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Regulation of Cardiac Fibroblast Metalloprotease Secretion

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Abstract

Matrix metalloproteases (MMPs) are proteolytic enzymes secreted by cardiac fibroblasts. Altered expression of MMPs plays an important role in congestive heart failure and other cardiovascular diseases[1,2]. Emerging evidence has led us to consider the role of phosphatidylinositol 4-kinase (PtdIns 4-kinase) in regulating MMP secretion in cardiac fibroblast cells[3-7]. PtdIns 4-kinase is a lipid kinase involved in various cellular processes including protein secretion. This project was designed to test the hypothesis that PtdIns 4-kinase controls MMP secretion. Techniques such as cardiac cell culture, transfection, and in-gel zymography were used for this experiment. Results demonstrated that PtdIns 4-kinase regulates MMP secretion. This research will provide insights in revealing molecular details of congestive heart failure and will contribute to the development of strategies against cardiovascular diseases.

Abbreviations used:

APMA – 4-aminophenyl mercuric acetate
CVD – cardiovascular disease
ECM – extracellular matrix
GFP – green fluorescent protein
HBSS – Hanks buffered saline solution
MMP – matrix metalloprotease
PtdIns - phosphatidylinositol
PtdIns 4-kinase – phosphatidylinositol 4-kinase
PtdIns-4P – phosphatidylinositol 4-phosphate
PtdIns $(4,5)P_2$ – phosphatidylinositol-4,5-bisphosphate
SDS – sodium dodecyl sulfate

Introduction

Since 1900, cardiovascular disease (CVD) has been the leading cause of death in the United States except in 1918. In a 2006 update, the American Heart Association estimated that more than 71,300,000 Americans have one or more forms of CVD. In particular, five million Americans are affected by one type of CVD called congestive heart failure[8]. Therefore, understanding the development of cardiovascular diseases at the molecular and cellular level was the motive for this study. By understanding the events that lead to cardiovascular diseases, we can develop specific interventions and treatments that could slow or reverse this process.

An area of growing research is the association of heart extracellular matrix (ECM) with congestive heart failure. The extracellular matrix consists of complex network of proteins and polysaccharides that forms a dynamic meshwork and occupies a significant portion of the extracellular space in mammalian tissues. This matrix is involved in many cellular functions including structural support to tissues and intercellular communication. The normal heart ECM is predominately composed of collagen polymers which serve as major contributors to structural integrity of tissues[9].

The cardiac fibroblast cells are mainly responsible for the synthesis of ECM collagen. In addition, fibroblasts regulate ECM collagen through the secretion of proteolytic enzymes called matrix metalloproteases (MMPs). These enzymes are constitutively expressed and play a critical role in controlling the composition and form of the ECM. However, improper MMP levels have been associated with several cardiovascular diseases. Previous studies have shown that serum MMP levels are significantly elevated in patients with congestive heart failure[1]. Further, increased MMP

activity is also associated with cardiomyopathy and contractile dysfunction[2]. Hence, the goal of this research is to determine the underlying mechanisms that regulate MMP secretion.

In this research project, the control of MMP secretion by phosphatidylinositol 4-kinase (PtdIns 4-kinase) was examined. PtdIns 4-kinase is a lipid kinase that regulates numerous cellular processes including vesicular transport, signal transduction, and cytoskeleton remodeling[10]. Its activity is primarily membrane associated and localized to the nuclear envelope, endoplasmic reticulum, Golgi, lysosome, and plasma membrane[10-12]. PtdIns 4-kinases function by converting phosphatidylinositol (PtdIns) into phosphatidylinositol 4-phosphate (PtdIns-4P). PtdIns-4P can directly interact with target proteins or be converted to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). PtdIns(4,5)P_2 is commonly known as a signaling molecule and a substrate for phospholipase C to produce inositol 1,4,5-trisphosphate and diacylglycerol.

Because of its tractable nature, the yeast *Saccharomyces cerevisiae* has proven to be a useful organism to study secretory protein transport. In addition, the mechanism of protein secretion in yeast is generally conserved in mammalian cells[13]. In 1999, Hama and colleagues demonstrated that yeast PtdIns 4-kinase is involved in Golgi-to-plasma membrane vesicular transport[3-5]. In addition, it has been shown that PtdIns 4-kinases have an important role in protein secretion in higher eukaryotes[6].

A previous work by Edgar Lee demonstrated that Wortmannin, a known inhibitor of PtdIns 4-kinase, decreased MMP secretion in ordinary fibroblast cells[7]. This preliminary study suggested that PtdIns 4-kinase may play a significant role in cardiac fibroblast cells. Therefore, we hypothesized that PtdIns 4-kinase regulates MMP

secretion in cardiac fibroblast cells. To observe the functional role of PtdIns 4-kinase, overexpressed wild type PtdIns 4-kinase (overexpressed wt) and overexpressed point-mutated PtdIns 4-kinase (overexpressed pm) were constructed. These recombinant genes created a dominant negative phenotype effect and allowed us to assess PtdIns 4-kinase activity. If PtdIns 4-kinase controls MMP secretion, then it provides a potential intervention in treating congestive heart failure and other cardiovascular diseases.

Materials and Methods

PtdIns 4-kinase dominant negative alleles

The dominant negative alleles were created using a *S. cerevisiae* PtdIns 4-kinase called Pik1. The Pik1 gene encodes for a hydrophilic protein (1066 amino acids) that is a homolog of the mammalian PtdIns 4-kinase. The other types of PtdIns 4-kinase in yeast are Stt4p, which has an unknown role in secretion, and LSB6, which does not seem to have an essential role[14]. The catalytic domain of Pik1 was subjected to PCR mutagenesis to generate a point mutation and cause it to be inactive. Mutated gene sequence was amplified using PCR and was inserted into a bicistronic mammalian expression vector (pIRES vector, Clontech) containing green fluorescent protein (GFP). A simultaneous expression of both the gene of interest and GFP is achieved and this allowed us to rapidly select transfected cells. The recombinant plasmids were digested and run using agarose gel electrophoresis to verify that the gene encoding PtdIns 4-kinase was integrated with the vector. Plasmids were amplified using *E. coli* DH5α and were purified by precipitation with polyethylene glycol.

Cardiac Cell Culture

One to two days old mice were anesthetized on ice and killed by decapitation. Hearts were isolated and the ventricular tissues were digested in 0.25% trypsin in Hanks buffered saline solution (HBSS) for 10-15 minutes and were incubated with shaking at 37 ^{0}C. For every 5 minutes, contents were forcefully shaken to assist further breakdown of the remaining fragments into singly dispersed cells. Contents were poured through a sterile funnel-fitted tube with gauze that was initially pre-wetted with 5 mL of serum

supplemented medium (90% Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum). Cells were centrifuged at 1,000 rpm for 10 minutes and were resuspended using serum supplemented medium. After 60 minutes at 37 ^{0}C, the non-adherent myocytes were aspirated and a new medium containing 500 ug/ml penicillin and streptomyocin was added to the flask. The adherent cells were predominately fibroblasts and were cultured at 37 ^{0}C in a CO_2 incubator until confluency (5-7 days) and passaged using standard trypsin-EDTA release.

Transfection

One day before transfection, 2-8 x 10^5 cells were plated in a 6-well plate containing growth medium without antibiotics. When cells are 80-90% confluent, they were transfected using Lipofectamine from Invitrogen. For each transfection sample, DNA and Lipofectamine were mixed and incubated for 20 minutes at room temperature to create DNA-Lipofectamine complexes. These complexes were added to each well containing cells and medium. Cells were incubated at 37 ^{O}C in a CO_2 incubator for 24-48 hours. After transfection, GFP expression was monitored using BioRad 1024 laser scanning confocal microscope.

In-gel Zymography

Cells were starved with serum-free media for 4-6 hours before collecting the media for secreted MMPs. Cells were then collected by scraping and were lysed using a vortex to determine retained MMPs in the cells. Equal volume samples were concentrated in Microcon Centrifugal Filter Devices, YM-10 (Millipore Corporation,

Bedford, MA 01730) and sodium dodecyl sulfate (SDS) sample buffer was added. In-gel zymography was used to detect MMP activities and measure MMP levels. Zymogram gels were prepared with 12% resolving or separating gel [Gelatin Type A, H_2O, 30% acrylamide mix, 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, TEMED] and 5% stacking gel [H_2O, 30% acrylamide mix, 1.0 M Tris (pH 6.8), 10% SDS, 10% ammonium persulfate, TEMED]. Samples were activated using 10mM of 4-aminophenyl mercuric acetate (APMA) in 0.1 M NaOH for 2 hours in an incubator at 37 ^{0}C. Samples were loaded and run at 120 mV (small gels) in 1x SDS Running Buffer. Gels were removed from glass plates and soaked for 45 minutes in 2.5% Triton X-100 on a shaker. Gels were incubated in digesting solution [1M Tris-HCl (pH 8), 1 M $CaCl_2$, 1mM $ZnCl_2$, H_2O] for 24 hours at 37 ^{0}C. After incubation, gels were stained with Coomassie Blue for about 15-30 minutes and destained with destaining solution [40% methanol, 10 % acetic acid, H_2O] until the bands of lysis became clear.

Results

GFP expression by cardiac fibroblast cells was observed using a confocal microscope. Transfection efficiency using Lipofectamine varied from 30-50% on all the samples (Figure 1).

Figure1. **GFP expression in cardiac fibroblast cells.** Fibroblast cells were transfected with either an empty vector (left), a vector containing an overexpressed wt (middle), or a vector containing an overexpressed pm (right).

MMP activities were observed using an in-gel zymography. Media samples were collected after starving the cells with serum-free media to determine levels of secreted MMPs. Additionally, the cells were collected to assess levels of retained MMPs inside the cells. Control media and control cell lysate samples showed a slight MMP activity at ~ 55 kDa, while both media samples of overexpressed wt and overexpressed pm had no MMP activity. In contrast, cell lysates of overexpressed wt and overexpressed pm demonstrated MMP activities at ~55 kDa. Additional MMP activities were detected on all cell lysate samples at ~22 kDa (Figure 2). A reasonable interpretation of these results is that an MMP activity at ~55 kDa represents MMP13 and MMP activity at ~22 kDa represents MMP12. MMP13 is a member of the collagenase family and is capable of

degrading the fibrillar collagens of type I, II, III, and V. MMP12 is also known as metalloelastase and is commonly found in macrophages[15].

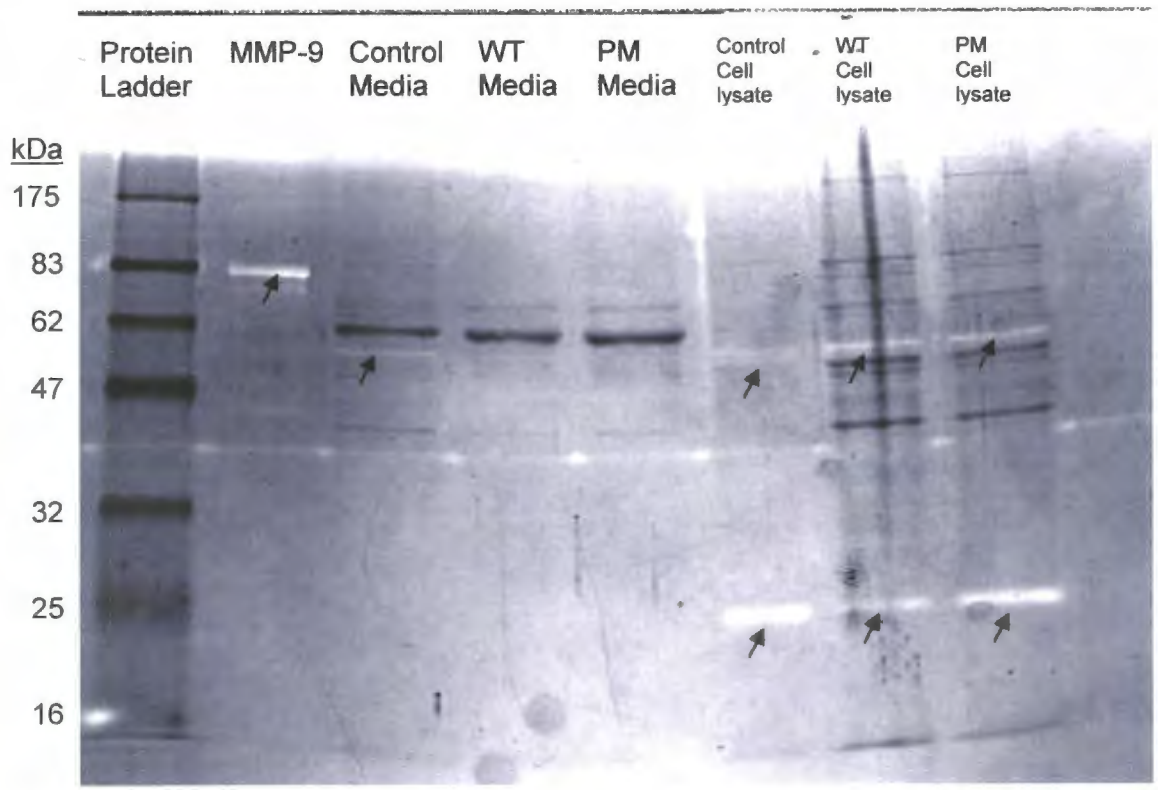

Figure 2. **In-gel zymography showing MMP activities.** The arrows point to white bands which indicate MMP activities while the dark bands represent other types of protein. The white bands around 40 kDa are background noises caused by the gel chamber. Media samples (Control, overexpressed wt, overexpressed pm) represent secreted MMPs while cell lysate samples (Control, overexpressed wt, overexpressed pm) represent MMPs retained in the cells.

Low levels of MMPs were detected on the gel due to the addition of SDS sample buffer containing β-mercaptoethanol in media and cell lysate samples. The β-mercaptoethanol is a reducing agent that severely denatures the MMP conformation which consequently prevents the MMP from refolding and becoming active after running the gel. As a result, MMP activities were either reduced or prevented from occurring.

Conclusions

Most phosphoinositide kinases are considered to be heterodimers containing both a catalytic and a regulatory protein subunits[12]. Previous reports have demonstrated that overexpression of an inactive version of a catalytic protein subunit of a phosphoinositide kinase created a dominant negative phenotype effect[16]. In this situation, the inactive catalytic protein subunits compete with wild type catalytic protein subunits. Consequently, this will decrease the overall phosphoinositide kinase activity. In a similar condition, overexpressing a wild type version of PtdIns 4-kinase creates an analogous effect with the dominant negative phenotype. It is unknown how this occurs, but it is suggested that forced overexpression of PtdIns 4-kinase in yeast and mammalian cells creates an imbalance in the membrane trafficking system and causes the Golgi to scatter[14]. Therefore, with the disruption of membrane trafficking, it is presumed that MMP secretion is hindered.

Our results confirmed that PtdIns 4-kinase regulates MMP secretion. As shown in Figure 2, the absence of MMP13 secretion in media samples for both the overexpressed wt and overexpressed pm indicated that MMP13 was not secreted outside the cell. Instead, MMP13 for overexpressed wt and pm were kept inside the cell. Therefore, we conclude that PtdIns 4-kinase activity is involved in Golgi-to-plasma membrane vesicular transport. Further, we conclude that when PtdIns 4-kinase activity is decreased or membrane trafficking is disrupted, MMP13 secretion decreases correspondingly. To confirm if overexpression of PtdIns 4-kinase scatters the Golgi in mammalian cells, Bodipy TR conjugated ceramide (Golgi stain) will be used to observe any changes in the Golgi and to check for vesiculation. Contrary to the results of

MMP13, MMP12 did not display a similar pattern; they were all seen in cell lysate samples. Since MMP12 is associated with macrophages, perhaps high concentration of macrophages existed in the cell culture.

In 1998, Tyagi discovered that high levels of specific MMPs (MMP1, MMP2, MMP9) were found under heart failure conditions[2]. This led us to predict that MMP1, MMP2, and MMP9 have an essential role in remodeling the ECM of the heart and will be highly secreted in mouse cardiac cells. However, our results indicate that MMP13 may play an important role in cardiac cell function and ultimately in cardiovascular diseases. A replication of this experiment will be completed to confirm our results. It is imperative that we use SDS sample buffer without the β-mercaptoethanol on all samples in order to enhance the detection of MMP activities. Quantitative analysis of MMPs will also be completed to determine the expression levels of MMPs.

This study will provide a greater understanding on the role of PtdIns 4-kinase in regulation of MMP secretion. By providing additional insights, this study will contribute to the likely development of new drugs for the treatment of cardiovascular diseases.

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