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Paracrine effects of hypoxic fibroblast-derived factors on the MPT-ROS threshold and viability of adult rat cardiac myocytes

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Abstract

Cardiac fibroblasts contribute to multiple aspects of myocardial function and pathophysiology. The pathogenetic relevance of cytokine production by these cells under hypoxia, however, remains unexplored. With the use of an in vitro cell culture model, this study evaluated cytokine production by hypoxic cardiac fibroblasts and examined two distinct effects of hypoxic fibroblast-conditioned medium (HFCM) on cardiac myocytes and fibroblasts. Hypoxia caused a marked increase in the production of tumor necrosis factor (TNF)- α by cardiac fibroblasts. HFCM significantly enhanced the susceptibility of cardiac myocytes to reactive oxygen species (ROS)-induced mitochondrial permeability transition (MPT), determined by high-precision confocal line-scan imaging following controlled, photoexcitation-induced ROS production within individual mitochondria. Furthermore, exposure of cardiac myocytes to HFCM for 5 h led to loss of viability, as evidenced by change in morphology and annexin staining. HFCM also decreased DNA synthesis in cardiac fibroblasts. Normoxic fibroblast-conditioned medium spiked with TNF- α at 200 pg/ml, a concentration comparable to that in HFCM, promoted loss of myocyte viability and decreased DNA synthesis in cardiac fibroblasts. These effects of HFCM are similar to the reported effects of hypoxia per se on these cell types, showing that hypoxic fibroblast-derived factors may amplify the distinct effects of hypoxia on cardiac cells. Importantly, because both hypoxia and oxidant stress prevail in a setting of ischemia and reperfusion, the effects of soluble factors from hypoxic fibroblasts on the MPT-ROS threshold and viability of myocytes may represent a novel paracrine mechanism that could exacerbate ischemia-reperfusion injury to cardiomyocytes.

Keywords

mitochondrial permeability transition-reactive oxygen species threshold; cardiac myocyte viability; cardiac fibroblasts; hypoxia; fibroblast-conditioned medium; fibroblast proliferation; apoptosis; necrosis; tumor necrosis factor- α

THE HEART FUNCTIONS AS A SYNCYTIUM wherein interactions between cardiac myocytes and nonmyocytes may occur directly through cell-cell contact or indirectly via the production of autocrine and paracrine mediators by resident cells (4, 11, 17, 21). In pathological states, intercellular communication mediated by cytokines and growth factors can profoundly influence the structural and functional remodeling of the heart (25, 26). Growing evidence suggests that cardiac fibroblasts are an important source of cytokines and growth factors that may exert paracrine effects on other cell types (13, 29, 30, 31). For example, it is reported that these cells produce peptide factors that induce myocyte hypertrophy (11, 21), whereas myofibroblasts from the site of infarction express vascular endothelial growth factor and its receptors that may contribute to angiogenesis (7). In spite of being the most abundant cell type in the heart, the pathophysiological roles of cardiac fibroblast-derived factors in a setting of hypoxia remain unexplored.

Hypoxia is a major factor influencing the extent of cell injury and response in myocardial ischemia and infarction. The response of different cell types to hypoxia depends on intrinsic adaptive mechanisms. In cardiomyocytes, hypoxia triggers mitochondrial permeability transition (MPT) and apoptosis (20, 27). Cardiac fibroblasts, on the other hand, are resistant to hypoxic injury (22), and it has been reported that hypoxia reduces DNA synthesis in these cells (1). Moreover, recent studies in our laboratory show that hypoxia causes reversible cell cycle arrest in cardiac fibroblasts (unpublished observations), which might help conserve energy stores and, possibly, prevent cell death. The present study examined whether factors released from cardiac fibroblasts in response to hypoxia may modify these diverse cellular responses to the primary stimulus.

This communication furnishes evidence for the first time that hypoxic fibroblast-derived factors may enhance the susceptibility of cardiac myocytes to reactive oxygen species (ROS)-induced MPT and compromise myocyte viability. The finding points to a novel paracrine mechanism that could contribute to cardiomyocyte injury in a setting of ischemia and reperfusion in which both hypoxia and oxidant stress prevail. Additionally, this study demonstrates that soluble factors from hypoxic cardiac fibroblasts may exacerbate the effect of hypoxia on cardiac fibroblast proliferation.

MATERIALS AND METHODS

All fine chemicals were from Sigma Chemical (St. Louis, MO). Tetramethylrhodamine methyl ester (TMRM) was from Molecular Probes (Eugene, OR). [³H]thymidine (sp act, 18 Ci/mmol) was from Bhabha Atomic Research Center.

Isolation of cardiac myocytes and fibroblasts

Single cardiac myocytes were isolated from young adult male Wistar rats (3–4 mo) by a standard enzymatic technique (5). Cells were suspended in HEPES-buffered solution containing (in mmol/l) 137 NaCl, 4.9 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 15 glucose, 20 HEPES, and 1.0 CaCl₂, pH 7.2, and seeded on MatTek plates at $\sim 4 \times 10^4$ cells/cm². Freshly prepared cells were used in the experiments. Handling of animals and experimental procedures were in accordance with National Institutes of Health guidelines for animal care

and use [Manual 3040-2 (1999)] with Institutional Animal Care and Use Committee approval.

Cardiac fibroblasts were isolated by enzymatic digestion of ventricular tissue from young adult male Wistar rats followed by preplating in medium 199 with 10% FBS for 150 min (18). Cells from passages 2 and 3 were used for the experiments, and their fibroblastic nature was confirmed by immunocytochemistry using antibodies against vimentin, Factor VIII, and desmin (18). The cultures were free of other contaminating cell types (99% purity).

Preparation of conditioned media

Hypoxic ($PO_2 \sim 0.75$ mmHg/5% CO_2) and normoxic ($PO_2 \sim 150$ mmHg/5% CO_2 “control”) conditions were generated using the BBL-GasPak system and GasPak envelopes from Becton Dickinson. The PO_2 of the hypoxic and normoxic media was ~ 23 and 113 mmHg, respectively, and the pH was comparable between the groups. Viability of cardiac fibroblasts was not affected by >24 h of hypoxic incubation, as assessed by flow cytometric analysis of the sub- G_0/G_1 population and Syto13/propidium iodide staining. To generate conditioned media, confluent cultures of cardiac fibroblasts in serum-free medium 199 were maintained under normoxic/hypoxic conditions for 24 h at $37^\circ C$, and the culture supernatants were collected, centrifuged to remove any debris, and used directly for the experiments without storage or dilution.

Measurement of the MPT-ROS threshold by confocal microscopy

The MPT-ROS threshold was determined by confocal microscopy, following the protocol described earlier (14). Briefly, adherent myocytes were incubated for >120 min with conditioned media in the presence of TMRM and imaged with an LSM-410 inverted confocal microscope (Carl Zeiss, Jena, Germany) (14). Time scans were recorded from mitochondria arrayed along individual myofibrils in a line-scan mode with excitation at 568 nm and emission at >590 nm. Image processing was done using MetaMorph software (Universal Imaging, Downingtown, PA).

Measurement of DNA synthesis

Subconfluent ($\sim 60\%$) cultures of cardiac fibroblasts, synchronized by serum deprivation for 24 h, were exposed to the conditioned media for 24 h with $2 \mu Ci/ml$ of [3H]thymidine and processed for determination of acid-precipitable radioactivity (18).

Assessment of cell viability

Myocytes were exposed to the test media for 5 h and then washed with HEPES buffer containing 1 mM Ca^{2+} . Following this, the cells were incubated with annexin V-fluorescein isothiocyanate conjugate at $37^\circ C$ for 20 min in a humid chamber, counterstained with propidium iodide, and observed under a confocal microscope (Zeiss-LSM). A minimum of 300 cells were counted per dish.

Measurement of cytokine levels

Cardiac fibroblasts were exposed to normoxic/hypoxic conditions for 24 h. Levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-4, IL-6, IL-10, and interferon- γ in the culture supernatants were determined using the rat cytokine/chemokine LINCOplex kit (Linco Research), following the manufacturer's protocol.

Statistical analysis

Following overall comparison by one-way ANOVA, Student's *t*-test was used to examine differences between experimental groups, and significance was determined at *P* = 0.05.

RESULTS AND DISCUSSION

Cytokine release from cardiac fibroblasts, evaluated by the multiplex assay, showed that significant amounts of cytokines are produced by these cells under normoxic and hypoxic conditions (Fig. 1). A fivefold increase in TNF- α production under hypoxia suggested that hypoxia is a proinflammatory stimulus for these cells. In the present study, levels of other cytokines remained unaffected by hypoxia. IL-1 β , IL-10, and interferon- γ were undetectable (Fig. 1). TNF- α has been implicated in the pathogenesis of several cardiovascular diseases, including myocardial infarction, chronic heart failure, atherosclerosis, and viral myocarditis (23). Dramatic elevations in TNF- α levels are observed following myocardial infarction (25), and strong experimental evidence supports an important role for the cytokine in myocardial remodeling following acute myocardial infarction and the progression to chronic heart failure (23). Moreover, TNF- α has been implicated in cardiac myocyte apoptosis (8). The finding that fibroblasts might also be an important intracardiac source of this cytokine under hypoxic conditions is therefore particularly significant. It has been reported that hypoxia causes a >10-fold increase in TNF- α production in adult rat cardiomyocytes as well (6).

Cytokine production by these cells, particularly the striking increase in TNF- α release under hypoxia, led us to evaluate the effects of hypoxic cardiac fibroblast-conditioned medium (HFCM) on the MPT in cardiomyocytes and myocyte viability.

Mitochondria serve as final arbiters of life and death of the cell, since they are involved in the generation of ATP and play a critical role in triggering apoptosis or necrosis (15, 28). A key mechanism of mitochondrial injury involves the MPT that causes diffusion of small molecules across the mitochondrial inner membrane, resulting in mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude swelling that in turn lead to ATP depletion and cell death (28). There is increasing interest in the phenomenon of MPT because of its predominant role as a common pathway underlying both necrotic and apoptotic cell death during ischemia-reperfusion (10, 12, 14, 16, 32).

Determination of MPT induction by ROS in intact adult rat cardiac myocytes loaded with TMRM using high-precision confocal line-scan imaging with controlled, photoexcitation-induced ROS production within individual mitochondria is facilitated by the rigid, lattice-like distribution of mitochondria in these cells (14, 32). Basically, a row of ~25 mitochondria is photoexcited by laser, which results in controlled, incremental production of ROS that is

accumulated to the level at which MPT is induced in these mitochondria. The average time required to induce MPT in these mitochondria is calculated as the MPT-ROS threshold (tMPT). Shortening of the tMPT reflects cell stress, and the degree of tMPT shortening reflects the degree of stress. Using this technique, we examined the effects of fibroblast-conditioned media on the MPT-ROS threshold in adult rat cardiac myocytes. It was observed that HFCM significantly reduces the tMPT compared with normoxic fibroblast-conditioned medium (NFCM) and medium control that were comparable (Fig. 2), showing that hypoxic fibroblast-derived factors enhance the susceptibility of cardiomyocytes to MPT induction by ROS. The observation led us to examine if HFCM per se would impair myocyte viability, beyond its effect on the MPT-ROS threshold.

Exposure of cardiomyocytes to HFCM for 5 h resulted in loss of rod-shaped morphology with a significant increase in the number of round myocytes (Fig. 3). The treatment also resulted in an upward trend in the number of annexin-positive cells (Fig. 4), suggesting that HFCM may induce apoptotic changes in myocytes. Figure 5, *top* and *bottom*, provides a qualitative depiction of compromised cardiomyocyte viability in response to HFCM. Exposure of cardiomyocytes to the conditioned medium for 2 h was also found to affect cardiomyocyte viability (data not shown), but the effects were more marked at 5 h of exposure. Because of the striking increase in TNF- α levels in HFCM compared with NFCM, experiments were performed to see whether, by adjusting the TNF- α level in NFCM to about that in HFCM, the former would replicate the effects of HFCM on myocyte viability. NFCM spiked with TNF- α at 200 pg/ml induced morphological changes, with a significant increase in the number of round myocytes (Fig. 3). The treatment significantly increased the number of annexin-positive cells (Fig. 4). Although not statistically significant, there was also an increase in the number of propidium iodide + annexin-positive cells (Fig. 4). The findings indicate that low concentrations of hypoxic fibroblast-derived TNF- α may promote cardiac myocyte damage. Confirmatory evidence that TNF- α contributes to the HFCM effects on cardiomyocyte viability would require the demonstration that its removal results in significant attenuation of the modest HFCM effects. Remarkably, NFCM spiked with TNF- α at 200 pg/ml did not affect the MPT-ROS threshold of myocytes (Fig. 6), suggesting that TNF- α per se may not be responsible for all of the effects of HFCM. Thus, although TNF may be a major contributor, the data suggest that it may not have an exclusive role in the expression of the effects of HFCM and that other factors may also be involved, independently of or synergistically with, TNF. The significant increase in the number of apoptotic cells, in the absence of an effect on the MPT-ROS threshold, in response to TNF- α is compatible with the concept that apoptotic cell death need not necessarily be linked to MPT, which is consistent with the current thinking that MPT may be central in necrosis but not necessarily in apoptosis (3, 24). Together, the observations indicate that hypoxic cardiac fibroblasts may release soluble factors that could negatively affect the MPT-ROS threshold and compromise cardiac myocyte viability. Although the loss of viability in response to HFCM and TNF- α seems not dramatic, the observed differences should be viewed in light of reports that low levels of cardiomyocyte apoptosis can be a causal component of lethal dilated cardiomyopathy and heart failure (9).

The findings could be of considerable significance in vivo. Molecular and genetic studies in rodent models have clearly demonstrated that cardiomyocyte loss contributes to ventricular

dysfunction and is a critical process in the pathogenesis of heart failure (9). This in turn has fueled interest in molecular mechanisms that regulate cardiomyocyte death. In this context, the findings of the present study point to the possible role of paracrine mechanisms in cardiomyocyte loss. In particular, because both hypoxia and oxidant stress prevail in a setting of ischemia and reperfusion, these observations point to a novel mechanism that could exacerbate ischemia-reperfusion injury. The findings suggest for the first time a potential link between fibroblast-derived factors and mitochondrial stability in cardiomyocytes under pathological conditions.

To ascertain if HFCM would exert significant effects on fibroblasts themselves, basal levels of DNA synthesis were measured in cardiac fibroblasts exposed to the conditioned media. A 46% decrease in [³H]thymidine incorporation over 24 h was observed in cells incubated with HFCM (Fig. 7). Interestingly, NFCM spiked with TNF- α at 200 pg/ml decreased DNA synthesis in the cells by ~32% (Fig. 8), implying a potential role for TNF- α in modulating cardiac fibroblast proliferation under hypoxic conditions. Ongoing investigations in this laboratory show that hypoxia per se causes reversible cell cycle arrest in cardiac fibroblasts (unpublished data), which may represent a mechanism by which energy stores are conserved in these cells under hypoxic conditions. Moreover, it has been postulated that exit from the cell cycle may protect cells from apoptosis (2). The effect of HFCM on DNA synthesis, as seen in this study, is qualitatively and quantitatively comparable to that of hypoxia per se and, in tandem with the direct effects of hypoxia on cell proliferation (unpublished observations), suggests that oxygen deprivation may not only induce cell cycle arrest but may also trigger the release of factors from these cells that may add to the direct effects of hypoxia.

To conclude, there is increasing appreciation of the contribution of cardiac fibroblasts to multiple aspects of myocardial function and pathophysiology, including cardiomyocyte excitability and phenotype (17, 19). The independent effects of hypoxia on the production of peptide growth factors and cytokines by cardiac fibroblasts, and their autocrine/paracrine actions in the heart are, however, poorly defined. Intrinsic difficulties in evaluating these in the ischemic heart in vivo include the existence of a heterogeneous cell population and the inability to produce a uniform hypoxic insult. In this context, the present study used an in vitro cell culture model to evaluate cytokine production by hypoxic cardiac fibroblasts and has uncovered a novel mechanism by which soluble factors from hypoxic fibroblasts reduce the threshold for MPT induction by ROS in cardiac myocytes, compromise myocyte viability, and reduce fibroblast proliferation. It is remarkable that the effects of soluble factors from hypoxic fibroblasts are strikingly similar to, and hence may amplify, the distinct effects of hypoxia per se on cardiac myocytes and fibroblasts (1, 20, 27). The findings provide a compelling rationale for investigating the multiple actions of cytokines and growth factors derived from cardiac fibroblasts in various pathological states.

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Fig. 1.

Effect of hypoxia on cytokine production by cardiac fibroblasts. Cytokine levels in the culture supernatants were determined following exposure of cardiac fibroblasts to normoxic/hypoxic conditions for 24 h. Values are presented as means $\pm$ SD of 5 dishes. * $P = 0.0017$.

Fig. 2.

Effect of cardiac fibroblast-conditioned media on the mitochondrial permeability transition (MPT) pore-reactive oxygen species (ROS) threshold in adult rat cardiomyocytes. Cardiac myocytes were incubated for >120 min with conditioned media in presence of the tetramethylrhodamine methyl ester (TMRM) and imaged with an LSM-410 inverted confocal microscope. Time scans were recorded from mitochondria arrayed along individual myofibrils in a line-scan mode with excitation at 568 nm and emission at >590 nm. Image processing was done using MetaMorph software. Values are expressed as means $\pm$ SE; “*n*” denotes the no. of myocytes. **P* < 0.001 vs. normoxic fibroblast-conditioned medium (NFCM).

Fig. 3.

Change in cardiomyocyte morphology (rod to round). Cardiac myocytes were subjected to the indicated treatments for 5 h. The percentage of round cells in each group is expressed as mean $\pm$ SE; *n* denotes the no. of dishes per group. **P* < 0.0001 vs. NFCM.

Fig. 4.

Effects of hypoxic fibroblast-conditioned medium (HFCM) and tumor necrosis factor (TNF)- α on viability of adult rat cardiomyocytes. Cardiac myocytes were subjected to the indicated treatments for 5 h, and viability was assessed by annexin/propidium iodide staining, as described under MATERIALS AND METHODS. Values are expressed as means $\pm$ SE; n denotes the no. of dishes. The population marked “indeterminate” was propidium iodide positive but annexin negative. * $P < 0.001$ vs. NFCM.

Fig. 5.

Annexin V-propidium iodide staining of cardiomyocytes exposed to fibroblast-conditioned media. Cardiomyocytes were exposed to NFCM (1) or HFCM (2) for 5 h and stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide as described under MATERIALS AND METHODS. *A*: overlay of the bright field and both fluorescence channels (annexin and PI); rod-shaped and rounded myocytes are seen. *B*: overlay of FITC-annexin V and propidium iodide fluorescence. *C*: propidium iodide fluorescence. *D*: FITC-annexin V fluorescence. The two fluorescence channels were obtained sequentially, using a c-APOCHROMAT 10x/0.45 water immersion lens. Annexin fluorescence was excited at 488 nm, and the emission was collected at 515–565 nm. Propidium iodide fluorescence was excited at 568 nm, and the emission was collected at >590 nm. Loss of myocyte viability upon exposure to HFCM is evident in 2. Necrotic cells have become permeable to annexin.

Fig. 6.

Effect of NFCM + TNF- α on the MPT-ROS threshold of cardiomyocytes. Cardiac myocytes were incubated for >120 min with NFCM + TNF- α (200 pg/ml) in the presence of TMRM, and MPT-ROS threshold was determined as described under MATERIALS AND METHODS. Values are expressed as means $\pm$ SD; n denotes the no. of myocytes. The difference was not statistically significant.

Fig. 7.

Effect of cardiac fibroblast-conditioned media on DNA synthesis in adult rat cardiac fibroblasts. Synchronous, subconfluent cultures of cardiac fibroblasts from young adult rats were exposed to the conditioned media in the absence of serum for 24 h with [³H]thymidine (2 μ Ci/ml) and processed for determination of acid-precipitable radioactivity. Values are presented as means $\pm$ SD of 5 dishes. Similar results were obtained in three separate experiments. * P = 0.0002.

Fig. 8.

Effect of TNF- α on DNA synthesis in cardiac fibroblasts. Synchronous, subconfluent cultures of cardiac fibroblasts from young adult rats were exposed to NFCM spiked with TNF- α at 200 pg/ml for 24 h. [3 H]thymidine (2 μ Ci/ml) incorporation in DNA was determined as described under MATERIALS AND METHODS. Values are presented as means $\pm$ SD of 5 dishes. Similar results were obtained in two separate experiments. * P =0.0006.