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AUGMENTED EXPRESSION OF ATRIAL MYOSIN LIGHT CHAIN 1 IN VENTRICULAR ANEURYSMS OF HUMAN: ENZYME IMMUNOASSAY FOR ATRIAL MYOSIN LIGHT CHAIN 1

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Summary: We established an enzyme immunoassay (EIA) for atrial myosin light chain 1 (ALC1) using monoclonal antibodies KA1 and KB1, which were specific for ALC1 and for both ALC1 and ventricular myosin light chain 1, respectively. The serum ALC1 levels of healthy subjects were 0.28 ± 0.14 ng/ml (mean ± SD). The tissue ALC1 levels of normal adult human atria were much higher than those of ventricles ($p<0.01$, 2,120 ± 1,200 in right atria, 2,180 ± 1,450 in left atria vs. 36.0 ± 20.2 in right ventricles, 37.7 ± 15.3 in left ventricles, ng/mg of proteins). The tissue ALC1 levels of ventricular aneurysms were significantly higher than those of normal ventricles ($p<0.01$, 206.7 ± 101.8). These results indicate that ALC1 is augmented in aneurysms and that the EIA provides a useful tool to investigate the roles of ALC1.

Myosin is a major contractile protein and composed of two heavy chains with two pairs of light chains (LCs) (1). The cardiac muscle LCs are classified into non-phosphorylatable, alkali type (LC1) and phosphorylatable, regulatory type (LC2) and there are at least two isoformes of LC1 and LC2: atrial LC1 (ALC1) and ventricular LC1 (VLC1), and ALC2 and VLC2, respectively (2). ALC1 and ALC2 are the major forms of LCs in atria, while VLC1 and VLC2 are in ventricles (2).

The expression of ALC1 is regulated by developmental and hemodynamic changes (3-8). We reported that ALC1, strongly expressed in fetal ventricles and weakly in adult ventricles (8), is strongly re-expressed in the overloaded human ventricles (4, 5, 8). Since these studies mainly showed the qualitative changes in ALC1, we intended to develop the quantitative assay.

In the present study, we established a sensitive and specific sandwich enzyme immunoassay for ALC1 and determined its levels in sera and tissues of normal humans and those in the ventricular aneurysms caused by myocardial infarction.

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Abbreviations:
ALC1, atrial myosin light chain 1; VLC1, ventricular myosin light chain 1; ALC2, atrial myosin light chain 2; VLC1, ventricular myosin light chain 2; LCs, light chains; POD, peroxidase.

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Materials and Methods

Preparation of Monoclonal Antibodies

Monoclonal antibodies, KA1 and KB1, were obtained from cloned hybridomas as previously reported (8). KA1 immunoreacted only with ALC1, while KB1 immunoreacted with both ALC1 and VLC1 and cross-reacted with skeletal muscle myosin light chain 1. Both antibodies were found to be IgG_1, k class.

Preparation of Enzyme Immunoassay

Non-immune mouse IgG_1 was purified from non-immune mouse serum (Kyudou Co., Kumamoto, Japan) using protein A-Sepharose 4L-CB (Pharmacia LKB Biotechnology AB; Upsala, Sweden). Non-immune rabbit IgG was purified from non-immune rabbit serum (Kyudou Co.) by DEAE-cellulose column chromatography after sodium sulfate precipitation. KA1 and KB1 were each purified from ascites fluids by protein A-Sepharose 4L-CB. Peroxidase (POD)-conjugated KB1-IgG_1 (KB1-IgG_1-POD) with maleimide were prepared as previously reported (8). Purified KA1-IgG_1 (0.11 mg/ml) was treated with 0.1 M Glycine-HCl buffer, pH 2.5, at room temperature for 10 min. After treatment, the pH was adjusted to 7.5 with 2 M Tris-HCl buffer, pH 8.0 (acid-treated KA1). Polystyrene balls (3.2 mm in diameter, Immuno Chemical Inc., Okayama, Japan) were coated with acid-treated KA1-IgG_1 (0.1 mg/ml) by physical adsorption.

Blood Sampling and Tissue Extraction Procedures

Blood was collected from the antecubital vein of healthy male subjects (n=10, 28.2 ± 2.8 yr., mean ± SD) after 1 hour rest. Blood samples were taken into plastic syringes and transferred to chilled siliconized disposable tubes, then immediately placed on ice and centrifuged at 4°C. An aliquot of serum were immediately frozen at -80°C until use.

Normal human adult hearts were obtained at autopsy from the patients (n=6, 4 men and 2 women, 71.2 ± 7.4 yr.) who died of 1) non-cardiac diseases, 2) no hypertension, and 3) no marked cardiomegaly (heart weight < 350g). The atrial myocardium was obtained from atrial free wall adjacent to atrial auricle and the ventricular myocardium was obtained transmurally from right free wall and left anterior wall. All of the specimens were obtained within 3 hours after death. The specimens of aneurysms caused by myocardial infarction were obtained from the patients who were underwent ventricular aneurysmectomy (left anterior wall, n=5, 4 men and 1 women, 70.2 ± 4.1 yr.). The specimens were immediately frozen in liquid nitrogen and stored at -80°C until extraction. The following extraction procedures were carried out at 4°C. The myocardium was minced, washed with 10 vols of buffer A (1 mM $NaHCO_3$, pH 7.0 1 mM EDTA, 0.5 mM PMSF and 5 mM 2-mercaptoethanol) and centrifuged at 23,000 g for 10 min. The pellet was washed with 10 volumes of buffer A and centrifuged at 23,000 g for 10 min. Then, the resulting pellet was homogenized with 20 vols of the modified Hasselbach-Schneider solution (0.6 M KCl, 0.1 M potassium phosphate buffer, pH 7.5 10 mM $Na_4P_2O_4$, 1 mM $MgCl_2$, 1 mM EDTA and 2 mM 2-mercaptoethanol) (9) by Ultra Turrax (setting max. speed for 30 sec. x 2, IKA-WERK, UK), stirred for 14.5 min and centrifuged at 4,000 g for 10 min. The supernatant was stored at -80°C until use. Protein concentration was determined by the method of Bradford with bovine serum albumin (BSA) as standard (10).

Recovery of ALC1 by Tissue Extraction Procedures

We extracted myosin from tissues with the modified Hasselbach-Schneider solution by different ratios of the solution to the tissue. Since the recovery of myosin was maximal over 10 of the ratio, myosin was extracted with 20 vols of the modified Hasselbach-Schneider solution in the present study.

After the tissue samples were homogenized with 20 vols of the modified Hasselbach-Schneider solution, the whole homogenates and the supernatants containing the same amount of protein were analyzed by immunoblotting with KA1, as previously reported (8). The immunoblot was densitometrically scanned by a dual wave length thin-layer chromatographic scanner (CS-910, Simadzu, Japan) with a recorder (Chromatopac, C-R2A, Simadzu, Japan). The recovery of ALC1 by the extraction procedures was 87.6 ± 7.1 % (n=8). Then values were corrected for the recovery of ALC1.

Enzyme Immunoassay for ALC1

To minimize serum interference or protein effect in the EIA, a higher concentration (a final concentration, 1.0 M) of NaCl or KCl was considered better to be used. NaCl was used for the standard curve and serum samples. Since we extracted myosin from tissue samples with the

modified Hasselbach-Schneider solution, we used KCl for tissue samples instead of NaCl. The higher concentration of NaCl or KCl had no effect on the standard curve.

Acid-treated KA1-IgG_1-coated polystyrene ball was incubated at 4°C for 24h with purified ALC1, test serum or the extract of tissue in a total volume of 150 μl. Purified ALC1 was dissolved in 10 mM sodium phosphate buffer, pH 7.5, containing 1.0 mg/ml BSA and 1.0 M NaCl to a final volume of 150 μl to obtain the standard curve of ALC1. Test serum or the extract of tissue (50 μl) was mixed with 100 μl of 10 mM sodium phosphate buffer, pH 7.5, containing 1.0 mg/ml BSA, 20 μg/ml of non-immune mouse IgG_1, and 1.45 M NaCl or 1.2 M KCl. After removal of the reaction mixture, the polystyrene ball was washed twice by addition and aspiration of 2 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The washed polystyrene ball was transferred to another clean test tube and incubated at 4°C for 24h with 50 ng of KB1-IgG_1-POD in 150 μl of 10 mM sodium phosphate buffer, pH 7.0, containing 1.0 mg/ml BSA, 0.4 M NaCl and 100 μg of non-immune rabbit IgG. The polystyrene ball was washed twice as described above and transferred to another clean test tube. POD activity bound to the polystyrene ball was assayed at 30°C for 60 min using 3-(4-hydroxyphenyl)propionic acid as substrate. Fluorescence intensity of the reaction was measured relative to 0.2 μg/ml quinine in 50 mM H_2SO_4 using 320 nm for excitation and 405 nm for emission in a Hitachi spectorofluorophotometer (F-3000, Hitachi Co., Ltd., Tokyo, Japan).

Expression of the Detection Limit of ALC1

The detection limit of ALC1 by the EIA was taken as the minimal amount of ALC1 which gave a bound peroxidase activity significantly in excess of that nonspecifically bound in the absence of ALC1 (background). The existence of a significant difference from the background was confirmed by the *t*-test ($p<0.01$, $n=10$).

Statistical Analysis

Values were expressed as mean ± standard deviation (SD). Distributed values were analyzed by performing one-way analysis of variance (ANOVA). If necessary, Scheffe's F-test was applied. $P<0.05$ was considered statistically significant.

Results

Standard Curve and Specificity

A typical standard curve for the EIA of ALC1 is shown in Fig. 1. The detection limit of ALC1 was 30 amol/tube. Since 50 μl serum samples were used, the detection limit of ALC1 was 0.6 fmol/ml (16.2 pg/ml).

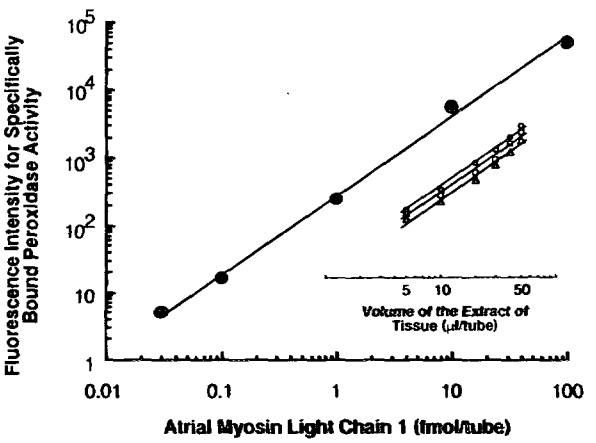

Figure 1. A typical standard curve of the EIA of ALC1.
The detection limit of ALC1 is 30 amol (0.81 pg)/tube. The EIA has no cross-reactivities with ALC2, VLC1, VLC2, and skeletal muscle and smooth muscle LCs. Open symbols indicate dilution curves of the tissue extract with a total volume of 150 μl for immunoreactions.

The EIA has no cross-reactivities with ALC2, VLC1, VLC2, and skeletal and smooth muscle LCs. The dilution curves of the extract of tissues were parallel with the standard curve of purified ALC1, when the volume of the extract of tissue used ranged from 5 to 50 μl in a total volume of 150 μl (Fig. 1).

Serum Interference and Assay Variation

The recoveries adding 0.3 - 90 fmol of ALC1 per tube to 50 μl of serum samples were 101.16 ± 4.70 - 106.33 ± 4.20 % (n=10). It indicated that serum of 50 μl in 150 μl of the assay mixture had no interference effect on the EIA within the ratio of serum to the amount of ALC1 used.

Precision was estimated at 3 different levels of ALC1 over the range of 0.3 - 90 fmol per tube. When 50 μl of serum was used, the coefficients of variation for within-assay and between-assay were 2.27 - 3.50 % (n=10) and 5.07 - 6.18 % (n=10), respectively.

Serum and Tissue Levels of ALC1 in Human

The serum and tissue levels of ALC1 in human are shown in Table 1. Within atria, there were no significant differences in the tissue levels of ALC1. Within ventricles, there were also no significant differences in the tissue levels of ALC1. The tissue levels of ALC1 in atria were about sixty times higher than those in ventricles. On the other hand, the tissue levels of ALC1 in the ventricular aneurysms caused by myocardial infarction were about six times higher than those in normal ventricles.

Discussion

This is the first report on the sandwich EIA for ALC1 in serum and tissues. The assay could be done within 3 days with sufficient reproducibility and would be much simpler than the radio immunoassay.

In the sandwich (two-site) immunoassay using mouse monoclonal antibodies, the presence of (anti-mouse immunogloblin) antibodies in some test sera may give false values by forming bridges between mouse monoclonal antibodies immobilized onto solid phases and labeled with

Table 1. Serum and Tissue Levels of ALC1 in Human

	n	ALC1
		(ng/ml)
Serum	10	0.28 ± 0.14
Normal Heart		(ng/mg of proteins)
Right atria	6	2,120 ± 1,200 *
Left atria	6	2,180 ± 1,450 *
Right ventricles	6	36.0 ± 20.2
Left ventricles	6	37.7 ± 15.3
Aneurysms	5	206.7 ± 101.8 *

Values are corrected for the recovery of 87.6 % by the extraction procedures and are expressed as the mean ± SD. *p<0.01 vs. normal ventricles.

enzymes (11). In 3 of 10 serum samples, values for serum ALC1 levels were higher in the absence of non-immune mouse IgG_1 than in the presence. These results indicate that the addition of non-immune mouse IgG_1 was essential to obtain reliable values of serum ALC1 level. The standard curve of ALC1 and the recoveries of ALC1 added to serum were not significantly affected by the addition of non-immune mouse IgG_1.

We previously reported by immunohistochemistry that ALC1 was strongly and homogeneously expressed in atria while it was only sporadically expressed in ventricles. There were no differences in the immunoreactivities of ALC1 between the right side and the left side of atria and ventricles (8). Interestingly, ALC1 was strongly re-expressed in the overloaded ventricles with old myocardial infarction (5). In this study, we showed by quantitative analysis that the tissue levels of ALC1 in atria were about sixty times higher than those in ventricles, and that there were no differences in the tissue levels of ALC1 between the right side and the left side of atria and ventricles. Furthermore, we clearly showed that the tissue levels of ALC1 in ventricular aneurysms caused by myocardial infarction were about six times higher than those in normal ventricles.

The EIA presented in this study is specific and sensitive for ALC1, and therefore we are able to assay ALC1 levels in sera and tissues. It may prove a useful tool to investigate the ontogenic, physiological and pathophysiological roles of ALC1.

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