Mast Cells Cause Apoptosis of Cardiomyocytes and Proliferation of Other Intramyocardial Cells In Vitro

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Background—Mast cells are multifunctional cells containing various mediators such as cytokines, proteases, and histamine. They are found in the human heart and have been implicated in ventricular hypertrophy and heart failure. However, their roles in pathogenesis of these diseases are unknown.

Methods and Results—Cultured cardiomyocytes from neonatal rats were incubated with mast cell granules (MCGs) for 24 hours. The highest concentration of diluted MCGs caused the death of 70% of cardiomyocytes. This cell death was proved to be apoptosis, as quantified by electron microscopy and biochemical criteria. MCG-mediated cytotoxicity was prevented by pretreatment of MCGs with protease inhibitors or a neutralizing antibody against rat mast cell chymase 1 (RMCP 1). RMCP 1 by itself was proved to induce cell death of cardiomyocytes. These results suggest that RMCP 1 contained in MCGs causes the death of cardiomyocytes. In contrast, MCGs induced the proliferation of intramyocardial cells other than myocytes. RMCP 1 was also proved to induce their proliferation.

Conclusions—Mast cells cause apoptosis of cardiomyocytes and proliferation of other intramyocardial cells via the activity of RMCP 1. Our results suggest that mast cell chymase may play a role in the progression of heart failure, because loss of cardiomyocytes and proliferation of nonmyocardial cells exaggerate its pathophysiology. (Circulation. 1999;100:1443-1449.)

Key Words: cells ■ remodeling ■ heart failure ■ apoptosis

Heart failure is a leading cause of death. When the heart is exposed to mechanical overload, many responses such as ventricular hypertrophy develop as a compensatory mechanism that ultimately breaks down if the overload persists for a long period of time. The mechanisms of decompensation remain poorly understood despite abundant investigative efforts.

Mast cells are multifunctional cells that contain various mediators such as cytokines, histamine, proteases, and leukotrienes. They are found in almost all major organs of the body and are involved in many types of inflammation and repair processes. Mast cells are also found in the human heart and have been implicated in cardiovascular diseases. It has been reported recently that mast cells are increased in number in the failing heart and that their density is higher in the hearts of patients with idiopathic dilated or ischemic cardiomyopathy than in normal hearts. In a rat model of acute myocardial infarction, mast cell density increased in the infarcted region and reached a maximum on day 21, when cardiac remodeling was ongoing. These observations suggest that mast cells in the human heart play a role in the progression of heart failure and cardiac remodeling.

In this study, we found that mast cells caused the death of cardiomyocytes and proliferation of nonmyocardial cells. Rat mast cell chymase 1 (RMCP 1) mediates these effects. Our results suggest that mast cells promote the progression of heart failure because loss of cardiomyocytes and proliferation of other intramyocardial cells participate in its pathophysiology.

Methods

Biochemicals

DMEM, trypsin/EDTA, and FBS were purchased from GIBCO BRL. Thymidine, compound 48/80, BSA, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, soybean-derived trypsin inhibitor (SBTI), histamine, cimetidine, and triprolidine were obtained from Sigma Chemical Co. Aprotinin was purchased from Boehringer Mannheim. Heparin sodium was purchased from Shimizu Pharmaceutical Co. Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries Co. Thiopropamide was purchased from Funakoshi Co, and collagenase was purchased from Worthington Biochemicals Co.

Cell Cultures

Primary cultures of neonatal cardiomyocytes were prepared by trypsin/EDTA digestion from the ventricles of 1- to 3-day-old...
Sprague-Dawley rats as described previously. In brief, cardiomyocytes were plated in serum-containing medium (DMEM, 7% FBS, 0.01% thymidine, 100 U/mL penicillin, and 10 mg/mL streptomycin) for 90 minutes to deplete the population of nonmyocardial cells. Nonattached cells were suspended in the same medium, plated onto 96-well plates, and cultured for 48 hours at 37°C in 5% air/5% CO₂. After plating, the culture medium was changed to serum-free medium (DMEM, 0.2% BSA, 100 U/mL penicillin, and 10 mg/mL streptomycin) and incubated for 48 hours. Thereafter, the cultured cells were used as cardiomyocytes.

Attached cells during preplating were cultured as nonmyocardial cells. They were maintained for 3 to 4 days and then passaged by treatment with trypsin/EDTA. They were plated at 2000 cells per well onto 96-well plates and cultured for 48 hours at 37°C in 5% air/5% CO₂. After plating, the culture medium was changed to serum-free medium (DMEM, 0.2% BSA, 100 U/mL penicillin, and 10 mg/mL streptomycin) and incubated for 48 hours. The nonmyocardial cell cultures consisted of >95% fibroblasts.

Collection and Isolation of Mast Cells

Rat peritoneal mast cells were isolated by the method of Sullivan et al. with minor modifications. Briefly, 8- to 9-week-old male Sprague-Dawley rats (Shizuoka Agricultural Cooperation Association) were used as the source of peritoneal mast cells. The peritoneal cavity of each animal was lavaged under sterile conditions with Tyrode’s buffer containing 0.1% BSA and 10 U/mL heparin. The peritoneal cells were pooled, centrifuged at 200g for 7 minutes at room temperature, and washed twice with Tyrode’s buffer containing 0.1% BSA. Aliquots (5 mL) of the cell suspension were layered on 2-mL columns of 38% BSA and centrifuged at 300g for 10 minutes. Mast cells were sedimented at the bottom. The mast cell fractions were collected, washed twice, and resuspended in DMEM. Cell viability was determined by trypan blue exclusion. Mast cells isolated by this procedure exceeded 90% in purity on the basis of staining with 0.05% toluidine blue O.

Preparation of Mast Cell Granules

Under sterile conditions, mast cell granules (MCGs) were prepared from purified mast cells by stimulation with compound 48/80. Optimal stimulation and release were achieved by incubating rat peritoneal mast cells (5×10⁵ cells/mL) for 10 minutes with 10 μg/mL compound 48/80 at 37°C. Thereafter, cold DMEM was added to stop the reaction and to dilute 4 times to a final concentration of compound 48/80 of 2.5 μg/mL. Aliquots of the cell suspension were used as 100% MCGs. All experiments were performed with compound 48/80 (2.5 μg/mL) in culture medium as a negative control.

Cell Viability Assay

Cell viability assay was performed by trypan blue exclusion assay as described previously with minor modifications. Briefly, cardiomyocytes or nonmyocardial cells were cultured with or without stimulants. Twenty-four hours later, the cultured cells were twice washed with PBS and treated with 25 μL collagenase trypsin for harvest. The protease reaction was terminated by addition of the medium containing trypan blue. Thereafter, trypan blue–excluded cells were counted; quadruplicate wells were counted for each experimental condition.

Cell survival was also analyzed by use of a nonradioactive cell proliferation assay system (MTT assay) consisting of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Pro-mega). Cardiomyocytes were cultured in quadruplicate in 96-well tissue culture plates, and MCGs were applied as described above. Results were read with an ELISA reader at 570 nm.

Effects of Protease on Cardiomyocyte Death by MCGs

To examine the role of proteases, MCGs were pretreated for 12 hours at 4°C with aprotinin (1.5×10⁻⁶ mol/L), SBTI (1.25×10⁻⁸ mol/L),11 or a mixture of protease inhibitors (0.5 mmol/L PMSF, 1 μg/mL peptatin A, and 10 μg/mL each of leupeptin, aprotinin, and SBTI).12 Aliquots of the inhibitor- or medium-treated MCGs were used to identify the role of the proteases.

RMCP 1, rat mucosal mast cell chymase (RMCP 2), and neutralizing antibody to them were purified as described previously.13,14 To examine the role of chymase, cardiomyocytes were cultured with RMCP 1 or RMCP 2. The role of RMCP 1 was also examined by incubating neutralizing chymase antibody with MCGs for 12 hours at 4°C. The antibodies were used at a concentration of 50 μg/mL. Aliquots of the antibody- or medium-treated MCGs were used to identify the role of RMCP 1 and RMCP 2.

Effects of Histamine Antagonists on Cardiomyocyte Death by MCGs

For experiments in which histamine receptor antagonists were used to inhibit MCG activity, the antagonists or solvent controls were preincubated with cardiomyocytes for 10 minutes, followed by addition of the stimuli.16 Triprolidine (10⁻⁶ mol/L) and thiopramide (10⁻⁶ mol/L) were dissolved in DMEM, and cimetidine (10⁻⁶ mol/L) was dissolved in DMSO, resulting in a final DMSO concentration of <0.01%.

BrdU Assay

Proliferation of nonmyocardial cells was analyzed with a nonradioactive cell proliferation assay system consisting of 5-bromo-2'-deoxyuridine (BrdU).17 Cells were cultured in quadruplicate in 96-well tissue culture plates, and MCGs or RMCP 1 was applied with BrdU as described above. Results were read with an ELISA reader at 450 nm.

Apoptosis Analysis

For analysis of apoptotic cells, a DNA fragmentation assay was performed with ApopLadder Ex (Takara Shuzo Co) and standard agarose gel electrophoresis. Fragmented and condensed nuclei in apoptotic cells were identified by staining of the cultured cells with propidium iodide and 4′,6-diamidino-2-phenylindole. Fragmented DNA was visualized by transmission electron microscopy.

Figure 1. Concentration-dependent effect of MCGs on cardiomyocyte survival. To examine effect of mast cells on their survival, cardiomyocytes were incubated for 24 hours with MCGs from 0.1% to 100%. Thereafter, trypan blue exclusion and MTT assays were performed. Values indicate percent of measurement under baseline conditions (without MCGs). *P<0.05 vs viability in absence of MCGs.
were dehydrated in an ethanol series and embedded in Polybed (Polyscience). Ultrathin (100-nm) sections were stained with saturated uranyl acetate and lead citrate and observed under a JEM-1200EX electron microscope (JEOL) at 100 kV.

Statistical Analysis
Differences between 2 groups were tested by unpaired 2-tailed Student's t test, with P < 0.05 considered statistically significant. Cell viability results are expressed as a percentage of baseline viability without MCGs. Values are presented as mean ± SE of 4 samples.

Results
MCGs Induce Cell Death of Cardiomyocytes
To examine the effect of mast cells on their survival, cardiomyocytes were cultured in the presence or absence of MCGs for 24 hours. MCGs caused death of cardiomyocytes in a concentration-dependent manner (Figure 1). The highest concentration of diluted MCGs caused death of ~70% of cardiomyocytes measured by trypan blue exclusion assay. Compound 48/80, which was used to prepare MCGs, did not influence cardiomyocyte survival (Figure 1, 0% MCGs versus DMEM alone).

Apoptosis Analysis
To determine whether cell death was apoptotic, the typical features of myocytic apoptosis were directly examined with Hoechst 33258 nuclear staining after the cardiomyocytes were cultured with MCGs for 24 hours. Chromosomal condensation and fragmentation were observed in a higher percentage of cardiomyocytes treated with MCGs (Figure 2A) than those treated with DMEM alone (Figure 2B) or DMEM containing 10% FBS (Figure 2C). In the cardiomyocytes cultured with MCGs, 40.5% of cardiomyocytes had apoptotic nuclei, whereas 51.4% were considered to be alive because they had intact nuclei. The remaining 8.1% were unclassified cells. This confirms that most cell deaths were apoptotic, even if one assumed that the unclassified cells were necrotic. DNA fragmentation assay to detect the presence of internucleosomal laddering in the genomic DNA, a hallmark of apoptosis, was also performed (Figure 3). DNA laddering was observed in samples from cardiomyocytes cultured with MCGs. In comparison, DNA laddering was not detected from control cardiomyocytes cultured in the absence of MCGs. Transmission electron microscopic analysis revealed that cardiomyocytes cultured with MCGs included several membrane-bound cellular fragments of various sizes with condensed cytoplasm and structurally well-preserved organelles (Figure 4). They were recognized to be compatible with apoptotic bodies. In the cardiomyocytes cultured in the absence of MCGs, these findings were not observed.
Effects of Mast Cell Protease on Cardiomyocyte Death
Because mast cells contain several mediators, including proteases, cytokines, and histamine, an attempt was made to identify the factor that caused cell death. To test whether mast cell proteases mediate the cytotoxic effect of MCGs, protease activity was inhibited by addition of a mixture of protease inhibitors, including PMSF, pepstatin A, leupeptin, aprotinin, and soybean trypsin inhibitor. Figure 5 shows the nearly complete protection against the cytotoxic effects of MCGs by the mixture of protease inhibitors. Tryptase, chymase, cathepsin G, and carboxypeptidase are the major proteases contained in mast cells, and their effects on other cell types are variable.2,3 To further examine which protease was cytotoxic, aprotinin and SBTI were incubated with MCGs. SBTI protected against cytotoxicity, but aprotinin was ineffective. These results suggest that chymase may mediate the cytotoxic effects of MCGs because, among the 4 enzymes described above, it is the only 1 inhibited by soybean trypsin inhibitor and not by aprotinin.11,19–21 To confirm that chymase causes their death, cardiomyocytes were incubated with RMCP 1 or RMCP 2 for 24 hours. RMCP 1 caused cardiomyocyte death in a dose-dependent manner, but RMCP 2 did not (Figure 6a). MTT assay performed on MCGs after incubation in the presence of neutralizing antibody to RMCP 1 revealed that the cytotoxic effects of MCGs had been prevented (Figure 6b). These results suggest that RMCP 1 contained in the MCG does cause cardiomyocyte death.

Effects of Histamine on Cardiomyocyte Death
Histamine, a potent mediator contained in mast cells, had no cytotoxic activity against cardiomyocytes (Figure 7a); accordingly, triprolidine (histamine H1 antagonist), cimetidine (histamine H2 antagonist), and thioperamide (histamine H3 antagonist) did not protect the cardiomyocytes against the cytotoxic activity of 50% MCGs (Figure 7b).
Tumor necrosis factor-α (TNF-α) is contained within mast cells and has been reported to cause cell death. In our experimental model, however, TNF-α did not decrease the number of cardiomyocytes of neonatal rats (data not shown). Therefore, the cytotoxicity of TNF-α contained in MCGs was not examined.

**MCGs and RMCP 1 Induce Proliferation of Nonmyocardial Cells**

In contrast, MCGs induced the proliferation of intramyocardial cells other than myocytes (nonmyocardial cells). Nonmyocardial cells were cultured with MCGs and DMEM containing 1% FBS for 24 hours. MCGs induced the proliferation of nonmyocardial cells as measured by both cell viability and BrdU assays (Figure 8a). Nonmyocardial cell cultures are composed of >95% fibroblasts, of which mast cells have been reported to promote proliferation via histamine, tryptase, and other mediators. Therefore, it is tempting to hypothesize that cardiac fibroblast proliferation was stimulated by MCGs.

To determine whether RMCP 1, which causes myocytic apoptosis, induces death or proliferation of intramyocardial cells other than cardiomyocytes, RMCP 1 was incubated with cardiomyocytes for nonmyocardial cells for 24 hours in the

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**Figure 6.** Effect of chymase and neutralizing antibody against chymase on MCG-induced cytotoxicity (MTT assay). a, Cardiomyocytes were incubated for 24 hours with RMCP 1 or RMCP 2 from 0.01 to 10 μg/mL. *P<0.05 vs viability in absence of chymase. b, MCGs preincubated for 12 hours at 4°C with neutralizing antibody to RMCP 1 or DMEM alone were cultured with cardiomyocytes for 24 hours. Values indicate percent of cell number measured under baseline conditions (without MCGs). *P<0.05 vs 50% MCGs.

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**Figure 7.** Effects of histamine and histamine antagonists on MCG-induced cytotoxicity. a, Lack of effect of histamine on survival of cardiomyocytes (MTT assay). Cardiomyocytes were incubated for 24 hours with histamine from 10^{-8} to 10^{-3} mol/L. b, Lack of effect of histamine (H1-, 2-, or 3-) antagonists against cytotoxicity of MCGs on cardiomyocytes (MTT assay). After 10-minute preincubation with triprolidine, cimetidine, thioperamide, or DMEM alone, MCGs were added to cultured cardiomyocytes and incubated for 24 hours. Values indicate percent of cell number measured under baseline conditions.
absence of FBS. RMCP 1 induced proliferation instead of death of nonmyocardial cells (Figure 8b).

**Discussion**

Results of this study indicate that MCGs cause apoptosis of cardiomyocytes and promote the proliferation of other cardiac cells; both effects are mediated by RMCP 1. It has been reported that mast cells are increased in number in myopathic human hearts and in the recently infarcted rat heart, along with an increase in their mediators such as tryptase and histamine. In addition, in the pressure-overloaded heart, chymase is activated, and apoptosis of cardiomyocytes and fibrosis have been observed. These observations suggest that mast cells and mast cell chymase are activated in the heart exposed to mechanical overload. Our study is the first to show that mast cells induce apoptosis of cardiomyocytes and proliferation of nonmyocardial cells via the activity of RMCP 1. These results suggest that mechanical overload activates cardiac mast cell chymase, resulting in myocyte loss and fibrosis. Loss of myocytes causes a decrease in contractile function of the heart, and fibrosis increases myocardial stiffness. Consequently, mast cells appear likely to cause both systolic and diastolic cardiac dysfunction and promote the pathophysiology of heart failure and cardiac remodeling.

Chymase was recently found to be present in the cytotoxic granules of cytotoxic lymphocytes and was implicated in perforin-mediated lysis. It is a serine protease of the granzyme family involved in apoptotic cell death. Cytotoxic T and natural killer cells are able to kill target cells through the action of granzyme. Although chymase has been known to be contained in mast cells, its cytotoxicity has not been reported. Our study is the first to show that mast cell chymase causes cell death.

The mechanism of cell death of cardiomyocyte and proliferation of nonmyocardial cells mediated by mast cell chymase is unclear, although 2 hypotheses have been formulated to explain how chymase may affect other cells. The first consists of a family of proteolytically cleaved receptors, known as the receptors of proteases. Three subtypes of protease-activated receptors have been cloned. Thrombin or tryptase cleave the amino-terminal extracellular extension of the intact and unactivated receptor, exposing the amino terminus, which then functions as a receptor agonist, binding to a region of the receptor and activating it. Although this receptor family has not been implicated in cell death, it may be a mediator of the cytotoxic action of mast cell chymase. The second mechanism is based on granzyme, a protease contained in cytotoxic T and natural killer cells that mediates their cytotoxicity for target cells. The pore-forming protein perforin punctures the target cell membrane, and granzyme is diffused into the target. Once in the cytosol, it initiates apoptosis by activating caspases. However, proliferation of nonmyocardial cells cannot be explained by this mechanism.

Several proteases are contained in mast cell granules, which have various effects on other cells. Tryptase, chymase, cathepsin G, and carboxypeptidase are the major proteases contained in mast cells. Of these proteases, chymase is most closely related to cardiovascular diseases. It is activated in pressure-overloaded hearts. Chymase is able to convert angiotensin I to angiotensin II independently of ACE and has a major role in the formation of angiotensin II. In addition, chymase activates interleukin-1β precursor and metalloproteinase. Through these functions, chymase has been thought to have a role in hypertrophy and fibrosis of the heart. Tryptase may be implicated in angiogenesis of hypertrophied heart because it induces tubular formation. It also induces proliferation of endothelial cells and proliferation of fibroblasts. Other proteases are known to have certain functions, although they are not fully clarified.
Mast cell cytotoxicity was examined by the MTT and trypan blue exclusion assays. These methods allow measurement not only of the number of apoptotic but also of necrotic cells. Therefore, the cytotoxicity measured by these methods does not directly reflect apoptosis. In our experiments, however, most cell deaths caused by MCGs were confirmed to be apoptotic by nuclear staining with Hoechst 33258 dye after 24 hours of culture with MCGs. This indicates that in this study, the MTT and cell viability assays mostly reflect apoptosis. That a few cardiomyocytes died of necrosis cannot be entirely excluded.

The actual concentration of mast cell chymase in the heart is unknown. Therefore, that the concentrations of MCGs and RMCP used in this study were physiological remains to be confirmed. However, cells in the immediate vicinity of mast cells are exposed to high MCG concentrations. So it is reasonable to assume that the concentrations used in our study are physiological.

In conclusion, we found that mast cells caused apoptosis of cardiomyocytes. This cytoidal effect was mediated by RMCP 1. In contrast, mast cells induced the proliferation of nonmyocardial cells. RMCP 1 was also proved to mediate this mitogenic effect. These results suggest that mast cells play a role in the progression of heart failure because loss of cardiomyocytes and proliferation of fibroblasts both participate in its pathophysiology.

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