Cardiac Mast Cells Mediate Left Ventricular Fibrosis in the Hypertensive Rat Heart

Scott P. Levick, Jennifer L. McLarty, David B. Murray, Rebecca M. Freeman, Wayne E. Carver, Gregory L. Brower

Abstract—Correlative data suggest that cardiac mast cells are a component of the inflammatory response that is important to hypertension-induced adverse myocardial remodeling. However, a causal relationship has not been established. We hypothesized that adverse myocardial remodeling would be inhibited by preventing the release of mast cell products that may interact with fibroblasts and other inflammatory cells. Eight-week-old male spontaneously hypertensive rats were treated for 12 weeks with the mast cell stabilizing compound nedocromil (30 mg/kg per day). Age-matched Wistar-Kyoto rats served as controls. Nedocromil prevented left ventricular fibrosis in the spontaneously hypertensive rat independent of hypertrophy and blood pressure, despite cardiac mast cell density being elevated. The mast cell protease trypsin was elevated in the spontaneously hypertensive rat myocardium and was normalized by nedocromil. Treatment of isolated adult spontaneously hypertensive rat cardiac fibroblasts with trypsin induced collagen synthesis and proliferation, suggesting this as a possible mechanism of mast cell–mediated fibrosis. In addition, nedocromil prevented macrophage infiltration into the ventricle. The inflammatory cytokines interferon-γ and interleukin (IL)-4 were increased in the spontaneously hypertensive rat and normalized by nedocromil, whereas IL-6 and IL-10 were decreased in the spontaneously hypertensive rat, with nedocromil treatment normalizing IL-6 and increasing IL-10 above the control. These results demonstrate for the first time a causal relationship between mast cell activation and fibrosis in the hypertensive heart. Furthermore, these results identify several mechanisms, including tryptase, inflammatory cell recruitment, and cytokine regulation, by which mast cells may mediate hypertension-induced left ventricular fibrosis. (Hypertension. 2009;53:1041-1047.)

Key Words: hypertension ■ collagen ■ cytokines ■ inflammation

Inflammatory cell recruitment is closely associated with the development of left ventricular (LV) fibrosis induced by hypertension. Hinglais et al first described small foci of inflammatory cells consisting of CD4+, CD8+, and OX-6+ lymphocytes, as well as ED-1+ macrophages, in perivascular regions of the left ventricle in 8-week–old spontaneously hypertensive rats (SHRs). By 12 months of age, fibroblasts were always colocalized with inflammatory cells, and all of the inflammatory cell types were increased in areas of fibrosis. Since then, other studies have substantiated this link between macrophages, and more recently T cells, and hypertension-induced LV fibrosis. However, an often-overlooked inflammatory cell in this process is the mast cell. Olivetti et al first reported increased mast cell density in the hypertensive right ventricle of rats after pulmonary artery constriction. Subsequently, Shiota et al found that cardiac mast cell density increased dramatically with age in the SHR, remaining above control levels at all of the time points examined, whereas Panizo et al showed a strong correlation between cardiac mast cell density and collagen volume fraction in the SHR left ventricle. However, these studies fell short of establishing a causal relationship. Hara et al made the observation that perivascular collagen was normal in mast cell–deficient mice after aortic banding; however, this was not the focus of their study and was only a qualitative assessment. Accordingly, the present study sought to establish whether mast cells are causally involved in the development of LV fibrosis associated with hypertension. To this end, mast cell stabilization with nedocromil was performed to test the hypothesis that mast cells mediate LV fibrosis. In addition, we sought to identify possible mechanisms by which cardiac mast cells induce remodeling in the hypertensive heart.

Methods

Experimental Design

All of the experiments were performed using adult male SHRs (n=26) and Wistar Kyoto (WKY; n=13) rats housed under standard environmental conditions and maintained on commercial rat chow and tap water ad libitum. All of the studies conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In addition, the protocol was

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erythrosinate,13 with mast cell density determined by dividing the number of mast cells that were stained with the mast cell–specific stain, pinacyanol diastase, by the area of the myocardial cross-section. Collagen volume fraction was determined as described previously,14 with 5-m-thick paraffin-embedded sections stained m-thick coronal sections with picrosirius red (0.1% Sirius Red F3BA in picric acid) after incubation in phosphomolybdic acid (0.2%). Perivascular areas were excluded from the analysis.

Immunohistochemical Identification of ED-1+ Macrophages

Antigen retrieval was performed by microwaving LV sections in 0.03 M sodium citrate buffer (pH 6.0) at 100°C for 24 hours before rinsing with Moscona’s salt solution and serum starvation for 24 hours in DMEM-F12. Medium was then replaced with DMEM with 1.5% FBS containing 100, 500, or 1000 µL/mL of skin recombinant tryptase (Promega) for 24, 48, or 72 hours. Collagen synthesis was determined by hydroxyproline analysis of collected medium as described by Edwards and O’Brien.16 Additional fibroblasts were grown on coverslips until 50% to 60% confluence for proliferation studies. The cells were then incubated with tryptase, at the concentrations described above, and 5-bromo-2′-deoxy-uridine (Roche) labeling reagent was added to the medium, under sterile conditions, for the final 4 hours of the 24-hour treatment period. The culture medium was then aspirated and the cells fixed with ethanol/glutaraldehyde for 1 hour at −20°C.−20°C. Anti-5-bromo-2′-deoxy-uridine mouse IgG antibody was added to the cells and incubated for 1 hour at 37°C. After washing, fluorescein isothiocyanate–conjugated antimouse secondary antibody was added to the cells for 1 hour at 37°C. Propidium iodide (Molecular Probes) served as a nuclear marker of all of the cells. The cells were mounted and examined for 5-bromo-2′-deoxy-uridine incorporation using a BioRad MRC-1024 confocal laser-scanning microscope at ×400 magnification.

Western Blotting

Western blotting, as described previously,17 was used to assess levels of myocardial tryptase in SHRs and WKY rats. Blots were probed using an antibody against mast cell tryptase (Santa Cruz Biotechnology) and imaged on radiographic film.

ELISA Analysis

Commercially available ELISA kits were used to determine myocardial levels of histamine (Neogen), tumor necrosis factor (TNF)-α (R&D Systems), interferon (IFN)-γ, interleukin (IL) 4, IL-6, and IL-10 (Alpco Diagnostics).

Statistical Analysis

All of the grouped data were expressed as mean±SD or SEM, as appropriate. Grouped data comparisons were made by 1-way ANOVA using SPSS 11.5 software (SPSS Inc). When a significant F test (P≤0.05) was obtained, intergroup comparisons were analyzed using Fisher’s protected least-significant difference posthoc testing.

Results

Biometrics and Myocardial Function

No differences in body weight were observed between WKY rats and SHRs (Table 1); however, there was a small but significant increase in SHRs with nedocromil. Table 1 also contains LV weights and LV weights indexed to body weight as measures of hypertrophy. SHR and SHRs with nedocromil both had significant LV hypertrophy in comparison with WKY by both measures. SHRs, but not SHRs with nedocromil, showed a trend toward a smaller end diastolic volume; however, this did not reach significance (Table 2). SHRs, but not SHRs with nedocromil, had a slightly smaller
right ventricle than WKY rats. Lung weights, measured as an indication of pulmonary edema, were similar among all of the groups. SHRs and SHRs with nedocromil had marked increases in systolic blood pressure, diastolic blood pressure, and mean arterial pressure compared with WKY rats (Table 1). Cardiac output, as a marker of LV function, was significantly increased in the SHRs and SHRs with nedocromil versus WKY rats. Hearts from SHRs as well as SHRs with nedocromil also had increased rates of contraction and relaxation (Table 2).

**Histological Analysis**

SHRs had significantly increased numbers of mast cells within the left ventricle; however, because of hypertrophy, there was no difference when expressed in terms of mast cell density (Table 3). Nedocromil caused a significant increase in mast cell number and density compared with both the SHRs and WKY rats. Collagen volume fraction was significantly increased in the SHRs compared with the WKY rats (Figure 1) and was normalized by nedocromil. The presence of ED-1\(^+\) macrophages in the left ventricle was examined as an assessment of the effect of mast-cell stabilization on inflammatory cell recruitment. These cells were identified in the hearts of WKY rats in low numbers, sparsely distributed, and always as single cells (Figure 2A). The density of ED-1\(^+\) cells was greater in the SHRs, and these cells were often found as small foci of cells (Figure 2B) or larger populations at sites of apparent necrosis (Figure 2C). Nedocromil largely prevented the accumulation of ED-1\(^+\) macrophages as foci or scar infiltrate (Figure 2D).

**Myocardial Histamine and Tryptase and the Effect of Tryptase on Isolated Adult Cardiac Fibroblasts**

Histamine was not increased in the SHRs or SHRs with nedocromil despite an increased number of mast cells. However, Western blot analysis showed significantly higher levels of tryptase in SHRs (Figure 3), which were normalized by nedocromil. Accordingly, the ability of tryptase to induce collagen synthesis from isolated adult SHR cardiac fibroblasts was examined. No significant increase in hydroxyproline concentration was noted in the first 24 or 48 hours at any of the concentrations examined (Figure 4A and 4B). Collagen synthesis was induced by 100 mU/mL, only after 72 hours of incubation time (Figure 4C and 4D). Cardiac fibroblast proliferation was increased by tryptase at all of the concentrations tested (Figure 4E). Nedocromil (10 \(\mu\)mol/L) did not alter tryptase-induced collagen synthesis from adult cardiac fibroblasts (data not shown).

**Myocardial Cytokines**

TNF-\(\alpha\), IFN-\(\gamma\), IL-4, IL-6, and IL-10 were measured to gain an understanding of whether, and to what extent, cardiac mast cells regulate cytokine production in the hypertensive heart. Although TNF-\(\alpha\) tended to be higher in the SHR, this did not reach significance (Figure 5A). However, there was a signifi-

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**Table 2. Myocardial Function**

<table>
<thead>
<tr>
<th>Groups</th>
<th>EDV, (\mu)L</th>
<th>(+dP/dt, \text{mm Hg})</th>
<th>(-dP/dt, \text{mm Hg})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>310.9±44.5</td>
<td>7218.0±1316.9</td>
<td>-5827.8±1852.6</td>
</tr>
<tr>
<td>SHR</td>
<td>288.9±57.7</td>
<td>12479.0±2139.8*</td>
<td>-13636.5±1343.9*</td>
</tr>
<tr>
<td>SHR+Ned</td>
<td>324.7±19.3</td>
<td>1274.0±1504.4*</td>
<td>-12530.2±1291.9*</td>
</tr>
</tbody>
</table>

*All of the values are mean±SD. EDV indicates end diastolic volume; \(+dP/dt, maximum rate of contraction; -dP/dt, maximum rate of relaxation; Ned, nedocromil.\)

\(*P<0.05\) vs WKY rats.

\(†P<0.05\) vs SHRs.

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**Table 3. LV Cardiac Mast-Cell Number and Density**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cardiac Mast-Cell No., per LV Section</th>
<th>Cardiac Mast-Cell Density, cells per mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>73.3±10.3 (4)</td>
<td>1.4±0.2 (4)</td>
</tr>
<tr>
<td>SHR</td>
<td>105.7±14.8 (6)*</td>
<td>1.6±0.2 (6)</td>
</tr>
<tr>
<td>SHR+Ned</td>
<td>138.5±32.4 (4)*†</td>
<td>2.2±0.6 (4)*†</td>
</tr>
</tbody>
</table>

*All of the values are mean±SD (n). Ned indicates nedocromil.\)

\(*P<0.05\) vs WKY rats.

\(†P<0.05\) vs SHRs.
significant increase in TNF-α in the SHRs with nedocromil. IFN-γ was elevated in the SHRs (Figure 5B) with mast-cell stabilization, preventing the increase. Myocardial IL-4 was also elevated in the SHRs (Figure 5C) and normalized by mast-cell stabilization. IL-6 (Figure 5D) was markedly lower in the SHR myocardium than the WKY rat, but was returned to normal levels by nedocromil. IL-10, a potential antiinflammatory cytokine, was also decreased in the SHR myocardium (Figure 5E). SHRs treated with nedocromil showed increased myocardial IL-10 levels.

Discussion

The data presented herein clearly demonstrate that mast-cell stabilization with nedocromil prevents the development of LV fibrosis in response to hypertension. Thus, this is the first study to demonstrate a causal relationship between cardiac mast cells and the regulation of fibrosis in the hypertensive heart. Previously, Panizo et al\textsuperscript{10} had identified a strong correlation between cardiac mast-cell density and collagen volume fraction ($r=0.87$) in the SHR, without establishing causation. Hara et al\textsuperscript{11} made the observation that perivascular fibrosis did not develop in mast cell–deficient mice with experimental hypertension; however, these observations were not the focus of their study, but rather a qualitative assessment made almost in passing. This study also identifies several potential mechanisms by which mast cells may mediate their profibrotic actions. Mast cells produce a variety of proteases, cytokines, growth factors, vasoactive agents, and other biologically active mediators that may influence tissue remodeling. One of these is the serine protease tryptase. Western blotting identified increased levels of myocardial tryptase in the SHR, and cultured adult SHR fibroblast studies revealed that these cells both synthesize collagen and proliferate in response to tryptase. Interestingly, however, whereas proliferation was induced at all concentrations, only the low concentration of 100 mU/mL was able to induce collagen synthesis. Likely, this concentration represents the high end of the concentration response to tryptase by these cells, and, as such, low levels of tryptase induce collagen synthesis, whereas higher levels are required for fibroblast proliferation in the SHR. Tryptase is known to induce proliferation of human fetal dermal fibroblasts,\textsuperscript{16} and induction of collagen synthesis by tryptase has been demonstrated in human skin\textsuperscript{19} and lung fibroblasts;\textsuperscript{20} however, this is the first study to demonstrate tryptase-induced modulation of adult cardiac fibroblasts, identifying this as a likely mechanism contributing to mast cell–mediated LV fibrosis.

It is unlikely that tryptase is the only mediator responsible, because other mast-cell products, eg, chymase and TGF-β, have been shown to induce neonatal cardiac fibroblast collagen synthesis and proliferation.\textsuperscript{21} Likewise, given the documented evidence that mast cells contain renin, it is highly likely that mast cells are involved in the local generation of angiotensin II in the hypertensive heart.\textsuperscript{22} Given that SHR myocardial histamine was not elevated in our study, it seems unlikely that it plays an important role in fibrosis. Similarly, myocardial TNF-α was not significantly elevated in the SHR. However, TNF-α can increase angiotensin II–stimulated production of collagen by increasing angiotensin II type 1 receptors\textsuperscript{25} and decreasing matrix metalloproteinase activity.\textsuperscript{24} Furthermore, fibrosis and hypertrophy were attenuated in TNF-α knockout mice that had undergone aortic banding.\textsuperscript{25} Thus, TNF-α could play a role in mast cell–mediated LV fibrosis. It is difficult to know whether the increased levels of TNF-α observed in our study after mast cell stabilization were the result of increasing production by other cells or, alternatively,
an accumulation of TNF-α inside mast cells that could not be released because of membrane stabilization.

The finding that mast-cell stabilization completely prevented LV fibrosis when other types of inflammatory cells have also been shown to be involved in the fibrotic process suggests intercellular signaling between these cells. Consistent with this supposition, we observed a decrease in ED-1 macrophage accumulation within nedocromil-treated hearts. IFN-γ and IL-4, which were both elevated in the untreated SHRs but not the SHRs with nedocromil, are classic markers of T-cell activation. Although the increased production of these cytokines may be directly derived from mast cells, it is likely that mast cells also regulate the T-cell population in the heart. In fact, mast-cell regulation of T cells is well documented in noncardiac settings. Nedocromil also prevented the marked decrease in myocardial IL-6 and IL-10 in the SHRs. Currently, the role of IL-6 in fibrosis is unknown with reports of decreased plasma IL-6 in the SHRs, increased myocardial IL-6 in 2-week- and 20-month-old SHRs, normal myocardial IL-6 in 3- and 12-month-old SHRs, and increased IL-6 expression in hypertensive deoxycorticosterone acetate salt rats. Thus, IL-6 levels may depend on the stage of remodeling. IL-10 is well known as having anti-inflammatory properties. Interestingly, upregulation of IL-10 using viral vectors in Dahl salt-sensitive rats prevented hypertension, normalized LV wall thickness and fractional shortening, inhibited fibrosis, and dramatically attenuated the increased heart weight:body weight ratio. We did not observe a decrease in blood pressure with nedocromil, despite myocardial IL-10 levels being increased. This is not surprising, because IL-10 was likely only locally elevated in the myocardium in our study versus a systemic upregulation in the study by Nonaka-Sarukawa et al. In addition, we did not see any reduction in LV hypertrophy, probably because of a lack of effect on blood pressure in our study. Intriguingly, the reduction in IL-10 levels in the hypertensive heart may serve as a stimulus for mast-cell activation, because Palaniyandi et al demonstrated, using an adenoviral vector, that upregulation of IL-10 inhibited mast-cell degranulation and prevented the increase in density in the heart in experimental autoimmune myocarditis.

Shiota et al had previously reported dramatic increases in cardiac mast-cell density in the SHRs at all of the time points investigated. However, although total cardiac mast-cell numbers were increased in our study, the accompanying hypertrophy resulted in mast-cell density being normal. Animals were 20 weeks of age (5 months; a time point not measured by Shiota et al), which does not rule out the possibility that density was increased at some point during the 12-week duration of these studies. At the time of sacrifice, our rats may have been in a period of normal mast-cell density, where the SHR is in a state of steady hypertension and compensated myocardial remodeling. In fact, in volume overload–induced myocardial remodeling, mast-cell density returns to normal during the compensated hypertrophy phase of the remodeling process. Interestingly, in our study, cardiac mast-cell density was significantly increased after treatment with nedocromil, which may represent a reaction by mast cells in response to being unable to release profibrotic mediators because of stabilization by nedocromil.

The prevention of LV fibrosis by nedocromil was also independent of changes in LV hypertrophy and blood pressure. At 20 weeks of age, the SHR has a well-compensated left ventricle that actually has superior rates of contraction and relaxation despite the presence of fibrosis and concentric remodeling. Ultimately, however, the heart is unable to maintain the high workload necessary to overcome the extensive fibrosis and increased stiffening of the ventricle and begins to transition to failure. So, although nedocromil did not prevent LV hypertrophy from occurring, it prevented fibrosis while allowing nonconcentric hypertrophy to develop. This has the effect of maintaining a more advantageous mass:volume ratio, which is necessary to overcome the increased afterload present in the SHR. Rates of contraction and relaxation were still high in the nedocromil-treated SHRs, probably to maintain cardiac output against the increased
afterload, but because of the improved remodeling, presumably this occurred at a lower workload. Systolic blood pressure, diastolic blood pressure, and mean arterial pressure were also unchanged by nedocromil. Although mast cell-derived products are capable of inducing both vasoconstriction and vasodilatation, these mechanisms do not seem to play a significant role in the development of hypertension.

**Perspectives**

This study establishes, for the first time, a causal relationship between mast-cell activation and LV fibrosis in the hypertensive heart. Furthermore, this study identifies several mechanisms by which cardiac mast cells potentially mediate fibrosis, including tryptase-induced initiation of collagen synthesis from cardiac fibroblasts and fibroblast proliferation, recruitment of other inflammatory cells, and regulation of both proinflammatory and anti-inflammatory cytokines. Thus, these findings reveal a previously underestimated role for mast cells as key mediators of multiple aspects of the fibrotic response in the left ventricle secondary to hypertension.

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**Disclosures**

None.

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