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BASIC PHARMACOLOGY

Preformed Angiotensin II is Present in Human Mast Cells

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Summary. Purpose: **The density of mast cells increases in the myocardium of patients suffering from heart failure. However, their function remains unclear. In this study, preformed angiotensin II (ANG II), a potent growth factor, was found to be contained in, and released by, human mast cells.**

Methods: **The human mast cell line (HMC-1) was incubated with 0 to 10^{-6} M calcitonin gene-related peptide (CGRP) or culture medium. The expression of renin-angiotensin system mRNA was examined using RT-PCR analysis. ELISA and immunohistochemistry with monoclonal antibody against human ANG II were performed to detect the presence of ANG II in HMC-1. The effect of CGRP on the expression of angiotensinogen mRNA was examined by quantitative RT-PCR analysis.**

Results: **Preformed ANG II was detected in a human mast cell line (HMC-1) which is a neoplastic cell line of mast cells by ELISA and immunohistochemistry. Presence of mRNA of angiotensinogen and renin was confirmed by polymerase chain reaction in HMC-1, while mRNA of angiotensin converting enzyme (ACE) was undetectable. Since myocardial mast cells are interfaced with nerve fibers and functionally associated with CGRP, the effect of CGRP on ANG II release from HMC-1 was examined. CGRP induced the release of ANG II and increased angiotensinogen mRNA in HMC-1.**

Conclusions: **The presence of preformed ANG II and gene expression of the renin-angiotensin system were detected in human mast cells. The release and synthesis of ANG II in mast cells was regulated by CGRP.**

Key Words. **mast cell, renin-angiotensin system, angiotensinogen, calcitonin gene-related peptide**

Introduction

Mast cells are multifunctional cells, which contain various bioactive mediators including proteases [1] and histamine [2]. They are found in the human heart [3], and their density increases in the myocardium of patients suffering from heart failure [4]. Their function, however, remains unclear.

The existence of a functional interconnection between immune and nervous systems has recently been proposed [5]. Accordingly, nerves interact with several types of cells associated with immune function, including mast cells [5,6], and myocardial mast cells are in communication with sensory nerves [6,7]. Neuropeptides contained in sensory nerve endings have been reported to activate mast cells *in vitro* [8,9]. As well, the functional interaction between mast cells and nerves has been reported [10,11]. Nerve stimulation causes mast cells stimulation and secretion. In particular, the activity of calcitonin gene-related peptide (CGRP) is associated with myocardial mast cell function [12], and its plasma concentrations are increased in the patients with heart failure [13] and acute myocardial infarction [14]. These observations are consistent with a neuron-cell interaction in the regulation of myocardial mast cells which, in turn, influences the pathophysiology of heart failure.

This study was performed to examine whether preformed angiotensin II (ANG II) is present in human mast cells and, if present, whether its release is associated with CGRP activity.

Materials and Methods

All experiments were treated in accordance with local institutional guidelines at all stages of the experiments.

Biochemicals

Isocove's modified Dulbecco's medium (IMEM), α-minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from GIBCO BRL (Gaithersburg, MD, USA). CGRP and α-thioglycerol were obtained from Sigma Chemical Co (St. Louis, MO, USA). Rabbit anti-human ANG II monoclonal antibody was supplied by Phoenix Pharmaceuticals, INC. (Belmont, CA, USA).

Cell cultures

The human mast cell line (HMC-1) [15] was developed from a patient suffering from mast cell leukemia, and kindly provided by JH Butterfield, MD (Mayo clinic, Rochester, MN, USA). HMC-1 was maintained in IMEM supplemented with 10% heat-inactivated FBS,

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1.2 mM α-thioglycerol, 4 mM L-glutamine, and penicillin, 100 U/ml, and streptomycin, 10 mg/ml [15].

Gene expression of angiotensinogen, angiotensin-converting enzyme and renin by polymerase chain reaction

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method, and its concentration was measured spectrophotochemically. First strand cDNA was synthesized using the SUPERSCRIPT Preamplification System for First-Strand cDNA Synthesis (GIBCO BRL, Gaithersburg, MD, USA). After 1:2.5 dilution, 1 μL of cDNA was amplified by PCR. Each PCR reaction contained 100 μmol/L of each dNTP, 0.5 μmol/L of each specific primer, 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% gelatin, and 0.25 U Taq polymerase (Perkin-Elmer Co, Foster City, CA, USA) in a 20 μL volume. A sense primer (F) and an antisense primer (R) were synthesized by use of the published cDNA sequences for each of the following: angiotensinogen, angiotensin-converting enzyme (ACE) and renin. The actual sequences of the oligonucleotides were as follows:

angiotensinogen F, 5′-TGACAGGATGGAAGACTGGCT-3′;
angiotensinogen R, 5′-GAGAGGTTTGCCTTACCTTGGA-3′;
ACE F, 5′-ATCACCGCGGAGAATGCAA-3′;
ACE R, 5′-TCCGTGAAGTTCTGCCAGATC-3′;
renin F, 5′-GCTCTTCGATGCTTCGGATTC-3′;
renin R, 5′-ATGTCCTGGCTGAGAAAGCCA-3′.

Each mRNA was analyzed by 50 cycles of amplification in a thermal cycler (Perkin-Elmer Co). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 60°C for 1 min. Ten μL of PCR fragments was separated electrophoretically on 2.0% agarose gels, stained with ethidium bromide, and photographed. Heart tissues were harvested from a cadaver autopsied for forensic investigations.

Preparation of cell-free supernatants and whole cell lysates

HMC-1 cells were resuspended in IMEM with 10% FBS at a density of 5×10^6/ml. Untreated cell suspensions were collected and centrifuged at 125 g for 7 min at 4°C. For whole cell lysates preparation, cell suspensions were sonicated and centrifuged at 200 g for 7 min at 4°C.

Immunohistochemistry

Light microscopy immunocytochemistry was performed by an alkaline phosphatase method as described previously with minor modifications [16]. Cell smears were prepared on glass slides and dried at room temperature for 30 min. The slides were fixed in 3% formaldehyde and 1% glutaraldehyde solution for 30 min on ice. After the cells were permeabilized with 0.8% Triton X-100 solution and blocked, the smears were incubated for 1 h with a rabbit antiserum to ANG II diluted 1:200 at room temperature. A control study was performed with healthy rabbit serum. The smears were incubated with Histofine Simple Stain MAX PO (Nichirei Co, Tokyo, Japan) and avidin-conjugated alkaline phosphatase (Nichirei Co) according to the manufacturer's protocol. A nuclear counterstain was performed with hematoxylin.

Stimulation of mast cells with neuropeptides

HMC-1 cells were resuspended in IMEM with 10% FBS at a density of 5×10^6/ml. Cells were preincubated for 1 h before addition of neuropeptides or culture medium. They were incubated with 0 to 10^{-6} M CGRP for periods ranging between 10 min and 6 h. At the end of incubation, cell suspension was centrifuged at 125 g for 10 min at 4°C. Supernatants were frozen at −80°C. Pellets were collected and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method. ANG II was assayed with an EIA kit (Peninsula Laboratories, Inc., San Carlos, CA, USA).

Quantitative RT-PCR analysis

Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and TaqMan PCR Core Reagent Kit (Perkin-Elmer Co) was performed according to the manufacturer's protocol. One ml of the first strand cDNA was used in the following assay. The following forward (F) and reverse (R) oligonucleotides, and probes (P) were used for the quantification of angiotensinogen; angiotensinogen F, 5′-TGACAGGATGGAAGACTGGCT-3′; angiotensinogen R, 5′-GAGAGGTTTGCCTTACCTTGGA-3′; angiotensinogen P, 5′-ACCCTGGCTTTCAACACCTACGTCCA-3′. Oligonucleotides and probes for 18S ribosomal RNA were purchased from Perkin-Elmer Biosystems. The conditions for the TaqMan PCR were as follows: 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

Statistical analysis

All results are expressed as mean ± SEM. Differences between 2 groups were tested by unpaired two-tailed Student's t test. Multiple comparisons among 3 or more groups were performed by one-way ANOVA and Fisher's exact test for post hoc analysis. Differences were considered statistically significant at $p < 0.05$.

Results

Expression of renin-angiotensin system mRNA in HMC-1

To examine whether human mast cells are capable of synthesizing ANG II, we first examined the HMC-1 for the expression of renin-angiotensin system mRNA

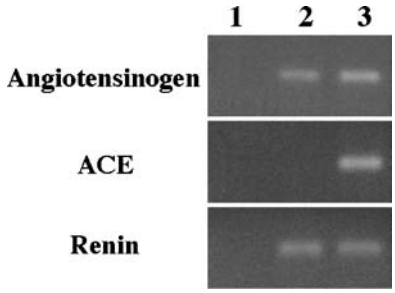

Fig. 1. *Detection of angiotensinogen mRNA, renin mRNA and angiotensin converting enzyme mRNA in HMC-1 by PCR: Lane 1, no DNA; Lane 2, HMC-1; Lane 3, Human heart tissue. mRNA encoding for angiotensinogen and renin is present, while ACE mRNA was not detected. cDNA from human heart was used as a positive control.*

using RT-PCR analysis. mRNA extracted from human myocardium was used as a positive control. The gene transcripts of renin and angiotensinogen mRNA were detected both in HMC-1 and human myocardium (Fig. 1). ACE mRNA was found in human heart tissue, but not in HMC-1.

Evidence of preformed ANG II protein in mast cells

ELISA and immunohistochemistry with monoclonal antibody against human ANG II were performed to detect the presence of ANG II in human mast cells. In ELISA, immunoreactive ANG II protein was detected in HMC-1 (Fig. 2). Supernatant of sonicated HMC-1 contained 105.1 ± 2.2 ng/10^{-6} HMC-1 cells (n = 3) of ANG II. On immunohistochemistry, ANG II was distributed predominantly in the cytosol of HMC-1 (Fig. 3A). Control experiments in which healthy rabbit serum was substituted for primary antibody showed no significant immunostaining (Fig. 3B).

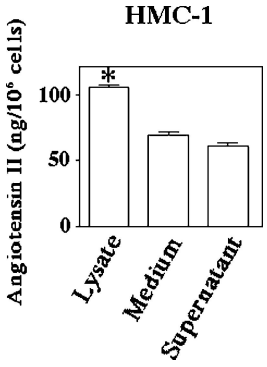

Fig. 2. *Detection of ANG II protein in human mast cells. Detection of immunoreactive ANG II in HMC-1. ANG II was detected in comparable amounts in the medium of HMC-1, which contains 10% fetal bovine serum, and in the supernatant.*

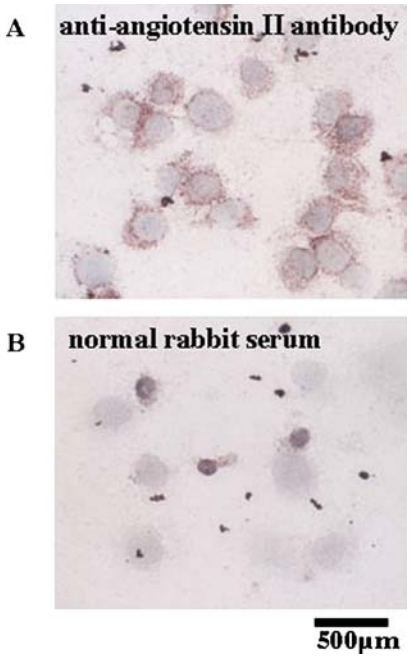

Fig. 3. *Immunohistochemical staining of HMC-1 with an antibody against ANG II (A) versus control serum (B). Note positive staining of ANG II in the mast cell granules of the cytosol in A. In B, both of mature and inmature cells of HMC-1 were observed. No significant immunostaing was shown in both cells.*

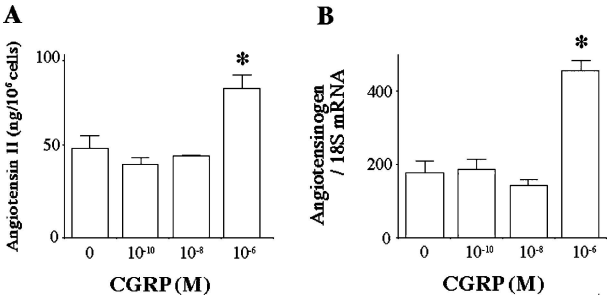

Fig. 4. *Effects of CGRP, 0 to 10^{-6} M, on the release of ANG II (A) and angiotensinogen mRNA in HMC-1 (B). * $p < 0.05$ vs incubation in absence of CGRP.*

CGRP promotes the production of ANG II in HMC-1

The cell-secreted ANG II as determined by ELISA in HMC-1 mast cells is shown in Figure 4A. HMC-1 cells were incubated with or without CGRP for 30 min, and ANG II contained in supernatants was examined by ELISA. CGRP induced the release of ANG II from HMC-1 cells in a concentration-dependent manner (Fig. 4A). In quantitative RT-PCR analysis, CGRP also increased angiotensinogen mRNA (Fig. 4B) Onset of upregulation of angiotensinogen mRNA was observed after 30 min of incubation with CGRP, and continued to

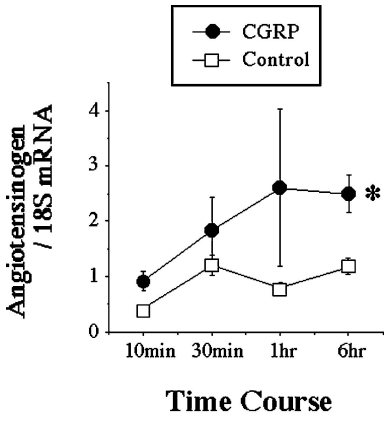

Fig. 5. *Time-dependent upregulation of angiotensinogen mRNA expression by CGRP. HMC-1 cells were left untreated, or were incubated with CGRP, 10^{-6} M, for 10 min, 30 min, 1 h or 6 h. * $p < 0.05$ vs control.*

increase thereafter, such that, after 6 h of incubation, mRNA levels in HMC-1 cells had increased 2- to 3-fold and were significantly higher than control (Fig. 5).

Discussion

This study reports 3 major new findings in human mast cells: (1) they contain preformed ANG II (2) they possess the gene expression of the renin-angiotensin system; and (3) CGRP, a potent neuropeptide, regulates their release and synthesis of ANG II.

Presence of ANG II in mast cells

ANG II is a potent growth factor which plays an important role in the development and pathophysiology of heart failure [17,18]. Accordingly, in controlled trials, angiotensin-converting enzyme inhibitors and ANG II type-1 receptor antagonists have prolonged the survival of patients suffering from heart failure [19,20]. In our experiments, no gene expression of ACE was found, in contrast to renin and angiotensinogen mRNA, which was detected. Others have reported the detection of chymase by RT-PCR in HMC-1 [21], and chymase is highly expressed in human cardiac mast cells [22–24]. Chymase is a serine protease which participates in inflammation and tissue remodeling [25–30]. Like ACE, human mast cell chymase possesses ANG II forming activity in the heart and blood vessels [31]. Therefore, we hypothesize that chymase might be implicated in the generation of ANG II, although it is generally thought that chymase exerts its angiotensin converting enzyme activity extracellulary. The mechanisms of the generation of angiotensin II in the mast cells should be further examined in the future.

Mast cells, found in nearly all organs, proliferate in several types of cardiovascular disorders [32], including myocardial hypertrophy [33] and failure [4], myocardial infarction [34], myocarditis [32], and atherosclerosis [35]. These cells contain various bioactive mediators such as cytokines, which play a critical role in the evolution of heart failure and myocarditis [36], and histamine, involved in a variety of cardiovascular diseases [37]. We have previously reported that mast cell chymase induces apoptosis of cardiac myocytes and proliferation of cardiac fibroblasts *in vitro* [30], which, respectively, cause systolic and diastolic dysfunction. These earlier observations have been extended by the finding of preformed ANG II in human mast cells, and the regulation of its release and synthesis by CGRP. This suggests that mast cells are a mobile renin-angiotensin system regulated by neural activity. As the number of mast cells increases, they may significantly contribute to the production of ANG II in heart disease [4,32–35].

Neural immunoregulation and mast cells

The production of ANG II regulated by CGRP, a potent neuropeptide contained in sensory C-fiber, suggests a neural participation in the synthesis of ANG II. Nerve fibers are interfaced with several kinds of cells of the immune system, including mast cells, monocytes, lymphocytes and macrophages [5,6]. They regulate the production of cytokines by macrophages in response to lipopolysaccharide, the mitogen-induced proliferation of lymphocytes, and the release if cytokines by lymphocytes [5]. Mast cells, a major cellular target of neural immunoregulation [5,6], are associated with neuropeptide-containing nerves in the heart [6,7], larynx [38], gastrointestinal and respiratory tracts [6,39,40], and skin [6]. Furthermore, CGRP contained in sensory C-fibers activates mast cells, increasing cardiac contractility and heart rate [12]. Changes in plasma concentrations of CGRP have been reported in congestive heart failure [13] and acute myocardial infarction [14]. These observations, along with the results of our experiments, suggest that CGRP released from **C-fibers** may regulate the production of ANG II by mast cells and, in turn, contribute to the development of cardiovascular disorders.

Limitations

In this study, we used HMC-1 which is a neoplastic cell line obtained from a patients with mast cell leukemia. It has been reported that HMC-1 cells differ from human mast cells isolated from diverse tissues. Therefore, some of the findings in our study may be due to the neoplastic aspect of mast cells, especially in the response to neuropeptides.

In conclusion, ANG II was found in human mast cells. CGRP induced its release and production, suggesting that neural regulation participates in the production of ANG II by mast cells. Stabilization of mast cells may represent a new approach in the management of some heart diseases.

Acknowledgments

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