in the IL-1β-mediated effect on cardiac fibroblast cell cycle kinetics could be further elucidated by taking advantage of genetically altered animals lacking expression of the p21 and/or p27 genes (98–101).

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2 F. Koudssi and C. S. Long, unpublished observations.
