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Original Article

Human cardiac fibroblast extracellular matrix remodeling: dual effects of tissue inhibitor of metalloproteinase-2

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ABSTRACT

Objective: Tissue inhibitor of metalloproteinase-2 (TIMP-2) is an endogenous inhibitor of matrix metalloproteinases (MMPs) that attenuates maladaptive cardiac remodeling in ischemic heart failure. We examined the effects of TIMP-2 on human cardiac fibroblast activation and extracellular matrix (ECM) remodeling.
Methods: Human cardiac fibroblasts within a three-dimensional collagen matrix were assessed for phenotype conversion, ECM architecture and key molecular regulators of ECM remodeling after differential exposure to TIMP-2 and Ala+TIMP-2 (a modified TIMP-2 analogue devoid of MMP inhibitory activity).
Results: TIMP-2 induced opposite effects on human cardiac fibroblast activation and ECM remodeling depending on concentration. TIMP-2 activated fibroblasts into contractile myofibroblasts that remodeled ECM. At higher concentrations (>10 nM), TIMP-2 inhibited fibroblast activation and prevented ECM remodeling. As compared to profibrotic cytokine transforming growth factor (TGF)-beta1, TIMP-2 activated fibroblasts and remodeled ECM without a net accumulation of matrix elements. TIMP-2 increased total protease activity as compared to TGF-beta1. Ala+TIMP-2 exposure revealed that the actions of TIMP-2 on cardiac fibroblast activation are independent of its effects on MMP inhibition. In the presence of GM6001, a broad-spectrum MMP inhibitor, TIMP-2-mediated ECM contraction was completely abolished, indicating that TIMP-2-mediated fibroblast activation is MMP dependent.
Conclusion: TIMP-2 functions in a contextual fashion such that the effect on cardiac fibroblasts depends on the tissue microenvironment. These observations highlight potential clinical challenges in using TIMP-2 as a therapeutic strategy to attenuate postinjury cardiac remodeling.

1. Introduction

Tissue inhibitor of metalloproteinase-2 (TIMP-2) is a critical regulator of tissue remodeling. TIMP-2 is constitutively expressed in most cell types [1] and has diverse actions within the extracellular matrix (ECM) microenvironment. TIMP-2 inhibits a broad spectrum of matrix metalloproteinases (MMPs) [2] and retains some biologic actions that are independent of its MMP-inhibitory activity [3,4]. The actions of TIMP-2 are complex and sometimes paradoxical. For example, TIMP-2 inhibits MMP-2 activity, but also facilitates cell surface activation of pro-MMP-2 by selective interaction with membrane type 1-MMP (MT1-MMP), forming a tri-molecular complex of TIMP-2/Pro-MMP-2/MT1-MMP [5]. MMP-2 plays a critical role in maladaptive ECM remodeling after cardiovascular injury. Given that TIMP-2 can both suppress and stimulate MMP-2 activity, and in light of its MMP-independent bioactivity, the role of TIMP-2 on cardiovascular cells and tissue remodeling is poorly understood.

Cardiac fibroblasts regulate ECM homeostasis through synthesis of ECM components and secretion of MMPs and TIMPs [6]. After myocardial infarction (MI), fibroblasts become activated to myofibroblasts. Ongoing and persistent myofibroblast activity disrupts ECM homeostasis and causes maladaptive post-MI cardiac remodeling. Myocardial TIMP-2 levels are reduced during the late phase of post-MI ventricular remodeling [7]. In a rodent MI model, TIMP-2 deletion worsened infarct expansion and ventricular dilatation [8], whereas myocardial TIMP-2 overexpression at the time of MI significantly improved survival and limited maladaptive cardiac remodeling [9]. We previously determined that vascular smooth muscle cell transplantation induced TIMP-2 expression and limited post-MI maladaptive remodeling [10]. These data and others suggest that TIMP-2, through its effects on ECM homeostasis, may be an important therapeutic target for antiremodeling strategies after cardiac injury. Paradoxically, Lovelock and colleagues [11] observed that TIMP-2 induced a profibrotic response in cultured murine cardiac fibroblasts.

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As compared to other TIMP species, TIMP-2 uniquely activated myofibroblasts and increased collagen synthesis. Before clinical translation of TIMP-2 therapeutically, the effects of TIMP-2 on cardiac fibroblasts and ECM remodeling should be determined.

We examined the effects of TIMP-2 on human cardiac fibroblast-mediated ECM remodeling within three-dimensional (3D) collagen matrices. We explored the MMP-independent actions of TIMP-2 using a modified analogue of TIMP-2 (Ala+) devoid of MMP-inhibitory activity while maintaining its tertiary protein structure [12]. This is the first study to outline the effects of TIMP-2 on fibroblast regulation and ECM remodeling in human cardiac cells.

2. Materials and methods

2.1. Human cardiac fibroblast isolation and expansion

Right atrial appendage biopsies ($N=6$) were obtained from consenting patients undergoing routine cardiac surgery at Foothills Medical Center (Calgary, Alberta). Patients underwent coronary artery bypass grafting or valve replacement surgery. No patients had extensive structural atrial remodeling. All experiments involving human tissue were approved by Conjoint Health Research Ethics Board at the University of Calgary and conform to the Declaration of Helsinki. Samples were minced and dissociated in 0.2% Collagenase Type II at 37°C in an Isotemp Dry Bath (Fisher Scientific) with gentle shaking. Cell suspension was collected and remnant tissue was removed using tissue strainer of 40 μm pore size (BD Falcon). Collected cells were centrifuged at 1500 RPM (rotations per minute) for 5 min at room temperature (RT). Cell pellet was subsequently seeded in complete medium composed of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum plus 50,000 units of penicillin and 50,000 mg of streptomycin. Cells were cultured in an incubator at 37°C with a 5% CO_2 atmosphere. Cells from passages 4 to 8 were used.

2.2. Characterization of human cardiac fibroblasts

Immunohistochemistry staining was used to characterize the cultured cells. Cells were seeded on coverslips prior to fixation with 4% paraformaldehyde (PFA) at RT for 20 min, then simultaneously blocked and permeabilized at RT for 1 h by incubation in blocking buffer (2% goat serum and 1% bovine serum albumin in 1× PBS) containing 0.1% Triton X-100. Next, coverslips were incubated in corresponding primary antibody, all at 1:500 dilution unless otherwise stated: mouse anti-fibronectin (Calbiochem), mouse anti-fibroblast surface protein (Sigma-Aldrich), rabbit anti-smooth muscle-22-a (1:200 dilution; Abcam), rabbit anti-vimentin, rabbit anti-discoidin domain receptor 2, rabbit anti-troponin I, rabbit anti-desmin or rabbit anti-von Willibrand Factor (Santa Cruz Biotechnology) at 4°C overnight with gentle shaking. After washing in blocking buffer, coverslips were then incubated in secondary antibody at 1:500 dilution, either Alexa Fluor 633 goat anti-mouse or Alexa Fluor 633 goat anti-rabbit, for 1 h at RT with gentle shaking. Lastly, coverslips were washed with blocking buffer and then mounted onto microscope glass slide in Prolong Gold Antifade Reagent (Invitrogen) containing DAPI for counterstaining of nuclear visualization. All fluorescent images were captured using confocal laser microscopy (LSM 5, Carl Zeiss Microscopy, Thornwood, NY, USA) and processed using Zen software (Carl Zeiss Microscopy).

2.3. Assessment of 3D collagen ECM remodeling

Cultured human cardiac fibroblasts were serum starved for 24 h. Cells were trypsinized and added to a liquid form of neutralized rat-tail type I collagen (BD Biosciences) at a final concentration of 1.8 mg/ml. The liquid gel mixture containing cells was kept on ice during the preparation. On a 24-well-plate, 400 μl of the liquid gel mixture containing 2.5×10^5 cells/ml was dispensed into each well and incubated at 37°C for at least 1 h to allow for gel polymerization. Immediately after polymerization, 500 μl of IMDM either alone [serum-free medium (SFM)] or containing 10 ng/ml human recombinant transforming growth factor (TGF)-beta1 (Gibco-Invitrogen, Frederick, MD, USA), 10 nM human recombinant TIMP-2 (Calbiochem, EMD Biosciences Inc., La Jolla, CA, USA), 10 nM Ala+TIMP-2 (gift from Dr. Stetler-Stevenson, NIH), 10 nM human recombinant TIMP-3 (R&D Systems Inc, Minneapolis, MN, USA), TIMP-2 (10 nM)+50 nM GM6001 (Calbiochem, EMD Biosciences Inc.) or 100 ng/ml human recombinant MMP-2 (R&D Systems Inc) was added to each well. MMP-2 was activated by incubating in 0.05 M borate (pH 9.0), 0.01 mM $ZnCl_2$, 5 mM $CaCl_2$ and 0.5 mM APMA (aminophenylmecuric acetate; Sigma) at 37°C for 30 min. Plates were further incubated overnight in a 37°C incubator with a 5% CO_2 atmosphere. To initiate ECM contraction, the cell–ECM constructs were released from the well wall using a sterile microspatula (Corning). Serial images of the ECM dimensions were obtained from the time of release (baseline) and at 24 h. Image J analysis software (NIH, USA) was used to measure the area of ECM contraction as a quantitative measure of ECM remodeling.

2.4. Cell number and viability

Cells were released from collagen ECM by incubating the constructs in 100 μl of 500 units/ml collagenase type II in 37°C water bath with constant agitation until the ECM was completely dissolved. Subsequently, the cell suspension was centrifuged at 1500 RPM for 5 min. Cell pellet was resuspended in 1 ml of IMDM and 0.1 ml of 0.4% trypan blue stain (GIBCO). Ten microliters of cell suspension was loaded onto a hemacytometer, and then the total cell number and the number of blue-stained cells were counted. Cell viability (%) was calculated using the formula as below:

$$\text{Cell viability (\%)} = [1.0-(\text{number of blue cells}/\text{number of total cells})]\times 100$$

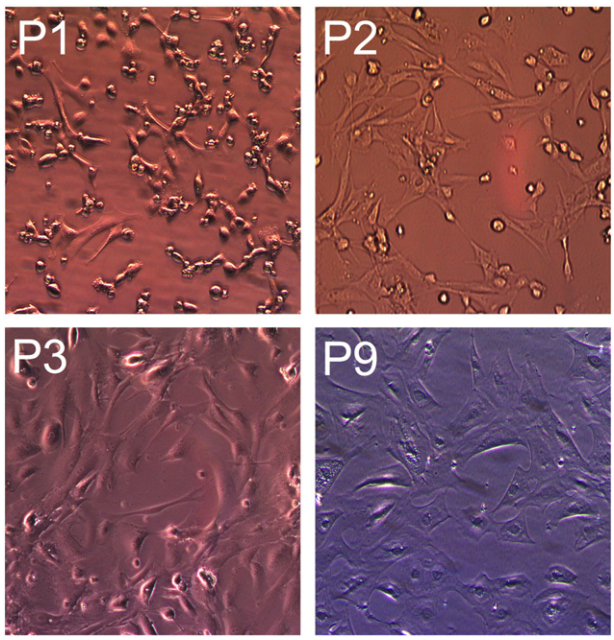

Fig. 1. Primary human cardiac fibroblasts morphology. Photomicrographs obtained from serial passages of human cardiac fibroblasts from the same isolation. Objective: 20×. Note that the changes in cellular morphology as cell passage increased. Scale bar=100 μm.