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Changes in Autoantibodies against β_1-Adrenoceptor and M_2-Muscarinic Receptor during Development of Renovascular Hypertension in Rats

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Abstract In an experimental rat's renovascular hypertension model, we studied the genesis of anti-cardiac β_1-adrenoceptor and M_2-muscarinic receptor autoantibodies in relation to the changes in immunological function during the development of renal hypertension. The biological activities of these autoantibodies were also examined. It was shown that after two weeks of operation both the frequency of occurrence and the titre of autoantibodies to cardiac β_1-adrenoceptor and M_2-muscarinic receptor were significantly increased as compared with the control of pre-treatment. The increased autoantibodies lasted for several weeks and then automatically decreased gradually to the pre-clipping level at 10 weeks. Meanwhile the ratio of CD_4^+/CD_8^+ was also undergone an initial increase followed by gradual recovery and correlated well with the changes in antibody titre. The biological effects of these autoantibodies displayed an "agonistic-like" activities on the beating frequency of cultured neonatal cardiomyocyte. It is suggested that autoimmune mechanisms are involved in the pathogenesis of renal hypertension and the cardiac receptor autoantibodies might be one of the mechanisms leading to cardiac dysfunction.

Key words autoantibody; renal hypertension; cardiac receptor; immunology

In recent years, autoantibodies to β_1-adrenoceptor and M_2-muscarinic receptor have been successively discovered in the sera of patients with dilated cardiomyopathy (DCM) [1–3]. Iwata *et al.* [4] found that in a similar protocol with 6-month myocardial hypertrophy, β_1-adrenoceptor receptor desensitization, increased G_i protein and G protein-coupled receptor kinase-5 expression were in association with myocyte disorganization and interstitial fibrosis. So far, investigation in this field has focused mainly on the etiologic role of these autoantibodies in the pathogenesis of DCM. Little information, however, is available as to the genesis of autoantibodies in the course of cardiac diseases. To clarify the relations of myocardial pathologic state to the genesis of anti-receptor autoantibodies may deepen our understanding of the role of autoimmune mechanism in the pathogenesis of some heart diseases including DCM. In view of the cardinal role played by rennin-angiotensin system (RAS) in the myocardial pathologic changes during renal hypertension, it will be of importance to see if RAS system is related to the genesis of autoantibodies to cardiac-G-protein-coupled receptors [5].

In this study, in rat's renal hypertension model, we studied the genesis of anti-cardiac receptor autoantibodies in relation to immunological function changes during the development of renal hypertension.

Materials and Methods

Animal preparation

Healthy albino Wistar rats (male, initially weighing 200–250 g, Shanxi Medical University) were randomly divided into two groups: experimental group (n=64) and control group (n=6). Renal hypertension model (2K1C) was set up by clipping the left renal artery. Sham operation was performed for control group. The caudal blood pressure was measured and 2 ml blood sample was taken there-

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after by cutting the tail before and 2, 4, 6, 8, 10, 12 and 14 weeks (n=5–8) post-treatment. Sera were separated and stored at –20 °C for use. At each time point, the cardiac function parameters (LVESP, LVEDP and $\pm dp/dt_{max}$) were measured.

SA-ELISA (enzyme-linked immunoabsorbent assay) [6]

Two peptides corresponding to the second extracellular loop of human β_1-adrenoceptor (197–222 aa, H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C) [1] and M_2-muscarinic receptor (169–193 aa, V-R-T-V-E-D-G-E-C-Y-I-Q-F-F-S-N-A-A-V-T-F-G-T-A-I-C) [2] were synthesized according to the solid-phase method developed by Merrifield [7] by using Applied Biosystems 430A automated peptide synthesizer (Applied Biosystems Inc., USA). The peptides were confirmed to be pure by HPLC analysis on a Vydac C-18 column and by amino acid analysis on an automated amino acid analyzer (Beckman Instruments Inc., USA).

Fifty microliters of the peptide (50 μg/ml) in 100 mM Na_2CO_3 solution (pH 11.0) was coated on NUNC (Kastrup, Denmark) microtitre plates overnight at 4 °C. The wells were then saturated with PMT [PBS supplemented with 3% skimmed milk (*W*/*V*), 0.1% Tween 20 (*V*/*V*, E. Merck, Darmstadt, Germany) and 0.01% thimerosal (*W*/*V*, Sigma, USA)] for 1 h at 37 °C. After washing the wells three times with PMT, 50 μl rat serum with different dilution was added and incubated for 1 h at 37 °C. After washing again three times with PMT, an affinity-purified biotinylated sheep anti-rat IgG_{H+L} antibody (1:1000 dilution, Jackson Immunoresearch Laboratories Inc., USA) was added and allowed to react for 1 h at 37 °C. After three times washing, the bound biotinylated antibody was detected by incubating the plates for 1 h with 1 μg/ml streptavidin-peroxidase (Sigma, USA). Then the plate was washed with PBS three times and the substrate [2.5 mM H_2O_2, 2 mM 2,2′-azino-di(ethyl-benzthiazoline) sulfuric acid (ABTS, Sigma, USA)] was added. Optical density was read after 30 min at 405 nm (A_{405}) by using a microplate reader (Molecular Devices Corp., USA). The positive reaction of the sera against the peptides was confirmed as reported by Liu *et al.* [8].

Determination of CD_3^+, CD_4^+ and CD_8^+ T cell

The measurement of T lymphocyte subtype was carried out by flow cytometry (FACScan, Becton Dickinson Co./Mountain View, USA) as described by Lyons *et al.* [9]. The mAbs used for combining T lymphocyte included FITC-labeled anti-rat CD_3^+, FITC-labeled rat CD_4^+, and PE-labeled rat CD_8^+ (Pharmingen Co., USA).

Culture of neonatal beating cardiomyocytes

Isolation and cultivation of neonatal heart cells were preformed as previously described [10]. Briefly, cells were dissociated from the minced ventricles of 1–2 days old Wistar rats (Shanxi Medical University) with 0.08% trypsin, and cultured in monolayer at a density of 800 cells/mm^2 in DMEM medium equilibrated with humidified air at 37 °C. The DMEM medium contained 10% heat-inactivated bovine neonatal serum and 2 μM fluorodeoxyuridine, which prevented the proliferation of nonmuscle cells. On day 3 or 4, the cells were incubated in 2 ml 10% heat-inactivated bovine neonatal serum-containing medium for another 2 h.

Seven to ten selected cells or synchronously contracting cell clusters per flask were counted for 1 min. This procedure was repeated twice in different cultures to yield results representing a total of up to 30 cell for each sample of a given autoantibodies, receptor agonist isoproternol or carbachol and receptor antagonist atropine or propranolol, which were added cumulatively.

Statistical analysis

The antibody titre was expressed as geometric mean ± SD. Other data were expressed as mean ± SD. Student's *t*-test and χ^2-test were used for statistical analysis. $P<0.05$ was considered to denote statistical significance, and $P<0.01$ was considered as highly significant.

Results

Changes in cardiac function

Along with the elevation of blood pressure after clipping, the compensatory increase in cardiac function was concomitantly occurred as shown in Table 1. After 2 weeks post-treatment, the cardiac function parameters LVSP and $+dp/dt_{max}$ which reflect the cardiac systolic function were significantly increased ($P<0.05$). Parameters $-dp/dt_{max}$ and LVEDP reflecting cardiac diastolic function were also increased after 2 weeks post-treatment, but decreased after 6 weeks.

Changes in occurrence frequency and titre of autoantibodies

After 2 weeks of clipping when the blood pressure was increased to a stable higher level, the frequency of occurrence of anti-β_1-adrenoceptor and anti-M_2-muscarinic receptor autoantibodies were also increased from 6.25%

Table 1 Changes in left ventricular function at different point after operation

Group	LVESP (kPa)	LVEDP (kPa)	$+dp/dt_{max}$	$-dp/dt_{max}$
Pre-clipping	13.51 ± 0.96	0.64 ± 0.19	264.50 ± 22.74	197.82 ± 17.62
2-week post-clipping	16.83 ± 1.54**	0.78 ± 0.28*	336.12 ± 32.54*	256.38 ± 26.05**
4-week post-clipping	16.89 ± 1.75**	0.85 ± 0.28**	365.92 ± 33.61**	264.45 ± 32.51**
6-week post-clipping	17.05 ± 1.32**	0.91 ± 0.23**	304.66 ± 35.21*	208.21 ± 29.68
8-week post-clipping	16.89 ± 1.38**	1.35 ± 0.28**	266.43 ± 28.64	180.68 ± 22.35
10-week post-clipping	17.31 ± 1.07**	1.36 ± 0.29**	247.31 ± 25.46*	174.53 ± 19.62*

Values were represented as mean ± SD (n=6–10). *P<0.05 vs. pre-clipping group; **P<0.01 vs. pre-clipping group.

Table 2 Changes in occurrence frequency and titre of autoantibodies against cardiac receptors in sera of rats in experimental group

Group	Anti-β_1-adrenoceptor		Anti-M_2-muscarinic receptor	
	Frequency (%)	Titre	Frequency (%)	Titre
Pre-clipping	6.25	1:(42.5 ± 15)	9.38	1:(46.2 ± 20)
2-week post-clipping	43.75*	1:(113.9 ± 42)*	59.38*	1:(132.2 ± 38)*
4-week post-clipping	75.86*	1:(124.5 ± 30)*	82.76*	1:(169.5 ± 37)*
6-week post-clipping	59.61*	1:(144.1 ± 42)*	80.77*	1:(172.1 ± 35)*
8-week post-clipping	50.00*	1:(132.8 ± 36)	59.10*	1:(136.5 ± 44)*
10-week post-clipping	31.82*	1:(108.8 ± 30)	65.00*	1:(93.9 ± 26)
12-week post-clipping	16.67	1:(62.4 ± 22)	33.33	1:(67.3 ± 26)
14-week post-clipping	12.50	1:(58.6 ± 17)	37.50	1:(69.6 ± 18)

Values were represented as geometric mean ± SD (n=5–8). *P<0.05 vs. pre-clipping group.

and 9.38% to 43.75% and 59.38%, respectively (P< 0.05). The antibody titres were increased from 1:(42.5 ± 1.5) and 1:(46.2 ± 2.1) to 1:(113.9 ± 1.7) (P<0.05) and 1:(132.2 ± 2.3) (P<0.05) respectively 2-week post-clipping, increased to peak 6-week post-clipping, decreased thereafter, and returned to pre-clipping level 10-week post-clipping (Table 2, Fig. 1).

Changes of T lymphocyte subtype

The ratio of CD_4^+/CD_8^+ increased from 1.76 ± 0.12 to 2.34 ± 0.25 after 2 weeks post-treatment (P<0.05) and attained a peak value after 6 weeks, which is correlated well with the titre of autoantibody (r=0.817, P<0.05 for β_1-adrenoceptor; r=0.786, P<0.05 for M_2-muscarinic receptor) (Table 2, Fig. 1).

Chronotropic effects on cultured cardiomyocytes

Both autoantibodies revealed stimulatory "agonist-like" activities on the corresponding receptor: the autoantibody

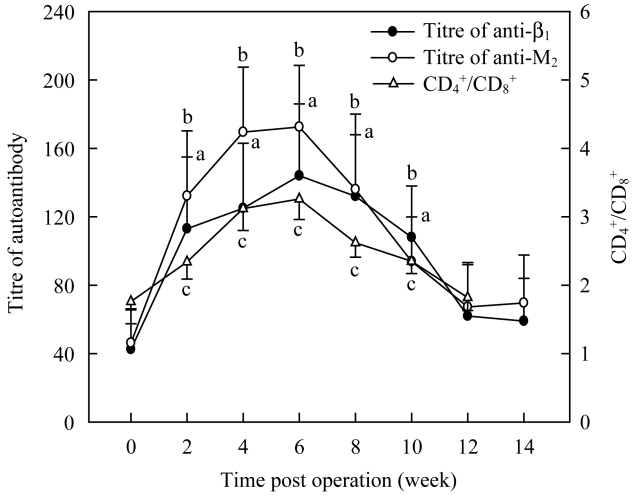

Fig. 1 Change of serum anti-β_1-adrenoceptor and anti-M_2-muscarinic receptor autoantibody titre and CD_4^+/CD_8^+ during the experimental period

Values are mean ± SD; ^aP<0.05, ^bP<0.05, ^cP<0.05 vs. pre-treatment group of corresponding measurement.

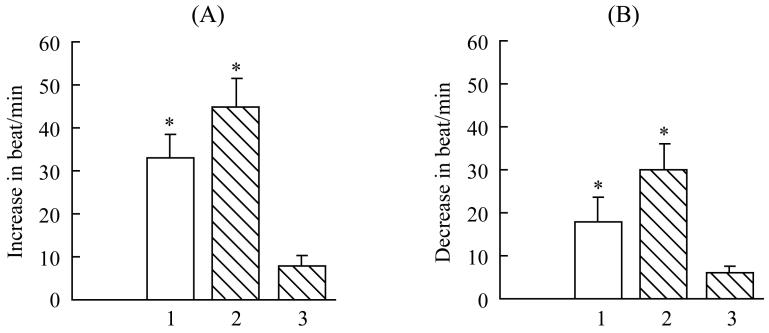

Fig. 2 Chronotropic effect serum containing autoantibodies on cultured neonatal rat cardiomyocytes

(A) Effect of anti-β_1-adrenoceptor positive sera. 1, anti-β_1 positive sera (1:400); 2, isoproterenol (10 μM); 3, anti-β_1 positive sera (1:400)+propranolol (1.5 μM). (B) Effect of anti-M_2-muscarinic receptor positive sera. 1, anti-M_2 positive sera (1:400); 2, carbachol (1.0 μM); 3, anti-M_2 positive sera (1:400)+atropine (1.5 μM).

of β_1-adrenoceptor increased the beating frequency and the autoantibody of M_2-muscarinic receptor slowed the beating frequency. These effects are comparable to those of 10 μmol isoprenaline and atropine, respectively (Fig. 2).

Discussion

There are growing evidences suggesting the involvement of cellular and humoral autoimmune mechanisms in the pathogenesis of some heart diseases, such as dilated cardiomyopathy, Chagas heart disease, spontaneous hypertension (SHR), etc. [1–4,10,11]. A variety of circulating autoantibodies has so far been detected in the sera of patients with heart diseases such as autoantibodies to myosin, mitochondrial adenine nucleotide translocator, cardiac receptors, etc. [12,13]. In considering that the whole heart activities are unexceptionally controlled and regulated by cardiac receptors, especially the predominant β_1-adrenoceptor and M_2-muscarinic receptors, autoantibodies to these cardiac receptors are among those of particular importance.

To clarify the conditions and mechanisms responsible for the abnormal occurrence of anti-cardiac receptor autoantibodies is an important aspect in assessing the involvement of immunological alterations in the pathogenesis or pathological implications of cardiovascular diseases [14]. In the present study, it was clearly shown that during the development of renal hypertension both the occurrence frequency and titre of autoantibodies against cardiac β_1-adrenoceptor and M_2-muscarinic receptor in the sera of experimental model animals were tremendously increased. However, autoantibodies generated in response to renal hypertension underwent automatically wax and wane process being returned to pre-clipping level after about ten weeks. The changes in the occurrence frequency and titre of autoantibodies during development of renal hypertension were closely related to the change of CD_4^+/CD_8^+ ratio suggesting that autoimmune mechanisms were involved in the formation of renal hypertension. Although the possible role of these autoantibodies in the pathogenesis of renal hypertension is not clear, the “agonist-like” effects of autoantibodies on the corresponding cardiac receptor [10–12] might in some way modulate the cardiac function and hence might be one of the causes leading to cardiac dysfunction in renal hypertension.

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Announcement

Since January 2005, *Acta Biochimica et Biophysica Sinica* (*ABBS*) will begin a four-year co-publishing schedule with the Blackwell Publishing Ltd.. The printed version will be produced by the Institute of Biochemistry and Cell Biology, and the online version by the Blackwell Publishing Ltd.. The online version of *ABBS* enrolled in the Blackwell Database Package will be freely available in 2005. On the website of *ABBS*, abstract in Chinese will be linked to each article.

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