Gene Expression of Cardiac Mast Cell Chymase and Tryptase in a Murine Model of Heart Failure Caused by Viral Myocarditis

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This study examined the gene expression of mouse mast cell proteases to clarify their role in the pathophysiology of viral myocarditis. Male DBA/2 mice were inoculated intraperitoneally with the encephalomyocarditis virus and the gene expression of mast cell chymase, mouse mast cell protease (mMCP)-4 and -5, and tryptase, mMCP-6, matrix metalloproteinase (MMP)-9 and type-I procollagen was measured by real-time quantitative RT-PCR analysis. The gene expression of mMCP-4, -5 and -6 mRNA was increased at 5 days, and continued to increase to day 14, coinciding with a prominent inflammatory reaction and extensive myocardial necrosis and fibrosis. The gene expression of MMP-9 was also increased, and there was a significant correlation between upregulation of mast cell proteases and MMP-9. The gene expression of type-I procollagen was increased at 5 days and continued to increase to day 14, suggesting that a fibrotic process had already begun during the acute stage of viral myocarditis. These findings suggest that mast cell chymase and tryptase participate in the acute inflammation and remodeling process of viral myocarditis. (Circ J 2003; 67: 881 – 884)

Key Words: Chymase; Mast cell; Metalloproteinase; Myocarditis; Tryptase

Vir al myocarditis is one of the most important causes of dilated cardiomyopathy (DCM), a major cause of heart failure, but its pathophysiology remains poorly understood. Mast cells play a major role in the pathogenesis of inflammatory diseases, such as bronchial asthma, bacterial peritonitis, rheumatic diseases and ulcerative colitis, producing several cytokines, including interleukin (IL)-1, IL-3, IL-4, IL-5, IL-6, interferon- (IFN-), and tumor necrosis factor (TNF-), that are mediators central to the development of inflammatory reactions.

Chymase and tryptase are the major serine proteases released by mast cells and these have been implicated in inflammation and tissue remodeling through extravasation of neutrophils, activation of matrix metalloproteinase (MMP) and IL-1 precursors, apoptosis of cardiac myocytes and proliferation of fibroblasts. They also stimulate epithelial and endothelial cells to release IL-8, which induces recruitment of granulocytes. These observations suggest that mast cell chymase and tryptase play a role in the pathogenesis of viral myocarditis and the present study was performed to clarify their involvement in the pathophysiology of heart failure caused by encephalomyocarditis virus (EMCV) myocarditis by examining their gene expression during the course of the disease.

Methods

Animal Preparations

Stocks of the M (myocardiotrophic) variant of EMCV were prepared as previously described and stored at −80°C. The 4-week-old male DBA/2 mice (Shizuoka Agricultural Cooperation Association) used in this study were treated in accordance with local institutional guidelines at all stages of the experiments. They were inoculated intraperitoneally with 0.2 ml EMCV in phosphate buffered saline (PBS) diluted to a concentration of 5 plaque forming units/ml. The mice were killed by cervical dislocation on days 5 and 14. Uninfected mice injected with 0.2 ml PBS were used as controls. Each group consisted of 5–11 animals. The hearts were dissected and immediately frozen and stored at −80°C.

Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis

Total RNA was isolated from the left ventricle by the acid guanidinium thiocyanate-phenol-chloroform method and the RNA concentration was measured spectrophotometrically. First-strand cDNA was synthesized using SUPERScript Preamplification System for FirstStrand cDNA Synthesis (GIBCO BRL, Gaithersburg, MD, USA). Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp, Foster City, CA, USA) was performed according to the manufacturer’s protocol. We used 2 ml of the first-strand cDNA in the following assay. The following forward (F) and reverse (R) oligonucleotides, and probes (P) were used for the quantification of mouse mast cell protease (mMCP)-4, mMCP-5, mMCP-6, MMP-9, membrane-type MMP (MT-MMP)-2,

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pro-α-2(I) collagen and glycolaldehydes-3-phosphate dehydrogenase mRNA;
mMCP-4 F, 5'-GAAGTGAAAAGCCTGACCTGC-3';
mMCP-4 R, 5'-CATGCTTTGTTGAACCCAAGG-3';
mMCP-4 P, 5'-TGCATCAGAGTCTTCAAGCCAGAGC-3';
mMCP-5 F, 5'-TTGCCAGCCTGTGAGGAAA-3';
mMCP-5 R, 5'-TACAGACAGGCCAGATCGCAT-3';
mMCP-5 P, 5'-CTGGAACTGGAATAGTGCAGGTTTGGTG-3';
mMCP-6 F, 5'-CGACATTGATAATGACGAGCCTC-3';
mMCP-6 R, 5'-ACAGGCTGTTTTCCACAATGG-3';
mMCP-6 P, 5'-CCCACCTCCTTATCTTCTGAAGCAAAGTGA-3';
pro-a-2(I) collagen F, 5'-GAGGACACCCCTTCTACGTTGTA-3';
pro-a-2(I) collagen R, 5'-CAGTCCAACAAGCATGCTGTGTA-3';
pro-a-2(I) collagen P, 5'-CAAACCTGGCTGCCACCACCATTGATGCTCTCTG-3';
MMP-9 F, 5'-TTGGTCTTCCCCAAAGACC-3';
MMP-9 R, 5'-TATCCACCGAGCTGGTCTCTCGAC ACTG-3';
MMP-9 P, 5'-AAAACCTTACACCTGACACCCACCAGTTGGA-3';
MT-MMP-2 F, 5'-ACTGACCTGCATGGAATCAGC-3';
MT-MMP-2 R, 5'-GAGGACACCCCTTCTACGTTGTA-3';
MT-MMP-2 P, 5'-TCTTCTGGGTCCGCTGAT-3';
GAPDH F, 5'-TTACACCACCATGGAGAAGGC-3';
GAPDH R, 5'-GGCATGGACTGTTCCATCAGTA-3';
GAPDH P, 5'-TGACACTGGCACCACCAACTGCTCAGT-3'.

The conditions for the TaqMan PCR were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

**Histological Analysis**

The hearts were fixed with neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 2 μm, and stained with hematoxylin–eosin for overall morphology and with Masson trichrome stain for the detection of connective tissue.

To determine the number of mast cells, the hearts were stained with toluidine blue. The total number of mast cells in a given section was calculated as cells/mm².

**Statistical Analysis**

All results are expressed as mean±SEM. Differences between 2 groups were tested by unpaired two-tailed Student's t test. Differences among more than 3 groups were analyzed by one-way analysis of variance with multiple comparisons by Fisher's least significant difference. Relationships between 2 variables were tested by linear regression analysis. Differences were considered statistically significant at p<0.05.
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Fig 3. Histopathologic findings in the hearts of mice inoculated with encephalomyocarditis virus. Before inoculation (A and D), on day 5 (B and E) and on day 14 (C and F); histopathologic examination was performed with hematoxylin-eosin stain (HE; A–C) or with Masson trichrome stain (MT; D–F) (×50; bar = 100 μm).

Results

Gene Expressions

We examined the upregulation of mMCP-4, -5 and -6, which are the counterpart of rat mast cell chymase 1, human chymase and human tryptase, respectively. The evolution of the relative concentrations of mast cell chymase, and mMCP-4 and -5 are shown in Fig 1. At 5 days after inoculation, the mRNA concentrations were already above baseline and at 14 days, they had each increased 3.8- and 4.1-fold, respectively (Fig 1A, B). Similarly, mast cell tryptase, mMCP-6, was significantly upregulated at 14 days after viral inoculation (Fig 1C).

The gene expression of MMP-9 mRNA and type-I procollagen was examined because they are key enzymes of the MMP family.14,21 MMP-3, in degradation and synthesis of ECM, activate MMP-3, an important constituent of the MMP family.14,21,22 Mast cell chymase and tryptase, which are implicated in the degradation and synthesis of ECM, activate MMP-3, an important constituent of the MMP family.14,21,22 MMP-3, in turn, activates other MMPs including MMP-9.21,22 Mast cell chymase induces the proliferation of cardiac fibroblasts and apoptosis of cardiac myocytes16 We have previously reported that IL-1, a prominent cytokine in cardiac remodeling,29 and is upregulated in the chronic stage of EMC viral myocarditis,27 and it has also been implicated in fibrosis after myocardial infarction.28 The human mast cell chymase, the counterpart of mMCP-5, which was upregulated in the present study, converts the precursor IL-1 to an active form,15 suggesting that mMCPs play a role in cardiac remodeling.

Histology

Cellular infiltration was observed at 5 days (Fig 3B) and at 14 days, the hearts of infected mice showed extensive myocardial cellular infiltration and necrosis (Fig 3C) compared with those of control mice (Fig 3A). The Masson trichrome stain revealed almost no fibrosis before (Fig 3D) or at 5 days (Fig 3E) after inoculation, but it became apparent at 14 days (Fig 3F).

Mast cells were detected in the hearts by toluidine blue staining. The number of mast cells was 1.8±0.3 cells/mm² in controls (n=4) and in the infected mice, 1.6±0.3 cells/mm² at 5 days (n=4), and 2.7±0.3 cells/mm² at 14 days after virus inoculation (n=6, p<0.05 vs at 5 days) (Fig 4).

The number of mast cells was increased significantly when myocardial fibrosis became apparent, and when the gene expressions of mMCP-4, -5, and -6, MMP-9, and type-I procollagen were markedly increased.

Discussion

In this study, mMCP-4, -5 and -6 were upregulated as early as 5 days after viral inoculation and had increased further at 14 days. MMP-9 was also upregulated at 14 days. This is the first study describing an upregulation of mast cell chymase, tryptase and MMP-9 during the course of viral myocarditis. In this model, cellular infiltration is regularly observed at 5 days after EMCV inoculation and by 14 days, signs of acute inflammation are more prominent, and myocardial fibrosis appears. From then on, fibrosis progresses, the cardiac chamber enlarges, and DCM is fully developed within 3 months after viral inoculation. This suggests that mast cell chymase and tryptase may be implicated in both the acute inflammatory reaction and the remodeling process associated with acute viral myocarditis.

The gene expressions of mMCPs and MMP-9 were significantly correlated in our study. MMPs represent the endogenous system of extracellular matrix (ECM) degradation and remodeling26 and are activated with the development of congestive heart failure.29 In end-stage DCM, the left ventricular content of MMP-3 and -9 increases, whereas that of MMP-2 remains unchanged compared with controls.29 In cardiac remodeling, degradation and synthesis of the ECM occur simultaneously, resulting in ventricular dilation. Mast cell chymase and tryptase, which are implicated in the degradation and synthesis of ECM, activate MMP-3, an important constituent of the MMP family.14,21,22 MMP-3, in turn, activates other MMPs including MMP-9.21,22 Mast cell chymase induces the proliferation of cardiac fibroblasts and apoptosis of cardiac myocytes.16 We have previously reported that IL-1, a prominent cytokine in cardiac remodeling, is upregulated in the chronic stage of EMC viral myocarditis,27 and it has also been implicated in fibrosis after myocardial infarction.28 The human mast cell chymase, the counterpart of mMCP-5, which was upregulated in the present study, converts the precursor IL-1 to an active form,15 suggesting that mMCPs play a role in cardiac remodeling.

Mast cells increase in number in the failing heart.29 In a rat model of acute myocardial infarction, the mast cell density reached its peak on day 21, when cardiac remodeling was ongoing.30 In the present study, the number of mast cells was increased at 14 days after virus inoculation when myocardial fibrosis became apparent. These observations suggest that mast cells participate in the progression of heart failure and cardiac remodeling. Recently, we studied the role of mast cells in the pathogenesis of heart failure in a mast cell-deficient mice model resulting from mutation of c-kit and left ventricular pressure overload.31 Left ventricular function was preserved, heart failure did not develop, and perivascular fibrosis was less prominent in the mast cell-deficient mice than in the wild-type. These observations suggest that mast cells play a critical role in the progression of heart failure. Shiota et al have reported that the gene expression and enzymatic activity of chymase increase in the hearts of cardiomyopathic hamsters.32 and in
chronically hypertensive hamsters, thus implicating the angiotensin II forming activity of chymase in the process of cardiac remodeling. However, in murine hearts, local angiotensin II is produced mainly by the angiotensin-converting enzyme, not by chymase. Therefore, we hypothesize that, in the mouse, mast cell chymase and tryptase play a role in cardiac remodeling via other enzymatic functions, including the activation of other effector molecules such as MMP, procollagen or IL-1, including the activation of other effector molecules such as MMP, procollagen or IL-1, including the activation of other effector molecules such as MMP, procollagen or IL-1.

In conclusion, the gene expression of mast cell chymase and tryptase was upregulated in the acute phase of viral myocarditis, and rose further in the subacute phase of heart failure. Their activation coincided with the development of myocardial necrosis and fibrosis, and correlated with the upregulation of MMP-9 and type-I procollagen. These findings suggest that mast cell chymase and tryptase participate in both the acute inflammatory reaction and in the remodeling process associated with acute viral myocarditis. The regulation of mast cell proteases may represent a new approach in the management of viral myocarditis and subsequent heart failure.

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