Aptamer Neutralization of Beta1-Adrenoceptor Autoantibodies Isolated From Patients With Cardiomyopathies

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Rationale: Autoantibodies directed against the beta1-adrenoceptor (beta1-AABs) have been proposed to drive the pathogenesis of idiopathic dilated cardiomyopathy (DCM), Chagas’ cardiomyopathy, and peripartum cardiomyopathy. For disease treatment, aptamers that bind and neutralize beta1-AABs could be significant.

Objective: We determined whether oligonucleotide-aptamers, selected to target human beta1-AABs directed against the second extracellular loop of the beta1-AAB, can neutralize these AABs and modulate their function in vitro.

Methods and Results: Using Monolex technology, we identified an ssDNA aptamer that targets human beta1-AABs. The neutralization potential of this aptamer against beta1-AABs isolated from patients with DCM, Chagas’ cardiomyopathy, and peripartum cardiomyopathy was analyzed using cultured neonatal rat cardiomyocytes by monitoring beta1-AAB induced cell toxicity and chronotropic cell responses. Aptamer addition reduced beta1-AAB induced cell toxicity and neutralized chronotropic beta1-AAB function in a dose-dependent manner. In the presence of aptamer neutralized beta1-AABs, cells remained fully responsive to agonists and antagonists, such as isoprenaline and bisoprolol. Both aptamer pretreated with a complementary (antisense) aptamer and a control scrambled-sequence aptamer were ineffective at beta1-AAB neutralization. Beta1-AABs directed against the first extracellular loop of the beta1-receptor and AABs directed against other G-protein coupled receptors were not affected by the selected aptamer.

Conclusions: A specific aptamer that can neutralize cardiomyopathy associated human beta1-AABs in vitro has been identified and characterized, providing a framework for future in vivo testing of this treatment option in animal experiments. (Circ Res. 2011;109:986-992.)

Key Words: aptamer ■ autoantibodies ■ beta1-adrenoceptor ■ cardiomyopathy ■ heart failure

Aptamers (synthetic, highly structured, single- or double-stranded oligonucleotide ligands) bind to their corresponding target molecules with high specificity, modulating the target’s function. Consequently, and because of their low immunogenicity and toxicity, some aptamers have already entered the clinical pipeline, including specific aptamers designed to protect the cardiovascular system from coagulation and thrombosis.

We present here, for the first time, in vitro experiments that demonstrate that an aptamer selected using established technology can specifically target and neutralize human autoantibodies (AABs) directed against the second extracellular loop of the beta1-adrenoceptor (beta1-AABs). Such AABs have been identified in patients with idiopathic dilated cardiomyopathy (DCM), Chagas’ cardiomyopathy, and peripartum cardiomyopathy and are believed to drive the pathogenesis of these diseases. In fact, the direct involvement of these AABs in disease pathogenesis was impressively demonstrated in animal studies, where transfer of beta1-AABs into healthy animals caused cardiac complications and DCM-like symptoms. This was recently supported by human studies, where beta1-AAB elimination via immunapheresis produced long-lasting clinical improvements in DCM and Chagas patients. Furthermore, a European multicentric clinical study (the Etiology, Titer-Course, and Survival Study) designed to enhance our knowledge of the roles of beta1-AABs in human heart disease and “promote endeavors to develop novel therapies targeting cardiac AABs” is ongoing.

To demonstrate the beta1-AAB inhibitory properties of the selected aptamer, we used cultured neonatal rat cardiomyocytes. To monitor the cytotoxic, apoptotic, and chronotropic...
responses of these cells to beta1-AABs isolated from patients with DCM, Chagas’ cardiomyopathy, and peripartum cardiomyopathy, cells were treated with beta1-AABs in the presence and absence of the aptamer. To control for specificity, the aptamer was also tested for its ability to neutralize AABs directed against the first extracellular loop of the beta1-AAB; or AABs directed against other G-protein coupled receptors, such as the alpha1-adrenoceptor (alpha1-AAB), angiotensin1-receptor (AT1-AAB), endothelin A-receptor (ETA-AAB), and muscarinic-2-receptor (M2-AAB). A selected, but inactive aptamer, as well as a scrambled sequence aptamer and complementary (antisense) aptamer, were also used for control experiments.

Methods

Human Autoantibodies Against G-Protein Coupled Receptors

Human IgGs containing beta1-, alpha1-, AT1-, ETA-, or M2-AABs were obtained from patients treated at the German Heart Center Berlin, or from Chagas’ patients recruited from Santa Bárbara Hospital, Sucre, Bolivia. The use of these IgGs for in vitro experiments was approved by appropriate authorities at the respective hospitals. All patients signed an informed consent form.

Aptamer Selection

Monoplex technology was used for aptamer selection. Specifically, a commercially available goat antihuman beta1-adrenoceptor antiantibody (Everest Ltd., UK) was covalently coupled to a carrier-column. In parallel, a DNA library consisting of a randomized 21-mer region, flanked by 21-mer primer binding sequences (TAC GAC TCA CTA TAG GGA TCC-(N21)-GAA TTC CCT TTA GTG AGG GTT), was preincubated with human and goat IgGs, both confirmed to be beta1-AAB negative, to remove aptamers with affinity for the nonvariable IgG region from the library. This preconditioned single-stranded DNA library was applied to the carrier-column and low affinity aptamer sequences were washed from the column using different buffer solutions (Tris buffer pH 7.3, 0.1% Tween-20, DMEM, 0.1% Tween-20, and phosphate buffer pH 7.5, 0.1% Tween-20). Tightly bound aptamers were then eluted from the column by target denaturation. Eluted aptamers were amplified by quantitative real-time PCR using a cycler (BioRad, Munich, Germany) and 2 pmol primer sequences. The resulting DNA was cloned and sequenced. Single aptamers were synthesized by Biotez GmbH (Berlin-Buch, Germany) and tested for beta1-AAB neutralizing properties. The random N21 sequence ACA GTA ACC GCG TGA GGT CGA (named aptamer 110) was found to be exclusively specific for beta1-AABs, which target the second extracellular receptor loop. For control experiments, a selected but beta1-AAB neutralizing inactive aptamer (N21, aptamer 109: AAA TTA AGT GTA TAA CCG TCA), a scrambled version of aptamer 110 (N21: AAG GCA TCG AAT CCG GAT GGC), and the antisense of aptamer 110 were used.

IgG Preparation

As recently described in detail, IgGs were prepared from patient serum by stepwise ammonium sulfate precipitation. Briefly, 1 mL of patient serum and 660 μL saturated ammonium sulfate solution were mixed (final concentration 40% ammonium sulfate) and incubated for 18 hours at 4°C. After centrifugation for 15 minutes at 6000 g, the pellet was resuspended in 750 μL phosphate-buffered saline solution (PBS), mixed with 750 μL saturated ammonium sulfate solution (final concentration 50% ammonium sulfate) and centrifuged again. After centrifugation, the pellet was resuspended in 700 μL PBS and dialyzed against a 100-fold volume of PBS for 72 hours. The dialysis membrane used (VISKING cellulose, type 27/32, Carl Roth Germany) had a MWCO of 14 kDa, ensuring loss of residual or coprecipitated low molecular weight molecules, including catecholamines (MW range 170–200 Dalton). Finally, the IgG concentration was adjusted to the serum concentration. Using this procedure, no changes in subtype composition (IgG 1–4) were observed compared to the original serum. The resulting IgG fractions were stored at −20°C.

Cell Cultivation

Neonatal Rat Cardiomyocytes

The hearts of 1- to 3-day-old rats were removed under sterile conditions and transferred to PBS containing penicillin/streptomycin (animal experiment license number: 411/96, Berlin, Germany). Ventricle tissue was separated, dissected in pieces and washed twice with 10 mL PBS containing penicillin/streptomycin, followed by once with PBS only. Ventricle pieces were then resuspended in 30 mL PBS containing 0.2% trypsin. After incubation for 20 minutes at 37°C, trypsinization was stopped with 10 mL ice-cold heat-inactivated calf serum. The resulting suspension was centrifuged at 130 g for 6 minutes and the pellet transferred to 20 mL of SM20-I medium. For cell count estimation, 100 μL of this suspension was added to 100 μL trypan blue solution. For cell culturing, 2.4–105 cells were resuspended in 2.0 mL of glucose containing SM 20-I medium, which was equilibrated with humid air and supplemented with 10% heat-inactivated calf serum, 0.1 M insulin, and 2 μmol/L fluoro-deoxyuridine (to prevent overgrowth of the myocytes by nonmyocytes), transferred to 12.5-cm2 Falcon flasks, and cultured as a monolayer for 4 to 8 days at 37°C. The medium was renewed after 2 days. Cardiomyocytes began to spontaneously beat after 2 days of culture.

Monitoring the Cytotoxic Beta1-AAB Response of Neonatal Rat Cardiomyocytes in the Presence and Absence of Aptamers

To test the cytotoxic effect of patient-derived AABs on neonatal rat cardiomyocytes and the impact of the selected aptamer on this effect, neonatal cardiomyocytes were incubated with either patient IgGs or the IgG/aptamer mixture for 72 hours. Specifically, 50 μL of the IgG preparation (or the IgG/aptamer mixture) was added to 2000 μL cell medium; and this treatment was repeated after 36 hours of incubation. After 72 hours after incubation, 200 μL of 0.05% trypan blue solution was added and cells were incubated at 37°C for 1 hour. After incubation, the medium was removed and cells were washed with PBS; both the total number of cells and the number of blue cells in the visual field (at ×100 magnification, approximately 30–50 cells per visual field) were counted. Cell counting was repeated for 4 fields and the percentage of blue cells was calculated for each visual field.

Monitoring the Apoptotic Beta1-AAB Response of Neonatal Rat Cardiomyocytes in the Presence and Absence of Aptamers

Incubation of cells with AABs premixed with and without aptamer was carried out adequately to the procedure described under the
“cytotoxic beta1-AAB response.” Seventy-two hours after incubation the cell media were decanted, cells were washed with 1 mL PBS followed by 1 mL binding buffer (Biotium Inc., CA). Afterward 1 mL binding buffer containing 20 μL FITC-Annexin V (Biotium Inc. CA) was added and the cells were incubated at 4°C for 10 minutes while slowly moving the flasks. The Annxin containing fluids were removed by suction, the cells were washed twice using 1 mL binding buffer each. Afterward 0.8 mL binding buffer were added and the cells were scraped using a cell scraper. The samples were immediately frozen and thawed only for measurement. The measurement of bound FITC-Annexin V (relative fluorescence) was run at a Shimadzu Spectrofluorophotometer RF 5001PC, settings: excitation, 495 nm, emission, 519 nm.

Monitoring the Chronotropic AAB Response of Neonatal Rat Cardiomyocytes in the Presence and Absence of Aptamers

**Assay Standardization and AAB Quantification**

To monitor the chronotropic response induced by patient-derived IgGs, the basal beating rate of the cardiomyocytes was recorded after 4 to 8 days of cell culture.

The following criteria were applied to select cells for beta1-AAB activity estimation: 1) the basal beating rate must be 100 to 160 bpm; and 2) cells stimulated with isoprenaline (10 μmol/L) for 5 minutes as a positive control must respond with a frequency increase of more than 45 to 50 bpm.

Patient IgG preparations were then added to the cardiomyocytes culture for 1 hour and the chronotropic response was monitored and compared to the basal beating rate (previously recorded). To identify and characterize the AAB activity, the bioassay was performed with or without specific blockers of the respective AAB-receptors: atropin (1 μmol/L) for the M2-receptor, propranolol (1 μmol/L) for the beta1- and beta2-adrenoceptor; bisoprolol (1 μmol/L) for the beta1-adrenoceptor-118.551 (0.1 μmol/L) for beta2-adrenoceptor; BQ 610 or BQ 123 (1 μmol/L) for the ETA-receptor; prazosine (1 μmol/L) for the alpha1-adrenoceptor; and Ibsartan or Losartan (1 μmol/L) for the AT1-receptor. Further characterization of the different receptor AABs was conducted using AAB-epitope-representing-peptides corresponding to the extracellular loops of the receptors.

**AAB/Aptamer Coincubation**

After estimation of basal beating rates, IgGs (50 μL) containing respective AABs were preincubated with active or control aptamers for 60 minutes and added to the cells. After 1 hour, cardiomyocyte beating rates were recorded.

In a second line of experiments, cells were first stimulated with AABs, followed by addition of the aptamers.

Both experimental designs and additionally electrophoretic mobility were used demonstrating basic characteristics of the aptamer 110 such as stability related to storage conditions, stability against freezing and thawing, stability in the presence of human and calf serum and specificity to beta1-AAB in presence of serum.

To exclude any aptamer-mediated effects on the cells, cells were also incubated with aptamers only and beating rates were recorded.

Cell sensitivity to typical beta1-receptor agonists and antagonists (isoprenaline, bisoprolol) was demonstrated by successive treatment in the presence of beta1-AABs and aptamer-inhibited beta1-AABs.

**Statistical Analysis**

The presented data are the arithmetic mean values±standard deviation (x±SD). Statistical analysis was performed using the SPSS software package (version 15); (SPSS Inc., Chicago, IL). For intergroup comparison, the 2-tailed Student t test was used for data analysis. Differences are significant at the level P≤0.05 (*), P≤0.01 (**), and P≤0.001 (***)

**Results**

**Aptamer Characteristics**

Storage of aptamer 110 for more than 1 year at −20°C as well as 1 hour at 37°C did not affect its biological activity against beta1-AABs. No reduced biological activity of aptamer 110 was also seen after 5 cycles of freezing and thawing and after the aptamer was incubated in serum for more than 1 hour. Additionally the electrophoretic mobility of aptamer 110 did not change after incubation in 50% human serum for 1 hour indicating the stability of aptamer 110. Full aptamer activity despite increasing serum concentrations excluded any significant binding of the aptamer 110 to serum proteins other than beta1-AABs.

**Cytotoxicity and Apoptosis of Beta1-AABs Are Reduced in the Presence of Aptamer**

As demonstrated in Figure 1A, trypan blue uptake by neonatal rat cardiomyocytes (which indicates the level of cell damage mediated by beta1-AABs) is clearly reduced by preincubation of the beta1-AABs with the selected aptamer. This holds also true for the beta1-AAB caused FITC-Annexin
V binding of the neonatal rat cardiomyocytes (Figure 1B). Preincubation of the beta1-AAB with aptamer 110 fully prevented the AAB caused FITC-Annexin V binding.

**Aptamer Treatment Inhibits Beta1-AAB-Mediated Chronotropic Effects**

Beta1-, beta2-, and AT1-AABs have a positive chronotropic effect on neonatal rat cardiomyocytes, whereas ETA- and M2-AABs have a negative chronotropic effect. Preincubation of beta1-AABs from DCM patients (50 µL of AABs containing IgGs diluted in 2000 µL cell medium was found to induce optimal chronotropy in prestudy experiments) with aptamer 110 (Figure 2A), or addition of aptamer 110 to cells that have been prestimulated with beta1-AABs (Figure 2B) reduced the chronotropic effect of beta1-AABs in a dose- and time-dependent manner, with complete inhibition observed in the presence of 100 µmol/L of aptamer 110. At this aptamer concentration, no toxic effects on the cells were detectable (Figure 1). Aptamer 110 also neutralized beta1-AABs isolated from patients with peripartum cardiomyopathy and Chagas’ cardiomyopathy (Figure 2C).

In control experiments, AABs directed against the first extracellular beta1-AAB loop or other G-protein coupled receptors, such as ETA-, alpha1-, beta2-, and M2-receptors, were unaffected by aptamer 110 (Figure 3A). Similar results were obtained when serum from Chagas’ cardiomyopathy patients, which contains a mixture of several AABs, was tested. Only beta1-AABs were neutralized by aptamer 110, whereas beta2- and M2-AABs were unaffected (Figure 3B).

In other control experiments, beta1-AABs were treated with either 1) the inactive aptamer 109 (selected together with aptamer 110 in the same run); 2) a scrambled version of aptamer 110; or 3) a mixture of aptamer 110 and its complementary antisense molecule (Figure 4). In all of these cases, no reduction in beta1-AAB-mediated chronotropic activity was detected, in contrast to results observed following aptamer 110 treatment.

**Sensitivity of Neonatal Rat Cardiomyocytes to Chronotropic Agonists and Antagonists Following Treatment With Beta1-AABs or Beta1-AABs Plus Aptamer**

In the presence of aptamer neutralized beta1-AABs, the sensitivity of neonatal rat cardiomyocyte beta1-adrenergceptor to agonist (isoprenaline) and antagonist (bisoprolol) remained intact (Figure 5). Specifically, columns 1, 2, and 3 show the cardiomyocyte response to only agonist (isoprenaline, 1 µmol/L) or only beta1-AABs (the response was not as strong as the response to isoprenaline), compared to the response from aptamer neutralized beta1-AABs, respectively. As demonstrated in column 4, an additional significant increase in beating rate was not observed in beta1-AAB prestimulated cells when subsequently stimulated with isoprenaline. In contrast, a full response, comparable to that seen in the presence of isoprenaline alone, was observed when beta1-AABs were first neutralized by aptamer 110 before treatment of cells with isoprenaline (column 5). In addition, the β-blocker bisoprolol showed full efficacy, fully blocking the isoprenaline response in the presence of aptamer neutralized beta1-AABs (column 6). The combination of isoprenaline with aptamer alone did not influence the efficacy of isoprenaline (column 7).

**Discussion**

Aptamers represent a class of chemicals with attractive, drug-like properties, which could potentially be very useful in the
treatment of a variety of diseases. Specific aptamers are currently in clinical use, or are being evaluated, for the treatment of macular degeneration and Myasthenia gravis, respectively. For the cardiovascular system, present efforts have focused on aptamer-based prevention of hypercoagulation and thrombosis. Thrombin in particular is seen as an attractive target for aptamer inhibition. In fact, antithrombin-aptamers (NU172) is currently in phase II clinical trials.

In this in vitro study, we present, for the first time, data suggesting that a well-selected aptamer is able to inhibit the cytotoxic, apoptotic, and chronotropic effects of beta1-AABs with high-specificity.

Based on in vitro investigations and animal experiments, beta1-AAB modified calcium- and cAMP-dependent signal transduction, and, consequently, beta1-AAB-mediated chronotropic, inotropic, and proapoptotic effects, have been proposed to be involved in the development of cardiomyopathy in subjects who present with beta1-AABs. Because of this, beta1-AABs are increasingly believed to be drivers of disease pathogenesis in DCM, Chagas’ cardiomyopathy and peripartum cardiomyopathy.

In support of this, the clinical significance of beta1-AABs in patients with Chagas’ cardiomyopathy and DCM has been demonstrated in both direct and indirect experimental and clinical studies. For example, Jahns et al immunized rats against the second extracellular loop of the beta1-receptor, to generate beta1-AABs. These rats developed progressively severe left ventricular dilatation and dysfunction. Furthermore, transfer of beta1-AAB positive serum from these rats into healthy, beta1-AAB negative rats induced cardiomyopathy. Moreover, elimination of beta1-AABs via IgG immunoapheresis, performed by Müller et al.13 as well as immunoapheresis specifically targeting beta1-AABs, as performed by Wallukat et al, resulted in long-lasting clinical improvements in DCM patients. In addition, beta1-AAB immunoapheresis was suggested by Labovsky et al for treatment of Chagas patients. Other therapeutic options currently under study include treatment with beta1-adrenoceptor epitope competing peptides, or peptides designed to neutralize beta1-AABs. However, only multicentric studies, such as the ongoing Etiology, Titer-Course, and Survival Study in Europe, have the potential to significantly extend our knowledge about the relevance of beta1-AABs in human heart disease, and the capacity to supply fundamental new strategies for the “development of novel therapies targeting cardiac specific AABs.”

In the present study we demonstrate the cytotoxic, apoptotic, and chronotropic effects of beta1-AABs, and the direct inhibition of these effects by neutralization of beta1-AABs with a selected aptamer. Our study provides evidence supporting the pathogenetic potency of beta1-AABs, and demonstrates for the first time that well-selected aptamers are able to reduce the pathogenetic potency of beta1-AABs.
Our results, coupled with the high specificity of aptamer 110 for beta1-AABs directed against the second extracellular beta1-receptor loop, as well as the low immunogenicity and toxicity of aptamers, suggest that aptamer neutralization of beta1-receptor loop, as well as the low immunogenicity and toxicity of aptamers. Therefore, our data provide an important framework on which to design future in vivo testing strategies.

Last but not least, aptamer 110 could be a useful biomedical research tool as a binder in beta1-AAB specific apheresecolumns or ELISA assays.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Autoantibodies directed to the second extracellular loop of the beta1-adrenoceptor are implicated in the pathogenesis of dilated cardiomyopathy, Chagas’ cardiomyopathy, and peripartum cardiomyopathy.

- Treatment strategies, especially those using peptide sequences that interact with beta1-AABs, are being investigated for removal or in vivo neutralization of beta1-AAB (immunoadsorption).

- The potential therapeutic utility of this approach might be restricted by the specificity of the peptides against DCM-specific beta1-AABs.

**What New Information Does This Article Contribute?**

- We show that a well-selected aptamer (single stranded oligonucleotide) binds and neutralizes beta1-AABs isolated from serum of patients with DCM, Chagas’ cardiomyopathy, and peripartum cardiomyopathy.

- Combined or successive treatment of cultured neonatal rat cardiac myocytes with beta1-AABs and aptamer abolished beta1-AAB induced pathogenic events such as cell chronotropy and apoptosis.

Stability of aptamers in vivo, their lack of immunogenicity in general and the ability to target beta1-AABs isolated from patients with different forms of cardiomyopathies, render aptamers as potential tools to neutralize potential harmful effects of beta1-AABs. The approach could provide for a new treatment strategy for patients with cardiomyopathies who have circulating beta1-AAB.
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