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Original article

Cardiac fibroblasts protect cardiomyocytes against lethal ischemia–reperfusion injury

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ABSTRACT

Roles of cardiac fibroblasts (CFs) in the regulation of myocardial structure and function have been emphasized in the last decade. Their implications in pathophysiological aspects of chronic heart diseases such as myocardial remodeling and fibrosis are now well established; however their contribution to the acute phase of ischemia–reperfusion injury still remains elusive. We hypothesized that CF may contribute to cardiomyocyte (CM) protection against ischemia–reperfusion injuries. Experiments performed on isolated neonatal rat CF and CM demonstrated that the presence of CF in co-cultures increases CM viability (58 ± 2% versus 30 ± 2% in control) against hypoxia–reoxygenation injury, in a paracrine manner. It was confirmed by a similar effect of hypoxic CF secretome alone on CM viability (51 ± 9% versus 31 ± 4% in untreated cells). These findings were corroborated by in vivo experiments in a mice model of myocardial infarction in which a 25% infarct size reduction was observed in CF secretome treated mice compared to control. Tissue inhibitor of metalloproteinases-1 (TIMPs-1) alone, abundantly detected in CF secretome, was able to decrease CM cell death (35%) and experiments with pharmacological inhibitors of PI3K/Akt and ERK1/2 pathways provided more evidence that this paracrine protection is partly mediated by these signaling pathways. In vivo experiments strengthened that TIMP-1 alone was able to decrease infarct size (37%) and were validated by depletion experiments demonstrating that CF secretome cardioprotection was abolished by TIMP-1 depletion. Our data demonstrated for the first time that CFs participate in cardioprotection during the acute phase of ischemia–reperfusion via a paracrine pathway involving TIMP-1.

1. Introduction

Cardiac fibroblasts (CFs) are well-recognized as active participants in both cardiac development and function [1,2]. Their primary role is to produce extracellular matrix proteins including interstitial collagens, proteoglycans, glycoproteins, matrikines and metalloproteinases (MMPs) [3]. By secreting soluble factors, they are implicated in intercellular cross-talks that contribute to regulate cardiomyocyte phenotype in response to various environmental changes [4]. For example, TGFβ [5], IL-6 [6] or angiotensin II produced by CF can induce cardiomyocyte hypertrophy. Several studies have shown their involvement in various cardiac pathological processes including adaptive response to ventricular pressure overload, inflammation or hypertrophy where they are involved in tissue remodeling and fibrosis [7].

It has been suggested that CF may also play a role in myocardial ischemia–reperfusion injury. Paracrine factors like FGF-1 or FGF-2 and more recently IL-33 produced by CF have been reported to be cardioprotective after myocardial ischemia–reperfusion [8,9]. Among the factors secreted by cardiac fibroblasts, tissue inhibitors of metalloproteinases (TIMPs) are endogenous proteases inhibitors. TIMP-1 is the most characterized TIMP isoform and recently a novel function of TIMP-1 distinct from MMP-dependent mechanisms was described. A direct biological activity able to exhibit anti-apoptotic characteristics in non-cardiac cell types such as breast epithelial cell line has been reported [10–12]. Reports suggest that the imbalance between MMPs and TIMPs plays a role in myocardial infarction and adverse remodeling [13–16].

However, the actual function of CF during acute myocardial ischemia–reperfusion remains unclear. Using both in vitro and in vivo experimental preparations, we present evidence that TIMP-1 secreted by cardiac fibroblasts can protect cardiomyocytes from lethal

Abbreviations: CFs, Cardiac fibroblasts; CMs, Cardiomyocytes; H, Hypoxia; H/R, Hypoxia–reoxygenation; I/R, Ischemia–reperfusion; MMPs, Metalloproteinases; R, Reoxygenation; Secr, CF secretome; Secr ΔTIMP-1, CF secretome depleted from TIMP-1; TIMPs, Tissue inhibitor of metalloproteinases.

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reperfusion injury, likely through the activation of the $ERK_{1/2}$ and the PI3 kinase-Akt signaling pathways.

2. Methods

All the experiments were performed in accordance with the recommendations of the European Ethical Committee (EEC) (2010/63/EU), the French National Ethical Committee (87/848), and the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) for the care and use of laboratory animals. Protocols were approved by the Lyon 1 University animal care committee under the references BH2012-64 and BH2012-65.

2.1. Isolation and enrichment of neonatal rat cardiomyocytes and cardiac fibroblasts

Primary cultures of neonatal rat cardiomyocytes (CMs) were prepared from 2 day-old rat hearts (Wistar, Charles River, France) as described previously [17]. Briefly, ventricles were washed and minced in cold PBS (Phosphate Buffer Saline) solution. A series of digestion by 0.3% trypsin (Trypsin 2.5% 10×, Life Technologies) was performed at 37 °C in a spinner bottle under a stirring platform. Supernatant from all cycles of trypsinization was centrifuged and cell pellets were resuspended in complete medium composed by DMEM F-12 (Life Technologies), 20% Gold Fetal Bovine Serum (PAA) and 1% Penicillin/Streptomycin (Life Technologies). Cells were then plated at 37 °C in humidified air with 5% CO_2.

Cardiac fibroblasts (CFs) were separated from CMs by differential plating. CMs were seeded at a concentration of 2.5×10^3 cells/mm^2 in the presence of a non-proliferative drug AraC (Cytosine-1-β-D-arabinofuranoside, Sigma-Aldrich) 5 μM to enrich CM population (Fig. S1A). Culture media of CMs and CFs were changed every 2 days. CF primary cultures were prepared each week to avoid in vitro differentiation into myofibroblasts (Fig. S1B).

Downregulation of TIMP-1 was performed using siRNA (Rn-TIMP1_3 from Qiagen) at 50 nM. CFs were transfected using DharmaFECT transfection reagent (Thermo Scientific) according to the technical recommendations. TIMP-1 depletion was validated by mRNA and protein level quantification by qPCR and Western blot (Fig. S4A and B). Twenty-four hours post-transfection, the medium was removed and CFs were subjected to hypoxia 48 h post-transfection for further experiments.

2.2. Hypoxia–reoxygenation (H/R)

H/R experiments were completed 4 days after cell isolation. The hypoxia insult combined a substrate and oxygen deprivation in Tyrode buffer (in mM: NaCl 130, KCl 5, HEPES 10, $MgCl_2$ 1, $CaCl_2$ 1, pH 7.4) by 3 h exposure to N_2 95% and CO_2 5% at 37 °C into a specifically manufactured bio-incubator (Verretech Laboratories, France) as previously validated [17]. Oxygen concentration, pH, and temperature were continuously recorded. "Reoxygenation" included exposure to nutrient-rich and serum-deprived fresh DMEM F-12 together with placement into a normoxic humidified incubator with CO_2 5% for 21 h. Sham group is maintained in complete medium and in normoxic conditions.

2.3. Transwell experiments

We set up an original model to investigate paracrine interaction between CMs and CFs. The first objective was to determine whether a crosstalk between the two cell types would play a role in the tolerance of CMs to H/R. To do so, we first performed H/R on a co-culture of CMs and CFs, separated by cell culture transwells (transwell, 0.3 μm pore size, Falcon, Becton Dickinson). The transwell is a semi permeable membrane separating both cell types to only allow paracrine interaction. CFs were seeded at 1.5×10^3 cells/mm^2 in cell culture transwells 24 h before applying the H/R protocol. CFs were positioned above CM culture dishes thereby allowing a paracrine interaction with CMs during all the sequence of H/R (Fig. 1A).

2.4. Cardiac fibroblast secretome experiments

A second objective was to assess whether this crosstalk might be mediated by factors secreted by CF during H/R. We then separated the two cell types and collected the fluids secreted by CF alone when exposed to 3 h of hypoxia (named CF secretome: Secr).

For each experiment, CF secretome was freshly prepared from 80% confluence CF subjected to 3 h of hypoxia by deprivation from serum, nutrients and oxygen in Tyrode buffer. After filtration (0.2 μm), the collected CF secretome was diluted into fresh deprived-serum DMEM F-12 (1:1 volume) and added at the beginning of reoxygenation into CM dishes. In control H/R experiments, Tyrode buffer was diluted into fresh DMEM F-12 (1:1 volume) (Fig. 1A).

2.5. Evaluation of cell death

At the end of the reoxygenation period, the medium was removed and cells were incubated with a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) 1.2 mM. After 90 min at 37 °C, formazan crystals were dissolved in DMSO (Dimethylsulfoxide, Sigma-Aldrich). Mitochondrial dehydrogenase activity, reflecting cell viability, was quantified spectrophotometrically at 550 nm (Multiskan Ex, Thermo Electron Corporation, Shanghai, China). Measurement of troponin I in the supernatant was used for specific evaluation of CM death according to the manufacturer's instructions (Architect *Stat* troponin-I, Abbott), using anti-troponin I primary antibody and acrinidium coupled secondary antibody [17]. Cell death assay by flow cytometry was performed using Annexin V and Propidium Iodide (PI) staining according to the manufacturer's instructions (Life Technologies). Analysis was performed according to FL1-H (relative fluorescence intensity of annexin V) and FL3-H (relative fluorescence intensity of PI) fluorescence channels. Results were expressed as percentage of PI or Annexin V positive cells reflecting cell death proportion in each group.

2.6. Western blots

Lysates were obtained by lysing cell monolayer with 1% Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 5 mM EDTA) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (PhosphoStop, Roche Diagnostics). Protein concentration was normalized by determination of total protein concentration using BCA (Bicinchoninic acid) method (Interchim). In each sample, 25 μg of total proteins migrated in sodium dodecyl sulfate 12% polyacrylamide gel (SDS-PAGE). After migration, proteins were blotted to PVDF (polyvinylidene difluoride) membrane by electro transfer (Trans-Blot Turbo Transfer, Bio-Rad). Proteins were detected after labeling by specific primary antibodies [rabbit anti Phospho-Akt (Ser473) and rabbit anti-Akt from Cell signaling technology, rabbit anti Phospho-ERK1/2, mouse anti ERK1/2 from R&D Systems, mouse anti-β1 integrin from BD Biosciences, goat anti-TIMP-1 from Santa Cruz] revealed by secondary HRP (Horseradish peroxidase) coupled antibodies. Revelation was obtained by addition of the reaction substrate (ECL plus kit and Western blotting detection system from GE Healthcare). The protein amount was determined using ImageLab software (Bio-Rad).

2.7. Cytokine array

Cytokine detection was performed according to manufacturer's instructions (ARY008, R&D systems). Briefly, CF secretome was pre-