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Effect of Curcumin on Matrix Metalloproteinases Screened in Norepinephrine Induced Cardiac Hypertrophy

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Abstract

Cardiac hypertrophy is a compensatory cellular response of the heart for an increased biomechanical stress which leads to a compromised cardiac output. Prolonged hypertrophy is known to be a major cause of cardiac failure. Various efforts are being made to develop strategies for reversal of hypertrophic conditions by targeting specific pathways. The family of matrix metalloproteinases (MMPs), which are responsible for remodelling of the extracellular matrix, are believed to be potential therapeutic targets. In this study, the screening and identification of putative MMPs involved in cardiac hypertrophy was done through a phylogenetic classification of all 23 human MMPs using distance based NJ and UPGMA methods. Nearest neighbours to MMP-2 and MMP-9, which have been recently reported in cardiac hypertrophy, were identified and investigated for similar functions through experimentation. Induction of hypertrophy through norepinephrine and its reversal by curcumin, a biophenolic compound derived from turmeric (*Curcuma longa*), was done in H9C2 cardiomyocyte cell line. Casein zymography was used to identify MMP activity and MMP-7 was identified as a potential protease functioning in cardiac hypertrophy, other than gelatinases (MMP-2 and MMP-9) previously reported. We thus report an increased activity of MMP-7 in conditions of induced cardiac hypertrophy, as well as the role of curcumin as an effective therapeutic agent for reversal of this condition.

Keywords: Curcumin, MMPs, MMP-7, Cardiac Hypertrophy, Norepinephrine

Introduction

Cardiac hypertrophy is a compensatory cellular response of the heart to various extrinsic and intrinsic stimuli that impose a hemodynamic burden for an increased cardiac output and a normalized environment. A prolonged existence of this process leads to maladaptive conditions that pose risk of deleterious outcomes such as heart failure, and sudden death [1]. The distinct responses characteristic of hypertrophy include an increased cell size, accelerated synthesis of sarcomeric and structural proteins, higher organization of sarcomere and re-expression of fetal genes (atrial natriuretic factor [ANF], beta-myosin heavy chain [β-MHC], skeletal alpha actin [SKA]) [2, 3]. A cascade of intracellular signalling pathways modulates the hypertrophic growth of cardiomyocytes [4, 5].

Extracellular matrix (ECM) is a major communication channel between cells. It acts as a mediator to regulate cellular proliferation, migration, adhesion and changes in gene expression during homeostasis and development [6]. The dynamic and metabolically active ECM of the heart interacts with cells of myocardium and undergoes remodelling during hypertrophy [7].

Matrix metalloproteinases (MMPs) are produced and secreted by cells of the myocardium and form component of ECM. These proteases are zinc-containing endopeptidases subdivided into six classes with their activity being regulated by Tissue inhibitors of metalloproteinases (TIMPs).

They are known to degrade other ECM proteins with substrate specificity [8, 9]. ECM integrity is maintained by a balance in the activity of MMPs and their inhibitors, TIMPs. The normal balance between activities of MMPs and TIMPs is lost in cardiac pathologies [10]. An enhanced ECM protein synthesis and thus increased MMP levels are observed during cardiac hypertrophy.

Recent studies have reported the presence of MMP-2 and 9 in cardiac hypertrophy [11]. MMPs are a large family of proteases (26 in vertebrates; 23 in humans) and a considerable amount of information is hidden in their structure that needs to be extracted using advanced biocomputational tools [12]. Analysis through these methods may provide a solution to the complex biological problems associated with the proteome of organisms. Phylograms highlight the evolutionary relationships and help to find link between sequence homology and functional characteristics of a protein. This sequence analysis may facilitate identification of the closely related neighbours that might be implicated in a disease.

In order to find out other potentially involved MMPs in cardiac hypertrophy, phylogenetic analysis was performed to screen the MMPs. Further studies were carried out on selected MMPs to confirm their role in hypertrophy. Norepinephrine (NE), an α-adrenergic agonist and a catecholamine, was used for the induction of hypertrophy under *in vitro* conditions. Curcumin (diferuloylmethane) is a natural polyphenol and the most active component of turmeric (*Curcuma longa*) giving it a characteristic yellow colour [13]. It plays a therapeutic role in cardiovascular diseases. Recent work has also reported its important role in cardiac hypertrophy with its ability to inhibit p300 histone acetyltransferase (p300 HAT) activity that plays a crucial role

in progression of hypertrophy and heart failure [14, 15, 16]. It was thus used as a reversal agent for cardiac hypertrophy. Studies were done to identify the effects of curcumin on the screened MMPs that can act as valid therapeutic targets.

Materials and Methods

Phylogenetic analysis of human MMPs

Complete sequences of 23 human MMPs were retrieved from SWISS-PROT, a public domain curated protein sequence database in FASTA format [17]. The sequences were aligned using multiple sequence alignment program ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [18]. Phylograms were constructed with the help of Neighbour Joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) methods and visualized in Treeview v1.6.6.

Cell culture and maintenance

Heart-derived H9C2 cardiomyoblast cells were obtained from National Centre for Cell Sciences (NCCS), Pune and cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with antibiotics (penicillin, streptomycin, gentamycin, amphotericin B), glucose, L-Glutamine,sodium bi-carbonate and 10% FBS (Sigma Aldrich, USA). Cells were maintained in humidified CO2 incubator (New Brunswick Scientific, USA) with 5% CO2 at 37oC and subcultured routinely at a split ratio of 1:3. These cells were treated with 2 μM norepinephrine (Sigma Aldrich, USA) in serum free DMEM with Insulin-Transferrin-Selenium (ITS) Supplement (Sigma Aldrich, USA) for 48 hrs to induce hypertrophy [19]. The cells were then harvested with Trypsin-EDTA (Sigma Aldrich, USA) and used for experimentation.

MTT assay for determination of *in-vitro* cytotoxicity of curcumin

In-vitro cytotoxicity of curcumin was determined using MTT assay [20]. Cells were treated with varying concentrations of curcumin (Sigma Aldrich, USA) for dose optimization. MTT (3-(4,5 dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, USA) was added to curcumin treated cells and the colour development was measured at 570 nm prior to addition of Dimethylsulfoxide (DMSO) (Sigma Aldrich, USA) in the ELISA plate reader (Bio-Rad). Cell viability was defined relative to untreated control cells as follows: Cell viability = absorbance of treated sample /absorbance of control.

Reversal of hypertrophic condition using curcumin

H9C2 cells were cultured in serum free DMEM media supplemented with ITS Supplement in presence of norepinephrine and curcumin at optimized concentrations for 48 hrs. This was followed by a morphological and biochemical analysis to see the effect of curcumin on hypertrophy.

Morphological analysis of cells

Cells were observed under an inverted microscope (Olympus) at 40X magnification and the images were captured. The cells were examined for an increased cell size

which was quantified in different fields using Image J software (National Institutes of Health) to see the presence of hypertrophy.

RNA isolation, cDNA synthesis, RT-PCR conditions

Total RNA was isolated from the cell pellet using TRIzol Reagent (Ambion) and the integrity of RNA was assessed on 1.2% agarose gel electrophoresis containing formaldehyde. The RNA was reverse transcribed using gene-specific ANF primers. cDNA was amplified by PCR using Taq Polymerase. β-Actin, was used as a loading control. The primers used for amplification:

ANF: 5'-CTGCTAGACCACCTGGAGGA-3' (F), 5'-AAGCTGTTGCAGCCTAGTCC-3' (R);

β-Actin: 5'-CATCGTACTCCTGCTTGCTG-3' (F), 5'-CCTCTATGCCAACACAGTGC-3' (R)

Extraction of total protein

Cell pellet was washed with ice cold 1X Phosphate buffered saline (PBS) solution. Total protein extraction buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Glycerol, NaCl, MgCl2, Triton-X 100, Phenylmethylsulfonyl fluoride (PMSF)) was added and cells were incubated at 4oC for 1 hr. Cells were centrifuged at high speed and low temperature. The supernatant was aliquoted and stored at -80oC. Total protein was quantified using Bradford estimation method [21].

Substrate Zymography

The total protein was solubilized in sample buffer containing 2% SDS without reducing agents [22]. A 10% polyacrylamide gel with casein (1mg/ml) as substrate was loaded with equal quantity of total protein samples and allowed to run. Gel was washed in 2.5% Triton X -100 for 1hr, 37oC after electrophoresis. This gel was then incubated in the incubation buffer (Tris pH=7.4, NaCl, CaCl2, Brij-45) for 48 hrs at 37oC. Staining of gel was done in 0.1% Coomassie brilliant blue R-250 solution in methanol and acetic acid for 1 hr followed by destaining.

Results

Phylogenetic analysis for screening of MMPs

Human MMP protein sequences were aligned using ClustalW2 and phylogram was constructed by NJ method (Fig 1). The phylogram was further validated using UPGMA method (data not shown). The evolutionary relationships and distances were established for this large family of proteases to identify closely related members of MMPs already reported in cardiac hypertrophy.

Sequence homology could possibly be related to similar functional characteristics of MMPs in a disease. MMP-7 and 20 were identified as neighbours to MMP-2 and 9 and investigated through experimentation.

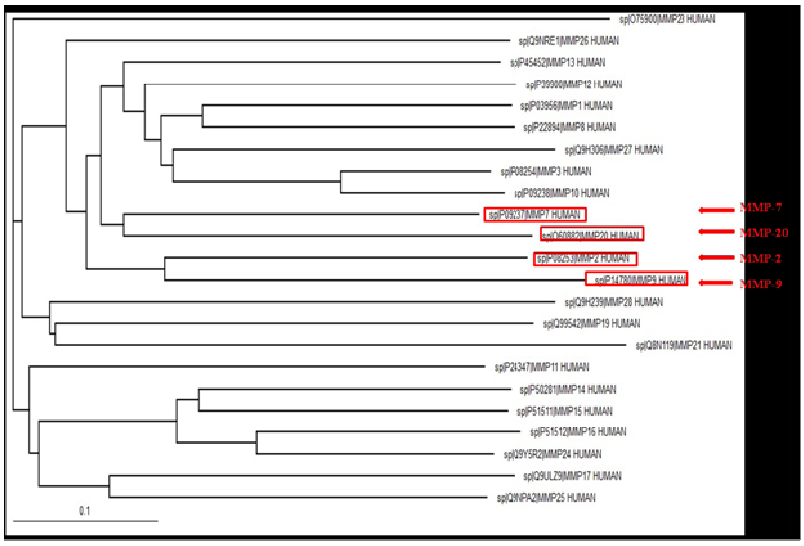

Figure 1: Phylogentic analysis of human MMPs: Phylogram of human MMPs were constructed using NJ method. Protein sequences were aligned using ClustalW2 multiple alignment tool. Phylogram was visualized in Treeview v1.6.6. MMP-7 and 20 were found as closely related neighbors of MMP-2 and 9.

Curcumin treatment prevents norepinephrine induced cardiac hypertrophy in H9C2 cells

Heart derived H9C2 cardiomyocyte cells were cultured above 95% viability that was confirmed using a hemocytometer by trypan blue staining. MTT assay was performed for dose optimization of curcumin dissolved in DMSO with no cytotoxic effects. A range of 2-20 µM dosage concentration was applied to cultured cells for 48 hrs. More than 95% viability of cells was observed up to a concentration of 8 µM beyond which a significant decrease in the cell viability was found (Fig 2).

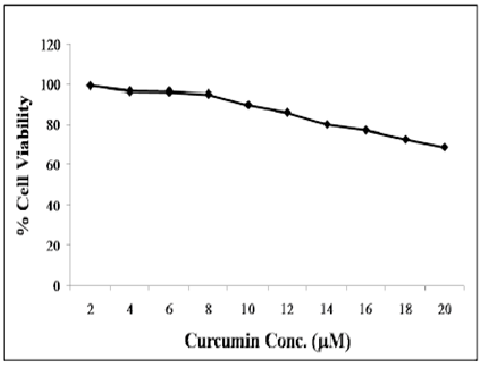

Figure 2: MTT Assay dose optimization of curcumin: Varying concentration of 2-20 µM were applied to optimize the safe dose of curcumin. More than 95% viablity was obtained at concentration above 8 µM.

H9C2 cells were treated with 8 μM curcumin in presence of 2 μM norepinephrine and cultured for 48 hrs. Morphological analysis indicates an increased cell size of norepinephrine treated cells while there was a reduced increase in the cell size of cardiomyocytes on treatment with curcumin in the presence of norepinephrine (Fig 3). Biochemical analysis shows an enhanced protein synthesis in presence of norepinephrine only and a reduction in increase of protein content was observed with curcumin treatment after norepinephrine induction (data not shown). Expression analysis by RT-PCR signifies a reduced ANF (fetal gene known as a hypertrophic marker) expression on curcumin treatment in comparison to its expression under hypertrophic conditions (Fig 4). Thus, this suggests that curcumin prevents norepinephrine induced cardiac hypertrophy.

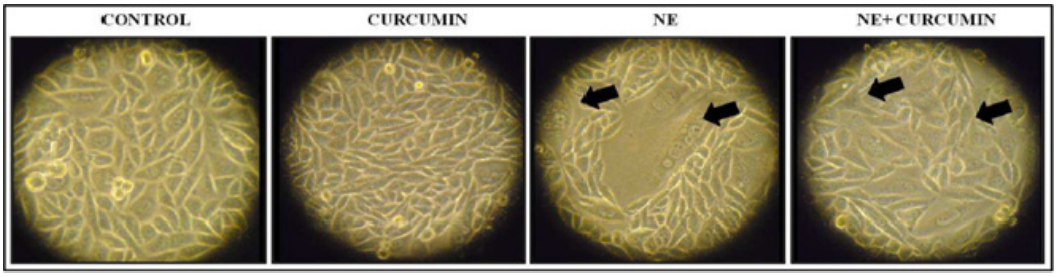

Figure 3: Morphological analysis of cells: H9C2 cells were treated with 8 μM curcumin (in DMSO) in presence of 2 μM norepinephrine and examined under inverted microscope. Comparison of cell size showed lesser increase in size of cells treated with curcumin in presence of norepinephrine than cells treated with norepinephrine only. Curcumin alone did not have a significant effect on cell size.

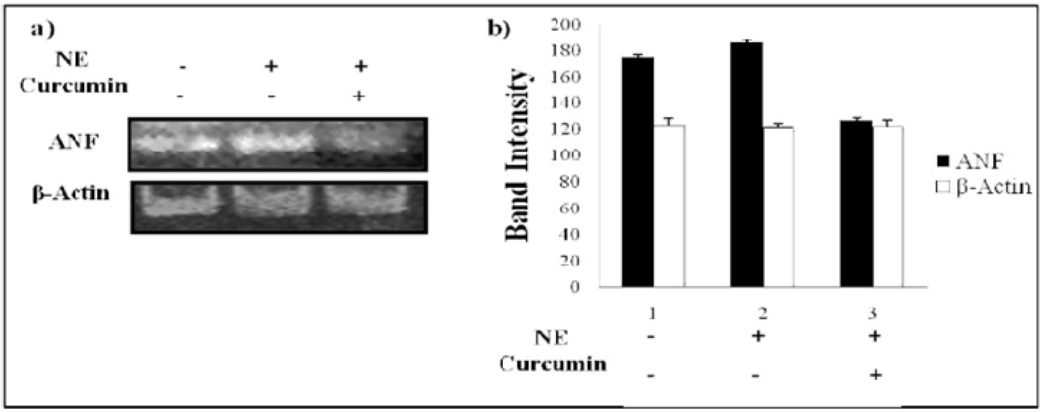

Figure 4: Expression analysis of hypertrophic marker gene by RT-PCR: ANF gene showed an enhanced expression under hypertrophic conditions. The expression was lowered in curcumin treated cells. The loading control, β-actin, showed a negligible change in expression and was constitutively expressed.

Casein degrading MMPs and effect of curcumin on MMPs in H9C2 cells

Casein substrate zymography was performed using total protein to investigate the presence of casein degrading metalloproteinases, MMP-7 and 20, screened through phylogenetic analysis, in H9C2 cells. The results indicate the existence of MMP-2 (proform-72kDa; active form- 66kDa) and MMP-9 (proform- 92kDa; active form-86kDa) that have already been reported in hypertrophy [23]. Moreover, the presence of an additional protease with activities shown at 28 and 19 kDa was also identified (Fig 5). Comparison of molecular weights with those of MMP family suggests MMP-7 as the newly identified protease. Thus, along with MMP-2 and 9, MMP-7 is also produced by H9C2 cells and may be playing a vital role in cardiac hypertrophy.

However, no activity of MMP-20 was found on the zymogram. An increased MMP activity in cells treated with norepinephrine while a decreased activity in cells treated with curcumin in presence of norepinephrine was also observed (Fig 4). Thus, the comparison of the activity of MMP in degrading the substrate indicates that curcumin may play a therapeutic beneficiary role and inhibit the increased MMP activity that causes an imbalance in ECM.

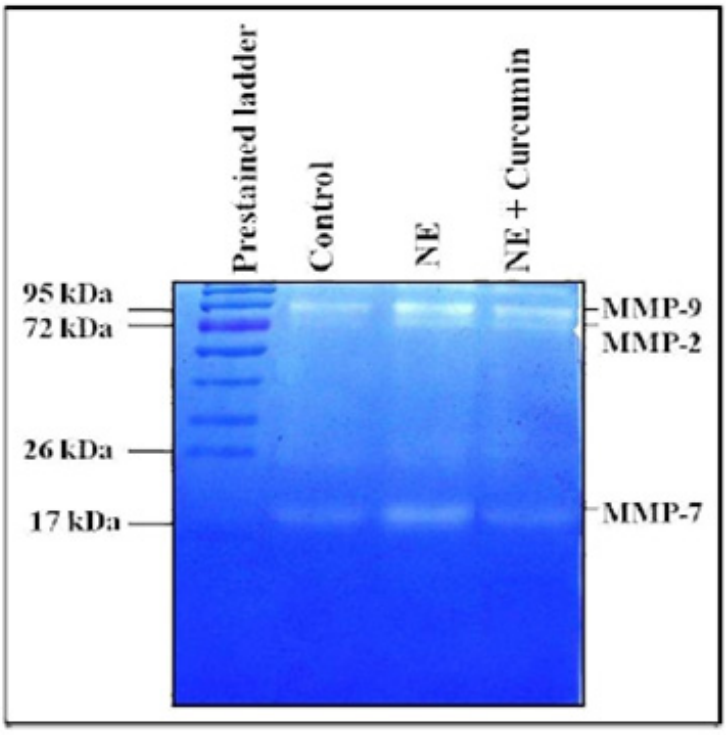

Figure 5: Casein zymography: Samples were separated on 10% SDS-PAGE gel with casein (1mg/ml). H9C2 cells produced proteases at 72 kDa (MMP-2) and 92kDa (MMP-9). Clear and faint bands of activity of a newly identified protease (MMP-7), at 17kDa and 26kDa, respectively, were also visible. Curcumin treated cells showed a decrease in MMP activity in comparison to cells treated with norepinephrine only.

Discussion

The incidence of occurrence of cardiac diseases is growing nowadays. Efforts to develop cardioprotective drugs that are safe and efficient are being undertaken. Indian medicinal plants have always been a subject of great interest. These plants are rich source of secondary metabolites and essential oils that are of therapeutic importance [24]. Moreover, many plant products have been reported to show cardioprotective properties. Curcumin is known to possess multifold medicinal properties and its potential role as a cardioprotectant is recently being investigated [25].

MMPs are expressed in minimal amounts under normal biological conditions. An imbalance in their regulation by TIMPS can bring about a significant rise in their levels in diseased tissues. In a span of two decades (1986-2007), a seven fold rise in scientific literature related to MMPs has been observed indicating their emerging role as potential clinical targets [26, 27]. Attempts to eliminate the detrimental role of these enzymes and retain the beneficial function can be of therapeutic interest [28]. Advancements in the field of bioinformatics have made complex data analysis, quick and simple. Use of biocomputational tools can help to unveil the information hidden in a structure and provide new biological insights for experimental studies.

Phylogenetic analysis facilitated screening of MMP-7 and 20 as the nearest neighbours of MMP-2 and 9. The close evolutionary relationship between these suggests that MMP-7 and 20 may also be implicated in this disease. Casein, a specific substrate for these potential MMPs was chosen for substrate zymography [29]. The presence of distinct bands at size 28kDa and 19kDa and its comparison with molecular weights of MMP family suggest this protease as MMP-7. Further studies, in presence of different protease inhibitors can be carried out to validate this result. Moreover, the downregulation of MMP activity in presence of curcumin further elucidates that this compound has a beneficiary role to control and inhibit the upregulated activity of these proteases that may cause an imbalance in the ECM. Future work can focus to identify other MMPs involved, through different substrate zymographies. Expression studies may additionally be done on the identified MMPs.

In conclusion, this study has helped to identify a novel MMP in *in-vitro* cardiac hypertrophy system and highlight MMPs as attractive therapeutic targets for development of potential preventive strategies using curcumin that serves a beneficial role in cardiac hypertrophy.

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