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Cardiac tissue inhibitor of matrix metalloprotease 4 dictates cardiomyocyte contractility and differentiation of embryonic stem cells into cardiomyocytes: Road to therapy☆

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ABSTRACT

Background: TIMP4 (Tissue Inhibitors of Matrix Metalloprotease 4), goes down in failing hearts and mice lacking TIMP4 show poor regeneration capacity after myocardial infarction (MI). This study is based on our previous observation that administration of cardiac inhibitor of metalloproteinase (~TIMP4) attenuates oxidative stress and remodeling in failing hearts. Therefore, we hypothesize that TIMP4 helps in cardiac regeneration by augmenting contractility and inducing the differentiation of cardiac progenitor cells into cardiomyocytes.
Methods: To validate this hypothesis, we transfected mouse cardiomyocytes with TIMP4 and TIMP4-siRNA and performed contractility studies in the TIMP4 transfected cardiomyocytes as compared to siRNA-TIMP4 transfected cardiomyocytes. We evaluated the calcium channel gene serca2a (sarcoplasmic reticulum calcium ATPase2a) and mir122a which tightly regulates serca2a to explain the changes in contractility. We treated mouse embryonic stem cells with cardiac extract and cardiac extract minus TIMP4 (using TIMP4 monoclonal antibody) to examine the effect of TIMP4 on differentiation of cardiac progenitor cells.
Results: Contractility was augmented in the TIMP4 transfected cardiomyocytes as compared to siRNA-TIMP4 transfected cardiomyocytes. There was elevated expression of serca2a in the TIMP4 transformed myocytes and down regulation of mir122a. The cells treated with cardiac extract containing TIMP4 showed cardiac phenotype in terms of Ckit+, GATA4+ and Nkx2.5 expression.
Conclusion: This is a novel report suggesting that TIMP4 augments contractility and induces differentiation of progenitor cells into cardiac phenotype. In view of the failure of MMP9 inhibitors for cardiac therapy, TIMP4 provides an alternative approach, being an indigenous molecule and a natural inhibitor of MMP9.

1. Introduction

The balance of extracellular matrix (ECM) is maintained by the interaction of MMPs (matrix metalloproteases) and TIMPs (tissue inhibitors of matrix metalloprotease) [1,2]. TIMPs are the endogenous inhibitors of MMPs in the cardiac ECM and the imbalance of MMP/TIMP is a hallmark of cardiovascular diseases including myocardial infarction [3–7]. The current study is based on our previous observation that cardiac inhibitor of matrix metalloprotease (~TIMP4) prevents adverse remodeling in volume overload heart failure mouse models and attenuates oxidative stress [8]. We have also established the role of extracellular matrix metalloproteases in end stage heart failure [9]. Initially, the function of TIMPs was confined to MMP inhibition due to limited available literature [10,11] however, emerging studies have indicated the role of TIMP in fine tuning ECM by TIMP mediated receptor signaling and regulating cardiac stem cell differentiation [12–14]. TIMPs consist of four family members (TIMP1–TIMP4) and TIMP4 is the least studied member. The role of TIMP4 binding partners, transcription factors and the signaling mechanisms in the context of heart failure, matrix remodeling and myocardial micro-environment is much needed to be explored.

TIMP4 is highly expressed in heart and mice with deficient TIMP4 succumb to induced myocardial infarction and have reduced survival rate [15]. In other words it can be inferred that, if TIMP4 is absent in the heart, it affects the regeneration capacity against myocardial infarction or the cardiac progenitor cells/stem cells fail to regenerate.

Abbreviations: AfCS, The Alliance for Cellular Signaling; ATCC, American Type Culture Collection Centre; BMP, bone morphogenetic protein; EB, embryoid bodies; ECM, extracellular matrix; GFP, green fluorescent protein; LIF, leukemia inhibitory factor; MCS, multiple cloning site; MEF, mouse embryonic fibroblasts; mESCs, mouse embryonic stem cells; MI, myocardial infarction; MMPs, matrix metalloproteases; PBS, phosphate buffered saline; ROS, reactive oxygen species; Serca2a, sarcoplasmic reticulum calcium ATPase 2; TBS, tris buffered saline; TGFβ, transforming growth factor β; TIMP, tissue inhibitor of matrix metalloprotease; vMHC, ventricular myosin heavy chain.

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However, in this context the studies are lacking on how TIMP4 regulates differentiation of cardiac progenitor cells and protection against myocardial infarction. The goal of this study is to understand the role of TIMP4 in cardiac function by exploring its effect on cardiomyocyte contractility and differentiation of embryonic stem cells into cardiomyocytes. Previous studies from our lab reported that MMP9 gene ablation induces survival and differentiation of cardiac stem cells into cardiomyocytes in the heart of diabetics [16]. In the study diabetic mice (insulin2 (+/−) akita) with MMP9 knock out were used and there was an increase in c-kit expression and survival in double knock-out mice which suggested improved survival and differentiation of stem cells. In another report from our lab we showed that MMP activation decreases myocyte contractility in hyperhomocysteinemia [17]. Hence overexpression of TIMP4 should augment contractility since TIMPs are the natural inhibitors of MMPs. We are looking into the mechanisms on how TIMPs can augment contractility by looking into calcium signaling gene serca2a (sarcoplasmic reticulum calcium ATPase2) and microRNA122a.

Embryonic stem cells provide an in vitro model system to study the factors that govern the differentiation of embryonic stem cells into cardiomyocytes. Defining these factors may help in elucidating the corresponding mechanisms in heart that regulate the differentiation of cardiac progenitor cells after myocardial infarction (death of myocardial tissue). Some of the factors that have been reported in promoting cardiogenesis include: 1) BMPs (bone morphogenetic proteins) that are members of transforming growth factor β (TGF β) superfamily [18,19]; 2) GATA transcription factors (1–6) and their co-activators Nkx-2 [20]; and 3) ventricular myosin heavy chain (vMHC) [21]. Binding of TGF β to the membrane receptors initiates signaling via MAP kinase pathway and phosphorylating smad family of transcription factors Nkx2.5 [22] which is helpful in regeneration. The transcription factor GATA-4 is a zinc finger protein with conserved domain WGATAR and binds to the DNA sequence CGATGG and AGATTA [20]. GATA-4 regulates the expression of cardiac specific genes troponin C, α-MHC and atrial natriuretic peptide [23,24] along with its co-activator Nkx2.5 [25].

Although the other members of TIMP i.e., 1, 2 and 3 are widely expressed, TIMP4 is specifically expressed in heart [26] and mice lacking other TIMP members are not as susceptible to MI as TIMP4 [15]. TIMP-3 has been reported to have beneficial effect in cardiac regeneration after MI [27], but Baker et al. [28] have reported TIMP 3 to cause apoptosis. Hence in the present study we hypothesize that TIMP4 helps in cardiac regeneration by promoting the differentiation of cardiac progenitor cells into cardiomyocytes and reducing contractile dysfunction by regulating serca2a through mir122a. To test this hypothesis, we transfected cardiomyocytes with TIMP4 in parallel with siRNA-TIMP4 and performed contractility studies. We checked the expression of serca2a and mir122a in the transformed cardiomyocytes. Additionally, we treated mouse embryonic stem cells (mESCs) with cardiac extract and cardiac extract minus TIMP4 to study the effect of TIMP4 on differentiation of mESCs into cardiac phenotype. This study provides insight into the role of TIMP4 in cardiomyocyte contractility and promoting cardiogenesis by using mouse embryonic stem cells as they are pluripotent, self-renewing and have the capacity to regenerate the defective myocardium.

2. Material and methods

2.1. Isolation and culture of mouse cardiomyocytes

For isolation and culture of cardiomyocytes, AfCS (The Alliance for Cellular Signaling) protocol (AfCS Procedure Protocol PP00000125 Version 1, 11/05/02) was followed. Proper approval was taken from the institutional IACUC (Institutional Animal Care and Use Committee) committee prior to animal experiments. Briefly, the heart was removed from C57BL/6 mouse, cannulated by aorta on Radnoti perfusion system and perfused with calcium free perfusion buffer for 4 min at 3 ml/min (the remaining blood was flushed from the vasculature and extracellular calcium was removed to stop contractions). The temperature of the perfusion system was maintained at 37 °C. Then the heart was perfused with myocyte digestion buffer containing Liberase blendzyme (Roche Molecular Biochemicals, Indianapolis, IN) for 8 to 10 min at 3 ml/min. The heart became swollen and turned pale. The heart was cut from the cannula below the atria and teased using forceps in a Petri dish containing 2.5 ml of myocyte digestion buffer. The solution was pipetted gently several times with a sterile plastic transfer pipette (2 mm opening) resulting in a homogenous suspension of myocytes. The suspension was transferred to two 15 ml tubes, one for plating and other for contractility studies. For contractility studies, the myocytes were allowed to settle down for 8–10 min and resuspended in myocyte stopping buffer 2 and calcium was reintroduced at a final concentration of 1.2 mM. The cells were counted using hemacytometer and found to be 90% rod shaped cells (Fig. 1a). The contractility studies were performed using ION OPTIX instrument (LLC, MA 02186, USA). Briefly, 1 ml of the resuspended myocytes were added to the FHD Microscope Chamber System provided by ION OPTIX having two electrodes and connected to the myopacer. The myopacer was set at 1 Hz frequency and 15 V. After the myocytes started contracting, the soft edge acquisition system recorded the contractions and the data was analyzed by ION wizard's analysis function. The myocyte isolation and contractility study was in agreement with Roth et al. [29] and Louch et al. [30]. For myocyte culture, the myocytes were resuspended after settling down in myocyte plating medium and the count was adjusted to 25,000 rod-shaped myocytes/ml of the plating medium in a 50-ml Falcon tube. The plating medium was equilibrated for 2 to 3 h at 37 °C in a 2% CO2 incubator. The cardiomyocytes were plated on laminin coated plates with concentration of laminin as 10 μg/ml. After plating, cardiomyocytes were transfected with TIMP4 plasmid for contractility studies.

2.2. Cloning of TIMP4 in DesRed2 plasmid

TIMP4 gene (GeneBank Accession Number BC064046) was cloned into pIRES2-DsRed2 plasmid (Clontech) which contains the IRES or internal ribosome entry site for enhanced mammalian expression and the DsRed2 gene for fluorescent red color detection (Fig. 1b). The TIMP4 mRNA (7846 bp) was amplified from mouse heart using forward and reverse primers containing restriction sites for EcoR1 and Sac II and cloned in frame into the vector MCS between EcoR1 and Sac II. The TIMP4 gene was confirmed by sequencing and restriction digestion. The expression of TIMP4 was checked by PCR, qRTPCR and Western blot in the transformed cardiomyocytes.

2.3. Transfection of mouse cardiomyocytes with eGFP, TIMP4 and contractility studies

The transfection of cardiomyocytes was performed using Lipofectamine 2000 (Invitrogen) at 2.5 μl and 1 μg of the plasmid DNA in OptiMEM medium (GIBCO). Lipofectamine (2.5 μl) and OptiMEM (22.5 μl) were mixed and incubated for 5 min at RT in an eppendorf tube. Similarly, DNA (1 μg–2 μl) and OptiMEM (23 μl) was mixed and incubated for 5 min at RT in another eppendorf tube. Then contents from both the tubes were mixed and incubated further for 5 min to form the liposomes. The resulting solution was then added to cardiomyocytes plated on laminin coated plates in OptiMEM. The transfection was standardized using the GFP plasmid (EF.GFP, plasmid 17616-Addgene). The siRNA transfection was done according to the manufacturer's protocol (Quiagen). The transfection was performed for 3 h, 6 h and 12 h and the expression of GFP, TIMP4 was checked at these time points using Western, Real time PCR and confocal imaging. The contractility studies were performed as explained above using the ION OPTIX (LLC, MA 02186, USA) as per the manufacturer's instructions. For calcium the myocytes were incubated with Fura-2 AM (diluted in incubation buffer