Evaluation of Immunomodulatory Biomarkers in a Pressure Overload Model of Heart Failure

Barry E. Bleske, Pharm.D., FCCP, Hyun Seok Hwang, M.S., Issam Zineh, Pharm.D., Michael G. Ghannam, and Marvin O. Boluyt, Ph.D.

Study Objectives. To characterize the immunomodulatory response in a pressure overload model of heart failure, and to further validate this animal model of human heart failure.

Design. Randomized, controlled, animal study.

Setting. Large university research facility.

Animals. Twenty-seven, male, Sprague-Dawley rats.

Intervention. The rats underwent either aortic constriction or a sham procedure.

Measurements and Main Results. Six months after the surgical procedure, echocardiographic measurements were obtained, the animals were sacrificed, and plasma samples were taken to measure concentrations of biomarkers. As six (40%) of the 15 rats in the aortic-constriction group died before the 6 months, only nine rats from this group underwent immunomodulatory evaluation. Compared with the sham procedure, aortic constriction increased the left ventricle:body weight ratio in the rats ($p=0.0016$) It also decreased the velocity of circumferential shortening ($p=0.08$) and increased myocardial expression of atrial natriuretic factor, β-myosin heavy chain, and fibronectin ($p<0.05$). Concentrations of the proinflammatory mediator interleukin (IL)-1β and the counterregulatory mediator IL-10 also significantly increased ($p<0.04$) in the group that underwent aortic constriction compared with the group that underwent the sham procedure. Nonsignificant increases (mean change ~50–180%) were also observed for IL-2, IL-6, and leptin concentrations.

Conclusions. In this classic animal model of heart failure, a systemic immunomodulatory response was evaluated after 6 months of pressure overload resulting in myocardial decompensation and, in some cases, mortality. The findings are similar to the immunomodulatory response that may be observed in human heart failure. These novel results further define this model of heart failure and suggest another aspect of its relevance to human heart failure with regard to pressure overload and the immunomodulatory response.

Key Words: aortic constriction, heart failure, cytokines, chemokines, interleukin, leptin, animal study.

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The pathophysiology of heart failure is complex, and our understanding of its underlying mechanisms has evolved throughout the years. A hemodynamic defect was previously considered to be the primary cause of heart failure, but this understanding evolved to include a neurohormonal aspect. Today, the pathophysiologic model of heart failure has further evolved to include an immunomodulatory component.[1] The broad spectrum of effects that neurohormones

have on the myocardium and the mechanisms that regulate this system in the setting of heart failure have been extensively studied over the last 20 years. The mechanisms and models of immunomodulatory molecules, such as cytokines, chemokines, and angiogenic factors in heart failure, have only recently gained notable attention. Furthermore, a few investigators have simultaneously explored a wide range of inflammation-related proteins as they relate to heart failure. To begin to address these issues, we evaluated the effect of sustained pressure overload on a wide array of systemic immunomodulatory proteins in a rat aortic-constriction model of heart failure. To our knowledge, this is the first time these variables were measured in this model at this time point in the progression of heart failure.

Methods

Animals

Male Sprague-Dawley rats were used for all experiments. The University Committee on Use and Care of Animals at the University of Michigan approved all animal protocols. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (revised in 1996).

Protocol

Six-week-old, male Sprague-Dawley rats weighing 250–275 g were obtained and maintained on a cycle of 12 hours of light and 12 hours of darkness. They had access to rat chow and water ad libitum. After a stabilization period and when the rats weighed a mean ± SD of 300 ± 10 g, 12 were randomly selected to undergo sham surgery, and 15 were randomly selected for aortic constriction.

In the rats that survived for 6 months after the procedures, echocardiographic measurements were taken, the animals were sacrificed, and plasma samples were obtained by directly aspirating the myocardium. Samples were immediately placed in tubes containing 0.05 ml of 10% disodium ethylenediaminetetraacetic acid and centrifuged. The resultant plasma samples was immediately frozen to -80°C until they were assayed.

From the College of Pharmacy (Dr. Bleske and Mr. Ghannam), and the Division of Kinesiology (Mr. Hwang and Dr. Boluyt), University of Michigan, Ann Arbor, Michigan; and the Department of Pharmacy Practice and Center for Pharmacogenomics, University of Florida College of Pharmacy, Gainesville, Florida (Dr. Zineh).

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Address reprint requests to Barry E. Bleske, Pharm.D., FCCP, College of Pharmacy, University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065.

Aortic Constriction

Aortic constriction was conducted as described previously.[2] The animals were given pentobarbital 30 mg/kg for anesthesia, and we performed a left thoracotomy, exposing the ascending aorta. A hemoclip was placed around the aorta 4–6 mm superior to the aortic valve, with the gap of the hemoclip applicator adjusted to 1.02 mm by using a thumbscrew. Previous experiments had established that a mean ± SD left ventricular–distal aortic pressure gradient of 46 ± 12 mm Hg resulted from constriction of this severity. When the clip was in place, pleural pressure was reinstated, and the wound was closed with coated polyglactin 910 sutures. The rats were ambulatory within 2 hours and were then returned to their cages.

Northern Blotting

Total RNA was isolated from the left ventricles, as described previously.[3, 4] Complementary DNA probes were synthesized and radiolabeled from a template by using the random prime method. The probes for atrial natriuretic factor and β-myosin heavy chain, fibronectin, glyceraldehyde 3-phosphate dehydrogenase, and the 18S ribosomal RNA were described previously.[3, 4] Oligonucleotides were radiolabeled by using terminal deoxynucleotide transferase with [α-^{32}P]deoxyadenosine 5′-triphosphate.

Measurement of Cytokines, Chemokines, and Angiogenic Factor

We evaluated immunoproteins that might plausibly contribute to the pathophysiology of heart failure (proinflammatory mediators) or to protection against it (antiinflammatory mediators). These proteins were chemokines (eotaxin, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, and interferon-γ–inducible protein-10), angiogenic and colony-stimulating factors (granulocyte and granulocyte-macrophage colony-stimulating factors and vascular endothelial growth factor), and prototypical proinflammatory and antiinflammatory cytokines (interferon-γ, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-18, tumor

necrosis factor-α, and leptin).

All analytes were measured by means of multiplex immunofluorescence cytometry (100 IS; Luminex Corp., Austin, TX) by using a commercially available kit (Lincoplex rat cytokine-chemokine immunoassay panel; Linco Research, Inc., St. Charles, MO). All plasma samples were assayed in duplicate and analyzed with analysis software (BeadView, version 1; Upstate, Charlottesville, VA).

Data and Statistical Analysis

Data are reported as medians and interquartile ranges unless otherwise noted. To make meaningful comparisons between the sham and aortic-constriction groups, we analyzed only those biomarkers for which at least 75% of the samples had concentrations above the lower limit of detection. For these analytes, samples with undetectable levels were treated as 0 pg/ml. On the basis of the 75% cutoff criterion, we excluded granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-5, IL-17, macrophage inflammatory protein-1α, tumor necrosis factor-α, and vascular endothelial growth factor from the analysis.

Because of the known nonnormal distribution of immunomarker concentrations, the Kruskal-Wallis test was used to compare differences in biomarker concentrations between the sham and aortic-constriction groups. A nonpaired *t* test was used to evaluate echocardiographic parameters and gene expression. A Fisher exact test was used to evaluate mortality. A p value less than 0.05 was considered to indicate a statistically significant difference.

All statistical analyses were performed with SPSS software, version 11.0 (SPSS Inc., Chicago, IL).

Results

By the end of the 6 months of pressure overload, six (40%) of 15 rats in the aortic-constriction group had died compared with none in the sham group (p=0.017). Therefore, immunomodulatory response was evaluated in only nine rats in the aortic-constriction group and in all 12 rats from the sham group.

Expression of the fetal-neonatal genes for atrial natriuretic factor and the β-myosin heavy chain, which are considered markers for pathologic hypertrophy and heart failure, was significantly elevated in the left ventricle of the rats with aortic constriction (Figure 1). Expression of fibronectin, a component of the extracellular matrix associated with myocardial fibrosis in heart failure, was also significantly elevated in the aortic-constriction group (Figure 2).[4] Aortic constriction produced a 51% increase in the left ventricle:body weight ratio (p=0.0016; Table 1) and decreased the velocity of circumferential shortening by 28% compared with the sham procedure (p=0.08). These findings were expected and demonstrated the progression of heart failure.

For most analytes, we observed no significant differences in plasma concentrations between the sham and aortic-constriction groups (Table 2). However, mean changes of approximately 50% to 180% were observed for IL-2, IL-6, and leptin. Significant differences were demonstrated between the groups for the prototypical proinflammatory

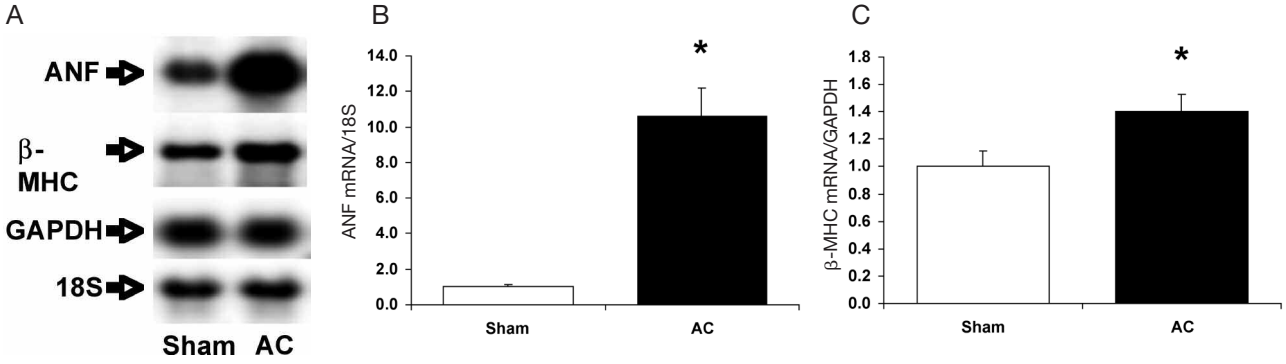

Figure 1. Expression of fetal-neonatal genes atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC), which are considered markers for pathologic hypertrophy and heart failure, in the left ventricle of rats 6 months after sham or aortic-constriction (AC) procedure. A representative northern blot is shown (panel A). Panel B is a bar graph of the ANF messenger RNA (mRNA):18S ratios (mean ± standard error) for the sham and the aortic-constriction groups. Panel C is a bar graph of the β-MHC mRNA:GAPDH ratios (mean ± standard error) for the two groups. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; 18S = 18S ribosomal RNA. *p<0.05.

Table 1. Biometric Data for the 21 Rats That Survived 6 Months After Surgery

Measure	Sham Group (n=12)[a]	Aortic-Constriction Group (n=9)[a]
Final body weight (g)	561 ± 22	555 ± 23
Weight of left ventricle (mg)	1021 ± 40	1500 ± 77[b]
Left ventricle:body weight ratio (mg:g)	1.82 ± 0.06	2.75 ± 0.20[b]

Data are mean ± standard error.
[a]The difference in the numbers of rats who died before 6 months in the sham (0) and aortic-constriction groups (6) was significant ($p<0.05$).
[b]The difference between groups was significant ($p<0.05$).

Table 2. Cytokine and Chemokine Concentrations 6 Months After Sham and Aortic-Constriction Procedures

Marker	Concentration (pg/ml)[a]	
	Sham Group	Aortic-Constriction Group
Eotaxin	71 (43–124)	52 (33–85)
Monocyte chemotactic protein-1	23 (15–35)	30 (25–34)
Inducible protein-10	50 (35–87)	49 (31–82)
Interferon-γ	184 (96–327)	137 (106–227)
Interleukin-1α	180 (136–377)	206 (129–295)
Interleukin-2	19 (9–41)	53 (16–177)
Interleukin-4	9.1 (3–31)	8.7 (4–25)
Interleukin-6	653 (272–1850)	1030 (272–1080)
Interleukin-9	24 (17.5–35)	36 (18–38)
Interleukin-13	696 (467–1102)	712 (285–897)
Interleukin-18	68 (48–95)	86 (60–289)
Leptin	4405 (2690–8090)	6580 (3070–9840)

Data are median (interquartile range).
[a]No significant differences were observed between groups.

IL-1β (Figure 3) and for the largely antiinflammatory IL-10 (Figure 4). Concentrations of IL-1β in the sham and aortic-constriction groups were 18 pg/ml (interquartile range 3–40 pg/ml) and 68 pg/ml (interquartile range 24–111 pg/ml), respectively ($p=0.039$). In addition, IL-10 concentrations were 4-fold higher in the aortic-constriction group than in the sham group (596 vs 146 pg/ml, respective interquartile ranges 398–904 vs 0–385 pg/ml, $p=0.038$).

Discussion

In this exploratory evaluation of the 6-month immunomodulatory response to aortic constriction, levels of the proinflammatory mediator IL-1β and the generally antiinflam-matory molecule IL-10 were elevated in animals exposed to cardiac-pressure overload compared with controls. These findings suggest that this classic model of aortic constriction not only produced the expected structural and functional changes in the myocardium due to pressure overload but also upregulated the immune system. The finding that levels of inflammation-related proteins increased helps to further define this important animal model and can direct further research efforts.

The major focus of research regarding the pathophysiology of heart failure has centered on neurohormonal activation (e.g., norepinephrine, angiotensin II) and, to a lesser extent, inflammatory proteins such as tumor necrosis factor-α and IL-6. However, levels of other immunomodulatory mediators have also been found to be elevated in heart failure and are now considered important to the development and progression of

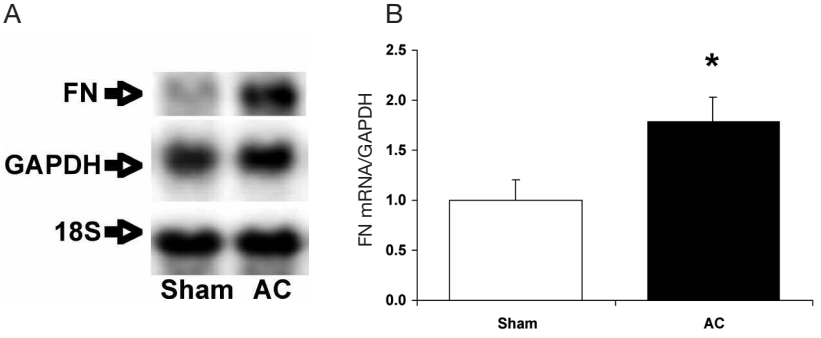

Figure 2. Expression of the extracellular matrix gene fibronectin (FN) in the left ventricle of rats 6 months after sham or aortic-constriction (AC) procedure. A representative northern blot is shown (panel A). Panel B is a bar graph of the FN messenger RNA (mRNA):GAPDH ratios (mean ± standard error) for the sham and the aortic-constriction groups. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; 18S = 18S ribosomal RNA. *$p<0.05$.

the disease. These additional mediators include but are not limited to IL-1β, IL-2, IL-18, and monocyte chemotactic protein-1.[1, 5–8] In addition, IL-10 may be an important counterregulatory immunomodulator in heart failure. A number of investigators have reported elevated levels of these mediators in patients with heart failure.[9–12]

The mechanisms by which these mediators contribute to the pathophysiology of heart failure have not been completely elucidated and are likely to be multifactorial. For example, immunomodulatory mediators may decrease cardiac contractility by means of a number of different mechanisms. Interleukin-1β may decrease contractility by increasing concentrations of ceramide and sphingosine, whereas IL-2 and IL-6 may increase concentrations of constitutively expressed nitric oxide synthase.[13–16] Other mechanisms may include uncoupling of the β-adrenergic receptor, modulation of the activity of calcium adenosine triphosphatase in the sacroplasmic reticulum, and oxidative stress.[6, 7, 15, 17, 18] In contrast to these mediators, IL-10 may have a protective function, which may include antioxidant effect and downregulation of proinflammatory immunomodulatory mediators such as tumor necrosis factor-α, IL-1, and IL-6.[19–21] In heart failure, IL-10 concentrations may be elevated in response to an increase in levels of catecholamines, tumor necrosis factor-α, IL-6, and perhaps to an increase in the rate of apoptosis.[22] The elevation in IL-10 observed in our study may account for the low concentrations observed for tumor necrosis factor-α and IL-6.

Overall, we observed a significant increase in concentrations of the immunomodulatory mediators IL-1β and IL-10 after aortic constriction. Moderate, nonsignificant increases were also seen for IL-2, IL-6, and leptin. Additional samples would likely have produced statistical significance. These findings suggest that activation of immunomodulators is important in the model of heart failure associated with chronic pressure overload and that they are similar to what has been observed in human heart failure.

A previous study of the effect of hemodynamic overload in mice for 6 hours to 35 days demonstrated somewhat different results.[23] The authors measured levels of IL-1β, IL-6, and tumor necrosis factor-α and reported acute increases at the 6 hours after aortic constriction. However, the same elevation was not observed at time points later than this. Reasons for the difference between the studies are not certain, but they may be related to the animal model, to the laboratory technique, and, perhaps most importantly, to the time of measurement. The long duration of our study may have been a factor, and it might have been most representative of chronic heart failure.

Our study had a number of important limitations. One was that we measured plasma concentrations and not tissue expression. However, human

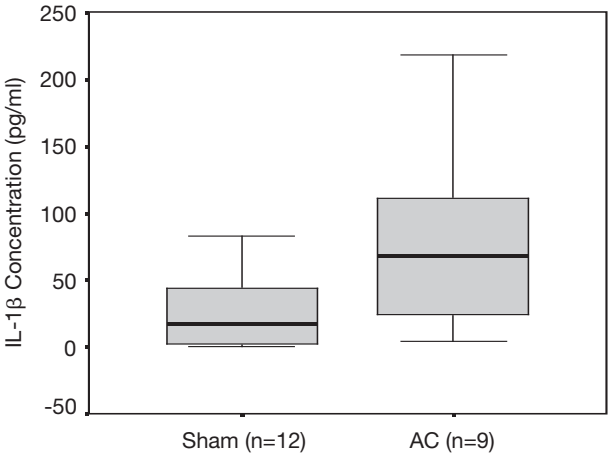

Figure 3. Median concentration of interleukin (IL)-1β in rats undergoing the sham versus aortic-constriction (AC) procedure (p=0.039). The intrabox line represents the median; the lower and upper box edges represent the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent 1.5 box-lengths from the upper and lower quartiles, respectively, or the maximum-minimum boundaries (whichever is smaller).

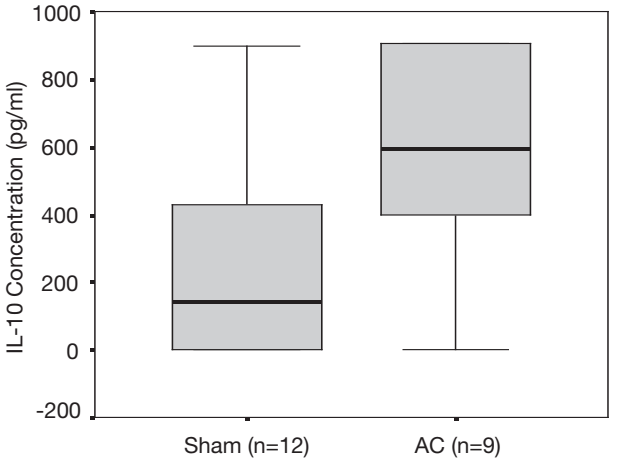

Figure 4. Median concentration of interleukin (IL)-10 in rats undergoing the sham versus aortic constriction (AC) procedure (p=0.038). The intrabox line represents the median; the lower and upper box edges represent the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent 1.5 box-lengths from the upper and lower quartiles, respectively, or the maximum-minimum boundaries (whichever is smaller).

studies have demonstrated elevated circulating levels of immunomodulators in patients with heart failure. Second, because we had limited numbers of samples and because this was the first exploration in this area, we applied a robust approach to determine the immunomodulatory effect on this commonly used animal model of heart failure, raising the issue of multiple testing. In light of this issue, the data should be evaluated as observational data needing confirmation. What is important is the overall picture of relatively large changes in immunomodulatory mediators in this animal model of heart failure that parallel findings in human heart failure.

Another issue was that we did not have serial measurements and could not describe the time course over which the immunomodulatory response may contribute to the development of heart failure. In theory, this response might be a major or minor contributor early in the development of heart failure, or it might be influential only late in the course of heart failure when the mortality rate is high.

Finally, although the immunomodulatory response is important to human heart failure, the etiology of heart failure in humans is rarely isolated to pure pressure overload. It is multifactorial and, at minimum, includes altered gene expression as well as structural, neurohormonal, immunomodulatory, and hemodynamic changes. Therefore, the relevance of human heart failure to our pressure overload model, which demonstrated typical structural changes and altered gene expression, is of concern. However, our novel results perhaps suggest increased relevance of this model to human heart failure now that we have observed an apparent immunomodulatory response.

Conclusion

This study demonstrated an immunomodulatory response after long-term aortic constriction in a rat model of heart failure. The results suggested that, in this classic animal model of heart failure, the immunomodulatory response may be an important component of the milieu creating the progressive deterioration of pump function. These findings help to further define this important animal model and may enhance its usefulness and applicability in studying heart failure.

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