Autoantibodies against G-Protein-Coupled Receptors Modulate Heart Mast Cells

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Mast cells are believed to be involved in myocardial tissue remodelling under pathophysiological conditions. We examined the effects of autoantibodies against G-protein-coupled receptors in sera of patients with heart diseases on myocardial mast cells in the cultured neonatal Sprague-Dawley rat heart cells. Cells collected at day 3 and 10 of the culture were preincubated with autoantibodies against α1-adrenoceptor and angiotensin II AT1-receptor, agonist phenylephrine and angiotensin II, and control IgG. The pretreated cultured cells were stained for selected mast cell markers tryptase, chymase and TNF-α. The cultured cells were also processed for observation with electron microscopy. The autoantibodies-treatment of the 3-day cultured cells caused both increased intensity of immunofluorescence (p < 0.05) and their enlarged diameters of the mast cells when compared to age-matched ones. In contrast, the fluorescence of preincubated 10-day-old mast cells was decreased compared with controls (p < 0.01). In control samples, the fluorescence of 10-day-old mast cells was significantly higher than that of 3-day-old ones (p < 0.001). Results of electron microscopy examination demonstrated there was an increased granulation of treated 3-day-old mast cells, while a degranulation of mast cells at day 10 of application. The results suggest the modulation effect of the autoantibodies against G-protein-coupled receptors on mast cells, indicating a potential functional link between the autoantibodies against G-protein-coupled receptors and the mast cells in progression of heart disease. Cellular & Molecular Immunology. 2007;4(2):127-133.

Key Words: mast cell, autoantibody, rat heart cell culture, immunofluorescence

Introduction

Mast cells represent a part of the immune system and are involved in many types of inflammation and repair processes. Mast cells as morphologically undetermined progenitor cells circulate in the blood and migrate through the vessel wall into tissue, where they ultimately reside and mature under the influence of local specific factors present in each tissue site (1, 2). Mast cells were also found in animal and human heart tissue (3-5) and, in addition, in cultured neonatal rat heart cells as well (6). Mast cells produce a variety of immune mediators, e.g., histamine, neutral proteases, growth factors and cytokines, which modulate different biological and inflammatory processes (5, 7). It is also known that the density of myocardial mast cells is higher in response to disorders like hypertension, ischemia-reperfusion, myocarditis, and dilated cardiomyopathy (4, 8-12) than in hearts without any cardiac pathology. The cell accumulation was simultaneously accompanied with elevated circulating and intracardiac immune mediators (13). Thus the data suggest that mast cells play an important role in the regulation of a functional state of the myocardium under normal (14), but mainly pathophysiological conditions, when the myocardium undergoes the process of structural remodeling (15). However, an immuno-modulatory capacity of mast cells in this process is not completely clear.

In addition to immunoglobulin (Ig) E-associated immune responses (16), mast cell activation and degranulation can be elicited by multiple mechanisms including signaling via Fcγ receptors, which are required for IgG antibody-related activation (17, 18). The ability of IgG-antibodies to activate mast cells indicates that the latter can provide an important cellular link between IgG-autoantibodies and eliciting and/or progression of inflammatory autoimmune diseases, including

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heart diseases (19, 20).

Several heart diseases were reported to be highly associated with functional IgG-autoantibodies (AABs) directed against G-protein-coupled receptors (21-26). These AABs have been identified in sera of patients with clinically diagnosed heart diseases. We hypothesized that the IgG-AABs against G-protein-coupled receptors could modulate myocardial mast cells via the corresponding G-protein-coupled receptors supporting their role in the progression of a heart disease. The aim of our study was therefore to examine the effect of IgG-AABs against α1-adrenoceptor and angiotensin II AT1-receptor isolated from sera of patients with cardiovascular disorders on mast cells present in cultured neonatal rat heart cells.

Materials and Methods

Patients
Blood samples were obtained from patients (women and men, mean age of 55.4 years) with primary hypertension, and pulmonary hypertension. Pre-eclampsia in women (ranging in age of 21-32 years) was defined by hypertension (blood pressure ≥ 140/90 mmHg) after week 20 of pregnancy. Control sera were from normotensive healthy blood donors (n = 5, mean age of 53.7 years). All procedures were performed in accordance with ethical standards. All patients gave written, informed consent as determined by national committee on human subjects.

Isolation of immunoglobulins
The immunoglobulin G fraction was isolated from 1 to 2 ml serum samples containing AABs against the α1-adrenoceptor and angiotensin II AT1-receptor responding well-established bioassay (23). IgG were precipitated by ammonium sulfate at a saturation of 40% as it was described previously (21). Briefly, after centrifugation the pellets were resuspended in the dialysis buffer (154 mmol/L NaCl, 10 mmol/L sodium phosphate, pH 7.2) and precipitated again to a final concentration of 50% ammonium sulfate. This procedure was repeated once. Thereafter, re-suspended immunoglobulins were extensively dialyzed against the dialysis buffer for 60 h. Immunoglobulins were transferred into phosphate buffer saline (PBS, pH 7.2) and kept frozen at -25°C until use.

Culture of neonatal rat heart cells
Neonatal rat myocytes were isolated and cultured as described by Wallukat and Wollenberger (21). Single cells were dissociated from the minced ventricles of 1-2-day-old Sprague-Dawley rats with 0.1% crude trypsin. For fluorescence, the cells were seeded onto coverslips with a density of 8 × 10^4 cells/ml in 100 μl Halle SM20-I medium (Biochrome, Berlin, Germany) at the bottom of 24-well plates. The medium contained 10% heat-inactivated bovine calf. After first 24 h of incubation, additional 400 μl of fresh medium was added to each well, and cells were further cultivated for additional days.

On day 3 and 10 the cells were incubated with the IgG-AABs against α1-adrenoceptor and AT1-receptor from sera of patients (12.5 μl/500 μl), control IgG sera (diluted 1:40 in 500 μl volume) as well as with corresponding agonists phenylephrine (10^6 mol/L) and angiotensin II (10^6 mol/L) for 1 hour at room temperature.

Immunofluorescence
The coverslips with cell cultures were fixed with methanol for chymase and TNF-α and with Carnoy’s fixative solution for tryptase. After blocking of non-specific binding sites in PBS containing 10% normal goat serum (Sigma) for 30 min, the cell cultures were stained using standard immunofluorescent protocols. Cells were incubated with primary antibodies for 2 h at room temperature: monoclonal anti-mouse chymase mast cell 3D5 (1:500, Biotrend, Germany), monoclonal anti-mouse mast cell tryptase A3A (1:100, Biotrend, Germany), polyclonal anti-rabbit TNF-α (1:100, Biotrend, Germany), diluted in PBS. The specificity of antibodies was controlled by omitting of primary antibodies. Detection of bound primary antibodies was performed with goat anti-rabbit and anti-mouse conjugated FITC and Cy 3(tm) (Jackson ImmunoResearch, Germany) at a dilution 1:50. Labeled samples were mounted with Citifluor (Plano Gmbh, Germany), examined under Zeiss Axioplan microscope equipped with appropriate filters.

Transmission electron microscopy
Control and pre-incubated cell cultures were routinely processes for electron microscopy: cells were fixed with 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 1 h at 4°C. After washed samples were postfixed with 1% OsO₄ for 30 min, dehydrated in a series of alcohol, infiltrated in propylene oxide and embedded to Epon 812. Ultrathin sections using ultra-microtome (LKB, Sweden) were cut with a diamante knife and mounted on nickel grids. Sections were stained with uranyl acetate and lead citrate.

Quantitative image analysis of mast cells in heart cell culture
Fluorescent signal of mast cells and their diameters were quantified and measured using morphometric software (AnalySIS, Soft Imaging System, Germany). For each selected mediator at least a minimum of 20 mast cells taken randomly were measured in each group: 3-day- and 10-day-old controls, after AAB-, agonists- and control IgG-application. Data in each group were added together and evaluated. Similar conditions of light and magnification were used for each measurement.

Statistical analysis
Differences in the mean values of data were evaluated using a one-tail Student’s t test. Criterion for significance was p < 0.05. Data were expressed in arbitrary units as mean ± standard error of the mean (SEM).

Results
AABs-induced modulation of immunofluorescent signal of
All selected mediators tryptase, chymase and TNF-α were detected in the mast cells of both 3-day- and 10-day-old control heart cell cultures (Figure 1). The quantitative analysis (Table 1) showed that the specific fluorescence in 3-day-old mast cells was lower than that in 10-day-old ones (172.1 ± 17.4 vs 452.1 ± 89.4, p < 0.001). Parallel, the diameter of 3-day-old mast was lower than that of 10-day-old ones (16.2 ± 2.9 μm vs 27.7 ± 6.2 μm, p < 0.001) (Table 2). The 10-day-old mast cells assayed by phase-contrast microscopy were full of granules suggesting the cell maturation (Figure 1).

The treatment of heart cell cultures with the AABs and agonists resulted in differences in the immunoreactivity of the mast cells. In contrast to 10-day-old heart cell cultures, the fluorescence of 3-day-old ones was compact (Figure 2). On the other hand, in 10-day-old mast cells only few immunostained granules were detected (Figure 2). These findings together with a phase contrast indicate a cell degranulation. Beside the granulated mast cells the treated 10-day-old cell cultures also contained non-degranulated ones. In addition, cell cultures preincubated with the AABs resulted in the appearance of TNF-α-immunoreactivity also

| Table 1. The immunofluorescence of mast cells present in rat heart cell culture |
|----------------------|----------------------|
|                      | 3D-MC                | 10D-MC               |
| Controls             | 172.1 ± 17.4          | 452.1 ± 89.4*        |
| Control IgG          | 190.6 ± 33.6          | 479.2 ± 101.5        |
| IgG-AAB              | 254.5 ± 50.1^        | 313.4 ± 92.7**^      |
|                      | 556.6 ± 81.2^b       | 36.2 ± 8.3**^b       |
| Agonists             | 273.9 ± 21.6^        | 343.9 ± 97.7**^c     |
|                      | 568.4 ± 79.9^d       | 40.6 ± 7.9**^d       |

Values are mean ± SEM. ^p < 0.001 vs control 3D-MC; *p < 0.005 vs control 3D-MC; **p < 0.01 vs control 10D-MC; *p < 0.01 vs control 10D-MC. ^a_degranulated mast cells; ^b_stimulated mast cells. Agonists: phenylephrine (10^-7 mol/L) and angiotensin II (10^-7 mol/L). Controls, nontreated cell culture; Control IgG, control sera of normotensive healthy blood donors; IgG-AAB, autoantibodies from sera of patients; 3D-MC, 3-day-old mast cells; 10D-MC, 10-day-old mast cells.
in the other cell types including cardiomyocytes (Figure 2).

Quantitative analysis demonstrated that the AABs and tested agonists against G-protein-coupled receptors caused an elevation of the immunopositivity of 3-day-old mast cell when compared to age-matched controls (254.5 ± 50.1 and 273.9 ± 21.6 vs 172.1 ± 17.4, p < 0.05) (Table 1). Likely, the diameter of treated 3-day-old mast cells increased (23.8 ± 2.5 μm, and 22.1 ± 2.9 μm vs 16.2 ± 2.9 μm, p < 0.05) (Table 2).

In contrast, the fluorescence of degranulated 10-day-old mast cells decreased compared with age-matched controls (Table 1) (313.4 ± 92.7 and 334.9 ± 97.7 vs 452.1 ± 89.4, p < 0.05). However, their diameter was higher than in controls (42.1 ± 4.9 μm and 46.3 ± 8.2 μm vs 27.7 ± 6.2 μm) (Table 2). Treated but non-degranulated 10-day-old mast cells increased their fluorescence (556.6 ± 81.2 and 568.4 ± 79.9 vs 452.1 ± 89.4, p < 0.05) as well as their diameter (36.2 ± 8.3 μm and 40.6 ± 7.9 μm vs 27.7 ± 6.2 μm, p < 0.05). No significant changes in fluorescence and diameter of mast cells were seen after treatment with control sera (Tables 1 and 2).

Subcellular markers of AAB-induced maturation and degranulation of mast cells

The ultrastructural analysis of mast cells showed that both 3- and 10-day-old control cell cultures contained heterogeneous populations of mast cells. The 3-day-old heart cell cultures contained mast cells in earlier stages of their maturation (Figure 3). A part of cells contained a moderate amount of cytoplasm, a rich network of rough endoplasmic reticulum, numerous mitochondria and a lot of small single progranules. Mast cells contained granules in several stages of completion. We also observed fused progranules with heterogeneous content of granules, forming dense cords, and few mature granules with uniform material. Due to the cells’ incubation with the AABs and agonists, the amount of all types of granules enhanced an indicating process of the cell maturation (Figure 3).

The 10-day-old control mast cells in contrast to 3-day-old ones became increasingly granulated corresponding to time-dependent maturation (Figure 4). Most mast cells were filled with a lot of dense, morphologically homogeneous granules, surrounded with a membrane. Due to increased granulation, intracellular cytoplasm was reduced, ribosomes were scattered and smaller mitochondria were seen occasionally. Non-membrane lipid bodies were observed in cytoplasm and displayed focal lucent areas (Figure 4). Only a small part of 10-day-old mast cells revealed ultrastructural markers of 3-day-old immature ones. Electron microscopy of 10-day-old mast cells treated with AABs and agonists showed signs of degranulation (Figure 4) manifested by granule exocytosis as well as without exocytotic degranulation. The latter was characterized with intragranular changes manifested by reduced electrondense material. Plasma membrane of 10-day-old mast cells frequently formed numerous short microvilli.

**Discussion**

The present study demonstrated immunohistochemically that the IgG-AABs against G-protein-coupled α1-adrenoceptor and angiotensin II AT1-receptor influence mast cell maturation and degranulation.

As mentioned above, functional IgG-AABs directed against G-protein-coupled receptors (adrenergic receptors, AT1- and muscarinic receptors) have been demonstrated in sera of patients with clinically diagnosed heart diseases e.g. dilated cardiomyopathy, chronic myocarditis, essential and pulmonary hypertension, Chagas’ disease and pre-eclampsia (21-26). The pathogenicity of the AAB has been demonstrated experimentally as well as clinically. The immunization of rabbits with peptides corresponding to the second extracellular loop of the β-adrenoceptor induced a dilatation of ventricles and the treatment of animals with...
β-adrenergic antagonists prevented heart disease (27). Moreover, the immunization of rats with β-adrenoceptor fusion proteins AABs induced a functional and structural modification of the heart corresponding to dilated cardiomyopathy (28). Cardiac remodeling was also observed in rats after chronic stimulation by AABs against α1-adrenoceptors (29). The removal of the AABs by unspecific and specific immunoabsorption led to an improvement of the cardiac function and an elevation of the survival rate (30-33).

Recent studies have indicated that an infection or environmental factors may be the primary etiological agents inducing heart injury (34). The inflammatory response associated with the generation of the AABs inappropriately attacking heart cells may have developed to an autoimmune disease. Our previous study (35) demonstrated an increased number of myocardial tryptase-, chymase- and TNF-α-positive mast cells in the heart tissue of patients at the end-stage of dilated cardiomypathy. Other groups confirmed our data (36). Thus, it was hypothesized that the mast cells could represent a critical link in the modulation of heart disease with autoantibodies. Several studies showed that the mast cell mediators possessed variable functions and therefore can influence the structure of the extracellular matrix of the heart via different ways. Tryptase was reported to modulate the development of interstitial fibrosis and in contrast, it can contribute to the degradation of connective tissue activating matrix metalloproteinase (37-39). Likewise, chymase can cleave structural proteins of the extracellular matrix via an activation of metalloproteinases and on the other hand, it can contribute to tissue fibrosis converting angiotensin I to angiotensin II, and releasing of FGF (38, 40). Furthermore, the mediator TNF-α is an inflammatory cytokine that induces the recruitment of inflammatory cells necessary to trigger autoimmune mechanisms (41, 42) and that is also involved in the induction of apoptosis (43).

To verify our hypothesis whether the AABs could influence the mast cells in the heart we treated heart cell cultures with the IgG-AABs directed against α1-adrenoceptor and AT1-receptors. The AABs were obtained from sera of patients with essential and pulmonary hypertension and pre-eclampsia, since they can represent an important risk factor for future cardiovascular disease (44, 45). In addition, we previously demonstrated that the AABs activated nuclear factor for future cardiovascular disease (44, 45). In addition, we previously demonstrated that the AABs activated nuclear factor NF-κB in cardiomyocytes (46), which is known to play a central role in coordinating the expression of genes controlling immune responses (47).

The profiles and properties of mediators expressed by mast cells in the heart differ in species (48). Therefore, firstly we detected the presence of proteases tryptase and chymase and TNF-α in control samples. In both 3- and 10-day-old cell cultures we observed immunopositive mast cells for all selected mediators indicating their synthesis by myocardial mast cells of Sprague-Dawley rat. Significant differences in fluorescence and diameters observed between both groups indicated the age-dependent process of mast cell maturation in cultures accompanied by increased synthesis of granule-bound mediators. Parallel, the electron microscopic analysis of mast cells demonstrated differences in granulation and variability in size and staining of granules in both groups. Differences in the fluorescent signals between 3- and 10-day-old cells indicated that a minimal age-dependent spontaneous degranulation of mast cells is possible. The latter was detectable at the 4-day-old myocyte cultures and did not rise with the time (6).

The increase in fluorescence and diameter of 3-day-old mast cells preincubated with AABs indicated the IgG-AAB-induced stimulation of granule-bound mediators’ synthesis and thus maturation of mast cells. The observed AABs-related maturation of immature mast cells thus could contribute to the increased number of mast cells in the heart tissue. On the other hand, the reduced fluorescence of 10-day-old mast cells after their preincubation with the IgG-AABs suggested the ability of AABs to induce mast cell degranulation associated with an release of mediators into the extracellular space of the myocardium where they can associate to its structural remodeling. Subcellular alterations like an increased amount of mature granules in treated 3-day-old mast cells as well as degranulation of treated 10-day-old ones supported the effect of the AABs against G-protein-coupled receptors on the stage of mast cells. Additional increase in fluorescence of a small part of non-degranulated mast cells in treated 10-day-old cell culture pointed out their further maturation and the presence of mast cells in different developmental stages.

The treatment of cells with phenylephrine, an agonist of α1-adrenoceptor and angiotensin II, an agonist of AT1-receptor induced similar responses like the IgG-AABs against α1-adrenoceptor and AT1-receptors supporting the specific effect of the AABs on mast cells. Moreover, both the AABs and the corresponding agonists induced activation and translocation of nuclear factor NF-κB into the nuclei of cardiomyocytes (46). The latter is known to stimulate expression of TNF-α as well (47). Correspondingly, our results demonstrated that in preincubated cell cultures TNF-α immunopositivity was displayed also in other types of heart cells including cardiomyocytes. That suggested that under pathophysiological conditions the cells of the heart may also express inflammatory mediators (49), contributing to the progression of inflammatory processes.

It was also shown that immunoglobulins are able to activate mast cells via Fcγ receptors during acute and also chronic non-allergic inflammatory responses (17, 18). The released mast cell mediators may locally enhance inflammatory events associated with an increase in the AABs generation or mediate cardiomyocyte function and extracellular matrix remodeling accompanied by a deterioration of the disease and thus the onset of cardiomyopathy (50). The AABs against α1-adrenoceptor and angiotensin II AT1-receptor as well as the corresponding specific agonists phenylephrine and angiotensin II modulated the process of mast cell maturation and degranulation, whereas the IgG-preparations of healthy controls had no effect on the stage of mast cells. Our results indicated that these processes could be specific via the G-protein-coupled receptors and may point to alternative pathways of mast cell activation.

Our experiments indicate that the agonist-mediated
activation of α1-adrenoceptor and angiotensin II AT1-receptor resulted in an activation of the maturation of immature mast cells and a degranulation of mature mast cells. Interestingly, we were also able to demonstrate that the IgG-AABs obtained from the sera of patients with hypertension, pre-eclampsia and pulmonary hypertension mimicked these effects. Based on these findings we assume that G-protein-coupled receptor AABs could play a role in the pathogenesis of heart diseases via the modulation of mast cell properties.

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