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# Functional Characterization of Cardiac Isoform of Alpha 2 Macroglobulin (CA2M): Identification of Non-Hypertrophic Domain

[1]Subbiah Ramasamy, [2]Pitchai Balakumar, [3]Manjeet Singh, [1,4]Andiappan Rathinavel,
[1]Koteshwara Ananthamurthy, [1]Tharmarajan Ramprasath and [1]Govindan Sadasivam Selvam
[1]Department of Biochemistry and Microbial Gene Technology, School of Biological Sciences,
Madurai Kamaraj University, Madurai, Tamilnadu, India
[2]Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India
[3]I.S.F. Institute of Pharmaceutical Sciences and Drug Research, Moga, Punjab, India
[4]Department of Cardio-Thoracic Surgery,
Madurai Medical College and Government Rajaji Hospital, Madurai, India

**Abstract:** Gene therapy is an emerging strategy for the treatment of cardiovascular disorders. However, the efficient vehicle for gene delivery into myocardium is inadequately available to treat cardiovascular disorders. Despite the fact that many non-invasive protocols are used in gene delivery, the *in vivo* gene transfer into the heart is less specific and toxic. Therefore, alternative safe targeting methods are needed to deliver the specific gene into myocardium. In the present study, we developed an efficient method of gene transfer using nonhypertrophic receptor binding domain of Cardiac isoform of Alpha 2 Macroglobulin (CA2M) into myocardium. CA2M is a serum marker protein, which is cardiac specific and induced during hypertrophy in rats. *In vivo* gene transfer by direct injection of the truncated forms of CA2M into myocardium was performed in rats. The expression study using western blotting and quantification-using sandwich ELISA have identified the Nonhypertrophic Receptor-Binding Domain (NhRBD) of CA2M. Thus, the NhRBD of CA2M may be used as a novel vehicle to deliver the therapeutic gene into myocardium.

**Key words:** CA2M, NhRBD, vehicle for cardiac gene delivery, functional characterization, non-hypertophic domain

## INTRODUCTION

Cardiac gene therapy holds as a potential molecular therapeutic approach for treating cardiovascular abnormalities (Williams *et al*., 2004). The vehicles used for targeted cardiac gene delivery such as adenoviral and c-type based viral vectors have been associated with cytotoxicity and triggering immune responses and thus limit their application (Wickham, 2000; George, 2003). Therefore, cardiac specific and non-toxic vehicles are required to be developed for cardiac gene transfer efficiently. Earlier studies from our laboratory has identified a novel high molecular weight serum protein of 182-kDa as Cardiac isoform of Alpha 2 Macroglobulin (CA2M) and suggested to be a specific biomarker of cardiac hypertrophy in rats and humans (Mariappan *et al*., 1994; Rajamanickam *et al*., 1998). Our further studies have suggested, that CA2M could be used as a diagnostic marker for cardiac diseases (Rathinavel *et al*., 2005). Moreover, our recent studies have confirmed that CA2M is a novel biomarker for cardiac disorders in patients with diabetes and HIV/AIDS (Annapoorani *et al*., 2006; Ramasamy *et al*., 2006). Since, CA2M is cardiac specific in nature; the nonhypertrophic domain of CA2M may be a potential vehicle for cardiac gene transfer. Therefore, the present study has been undertaken to explore the non-hypertrophy inducing functional domains of CA2M to develop the novel vehicles for transferring the therapeutic genes into myocardium.

## MATERIALS AND METHODS

**Pressure overload-induced cardiac hypertrophy in rats:** The Institutional Animal Ethical Committee approved the experimental protocol used in the present study. Young Wistar albino rats were used in present study. Six groups were employed in present study and each group comprising of 8-10 animals. The pressure overload-induced cardiac hypertrophy in rats was produced using aortic banding for 21 days (Mariappan *et al*., 1994). The sham-operated animals were subjected to same surgical procedures except aortic constriction.

**Corresponding Author:** Govindan S. Selvam, Department of Biochemistry, School of Biological Chemistry, Madurai Kamaraj University, Madurai-625 021, India

**Cloning and sequencing of N-terminal, middle fragment and C-terminal Receptor Binding Domain (RBD) of CA2M:** The primers for functional specific domains of CA2M were designed. The N-terminal primers are 5'GCATGCGGGAAGCACAG3'(sense)and5'CCATGGAC AGACTCCAAGGA3' (antisense), middle fragment primers are 5'TAACCATGGGTCCAGAGACAA3'(sense) and 5'GTCGACTCAGGCATTTCCATA5'(antisense) and C-terminal RBD primers are 5'CGCGCATGCATGCCCT ATGGCTGTGGAGAA CAG3'(sense) and 5'GCTGTCGACTCAGGCATTTCCATAATCTGTGCTG3' (antisense). The PCR protocols include denaturation at 94°C for 30 sec followed by annealing at 58°C for 50 sec and extension at 72°C for 60 sec. Thirty five cycles of reactions were performed with an initial denaturation for 4 min and followed by final extension at 72°C for 7 min. The resulting PCR products were resolved on 2% agarose gel. The three PCR yielded products were initially cloned in p-GEM-T Easy vector (Promega, Madison, USA). Using Not I restriction site they were transferred into pcDNA3.1(-) (Invitrogen, USA), a mammalian expression vector. The sequences for N-terminal, middle fragment and C-terminal RBD of CA2M were analyzed and submitted to NCBI Genbank under accession number of AY919611, AY921651 and AY887133, respectively.

**Preparation of plasmid dna for injection:** The plasmid DNA was prepared using the column method (Plasmid mini kit- Qiagen, Heidelberg, Germany). For injection, 100 μg of plasmid DNA was suspended in 100 μL of normal saline. The functional specific domain constructs of CA2M in pcDNA3.1(-) were injected into myocardium of normal rats to assess the development of cardiac hypertrophy. The full-length CA2M in pcDNA3.1(-) vector was used as a positive control. The animals were sacrificed at end of 3 weeks and the ratio of left ventricular weight to body weight (mg $g^{-1}$) (Balakumar and Singh, 2006a, b) and CA2M level (Mariappan *et al*., 1994) were measured to assess the development of cardiac hypertrophy.

**Western blot analysis and quantification of CA2M levels using sandwich ELISA:** The CA2M from the serum of rats was purified and anti-rat CA2M antiserum was raised as previously described (Harlow and Lane, 1998; Rajamanickam *et al*., 1998). The immuno-cross reactivity between rat CA2M and anti-rat CA2M antibody was tested by western blot analysis and the quantification of CA2M level was carried out using sandwich ELISA.

**Statistical analysis:** Results were expressed as mean±S.E.M. The data obtained from various groups were statistically analyzed using one-way ANOVA followed by post hoc analysis using Tukey's multiple range test. A p value <0.05 was considered to be statistically significant.

## RESULTS

The LVW/BW ratio and CA2M level were found to be high in aortic-constricted group when compared with sham group. This result indicates that cardiac hypertrophy has been developed in aortic constricted rats. Moreover, the LVW/BW ratio and CA2M level were observed to be increased in rats subjected to cardiac injection of full-length cDNA of CA2M, N-Terminal

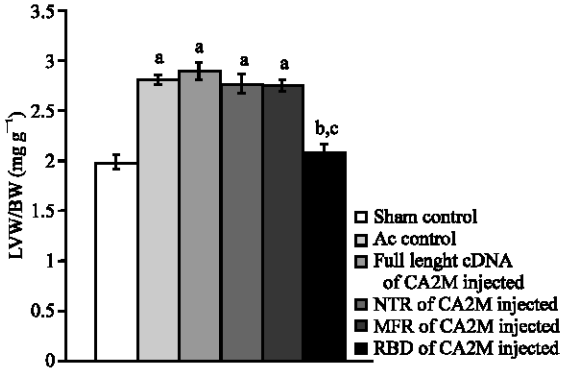


Fig. 1: Bar diagram shows the ratio of left ventricular weight to body weight (mg $g^{-1}$). Results were expressed as mean ± SEM. AC indicates aortic constricted; NTR indicates N-terminal region; MFR indicates middle fragment region and RBD indicates receptor-binding domain. a = p<0.05 Vs sham control; b = p<0.05 Vs AC control; c = p<0.05 Vs full length cDNA of CA2M injected group

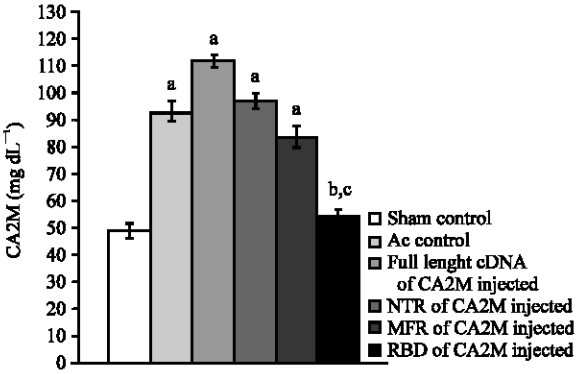


Fig. 2: Bar diagram shows the levels of Cardiac isoform of Alpha 2 Macroglobulin (CA2M) in various groups. Results were expressed as mean ± SEM. AC indicates aortic constricted; NTR indicates N-terminal region; MFR indicates middle fragment region and RBD indicates receptor-binding domain. a = p<0.05 Vs sham control; b = p<0.05 Vs AC control; c= p<0.05 Vs full length cDNA of CA2M injected group

Table 1: Shows the ratio of left ventricular weight to body weight (mg $g^{-1}$) and ELISA values of Cardiac isoform of Alpha 2 Macroglobulin (CA2M) in various groups. Results were expressed as mean ± SEM. AC indicates aortic constricted; NTR indicates N-terminal region; MFR indicates middle fragment region and RBD indicates receptor-binding domain. a = $p<0.05$ Vs sham control; b = $p<0.05$ Vs AC control; c = $p<0.05$ Vs full length cDNA of CA2M injected group

| | Sham control | AC control | Full length cDNA of CA2M injected | NTR of CA2M injected | MFR of CA2M injected | RBD of CA2M injected |
|---|---|---|---|---|---|---|
| LVW/BW (mg $g^{-1}$) | 1.97±0.06 | 2.78±0.03[a] | 2.86±0.072[a] | 2.71±0.081[a] | 2.7±0.043[a] | 2.06±0.071[b,c] |
| CA2M (mg $dL^{-1}$) | 49.2±2.9 | 93.7±3.6[a] | 112.6±2.3[a] | 97.1±2.72[a] | 82.8±3.54[a] | 53.3±2.78[b,c] |

Region (NTR) of CA2M and growth factor encoded Middle Fragment Region (MFR) of CA2M. It further shows, that cardiac hypertrophy has been developed in these groups. On the other hand, no cardiac hypertrophy has been developed in rats injected with RBD of CA2M since the LVW/BW ratio and CA2M level were not altered when compared with sham group (Fig. 1 and 2). The ratio of left ventricular weight to body weight (mg $g^{-1}$) and ELISA values of Cardiac isoform of Alpha 2 Macroglobulin (CA2M) obtained from various groups were summarized in Table 1.

## DISCUSSION

Gene therapy is an emerging strategy for the treatment of cardiovascular disorders (Isner and Losordo, 1999). The adenoviral and c-type based vectors have been currently employed in targeted gene delivery into myocardium (Wickham, 2000). However, their applications have not been satisfactory due to their less efficiency and more toxicity (George, 2003). Moreover, these vectors have been noted to trigger immune responses (Wickham, 2000; Roth *et al*., 2002). Therefore, developing novel non-viral vehicles for cardiac specific gene transfer without producing toxicity and immune responses would be a clinically mandatory one in cardiovascular therapeutics. In the present study, we have demonstrated that the Nonhypertrophic Receptor-Binding Domain (NhRBD) of CA2M may be used as a novel vehicle for gene delivery into myocardium. Earlier study in our laboratory has shown that CA2M is an early diagnostic marker of cardiac hypertrophy (Mariappan *et al*., 1994; Rajamanickam *et al*., 1998). Further, our laboratory has reported that *in vivo* administration of purified protein of CA2M or cDNA of CA2M has induced cardiac hypertrophy in rats (Rajamanickam *et al*., 2001; Rajan *et al*., 2003). Moreover, *in vivo* administration of polyclonal antibody raised against CA2M abolished the development of cardiac hypertrophy by down regulating the expression of beta-MHC and MLC-2 in rats (Rajamanickam *et al*., 2001). Recently, we have confirmed that CA2M is a novel diagnostic marker for all cardiac diseases and a new biomarker for myocardial infarcted diabetic patients (Rathinavel *et al*., 2005; Annapoorani *et al*., 2006). Finally, we have suggested that CA2M is a cardiac specific protein and strongly shown that CA2M is an early diagnostic marker for cardiac manifestations in AIDS patients (Ramasamy *et al*., 2006). The results obtained in the present study have pointed out the fact that the receptor-binding domain of CA2M is a non-hypertrophic domain. Since CA2M is cardiac specific in nature, the Nonhypertrophic Receptor-Binding Domain (NhRBD) of CA2M could be exploited non-invasively to target and specifically express genes of our interest into myocardium. Further, a study exploring the functional characterization and applications of NhRBD of CA2M as a novel vehicle for cardiac gene transfer is currently underway.

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