Myocardial Osteopontin Expression Coincides With the Development of Heart Failure


Abstract — To identify genes that are differentially expressed during the transition from compensated hypertrophy to failure, myocardial mRNA from spontaneously hypertensive rats (SHR) with heart failure (SHR-F) was compared with that from age-matched SHR with compensated hypertrophy (SHR-NF) and normotensive Wistar-Kyoto rats (WKY) by differential display reverse transcriptase–polymerase chain reaction. Characterization of a transcript differentially expressed in SHR-F yielded a cDNA with homology to the extracellular matrix protein osteopontin. Northern analysis showed low levels of osteopontin mRNA in left ventricular myocardium from WKY and SHR-NF but a markedly increased (≈10-fold) level in SHR-F. In myocardium from WKY and SHR-NF, in situ hybridization showed only scant osteopontin mRNA, primarily in arteriolar cells. In SHR-F, in situ hybridization revealed abundant expression of osteopontin mRNA, primarily in nonmyocytes in the interstitial and perivascular space. Similar findings for osteopontin protein were observed in the midwall region of myocardium from the SHR-F group. Consistent with the findings in SHR, osteopontin mRNA was minimally increased (≈1.9-fold) in left ventricular myocardium from nonfailing aortic-banded rats with pressure-overload hypertrophy but was markedly increased (≈8-fold) in banded rats with failure. Treatment with captopril starting before or after the onset of failure in the SHR reduced the increase in left ventricular osteopontin mRNA levels. Thus, osteopontin expression is markedly increased in the heart coincident with the development of heart failure. The source of osteopontin in SHR-F is primarily nonmyocytes, and its induction is inhibited by an angiotensin-converting enzyme inhibitor, suggesting a role for angiotensin II. Given the known biological activities of osteopontin, including cell adhesion and regulation of inducible nitric oxide synthase gene expression, these data suggest that it could play a role in the pathophysiology of heart failure. (Hypertension. 1999;33:663-670.)

Key Words: osteopontin ■ heart ■ heart failure ■ rats, inbred SHR ■ hypertrophy

The spontaneously hypertensive rat (SHR) has been proven to be a valuable model for studying the transition from myocardial hypertrophy to failure.1– 4 In these animals, a period of several months of compensated hypertrophy is followed by the development of heart failure (SHR-F). We have shown that in comparison to SHR with compensated hypertrophy (SHR-NF), the transition is associated with pathological evidence of cardiac decompensation, ie, presence of pleuropericardial effusions, atrial thrombi, and right ventricular (RV) hypertrophy with reduced contractile function and increased stiffness of papillary muscle, increased interstitial fibrosis, and increased expression of atrial natriuretic peptide (ANP), collagen, fibronectin, and transforming growth factor-β1 mRNAs.3,5–8

In an attempt to identify genes that are differentially expressed coincident with the transition from hypertrophy to failure, we used differential display reverse transcriptase–polymerase chain reaction (RT-PCR) to compare RNA isolated from the left ventricles (LV) of SHR-F with that from age-matched SHR-NF and Wistar-Kyoto rats (WKY).9 One of the transcripts that was differentially expressed in myocardium from SHR-F was for osteopontin, an extracellular matrix protein that can act as an adhesion molecule, affect cellular function by interacting with integrins, and modulate the expression of inducible nitric oxide synthase.10–12

Although it has recently been shown that osteopontin can be expressed in myocardium in response to necrotic injury13 or short-term pressure overload,14,15 its expression has not previously been associated with the development of myocardial failure. This report describes the differential expression and localization of osteopontin in the myocardium of both SHR and aortic-banded rats coincident with the development of pathological evidence of cardiac decompensation. Since angiotensin II (Ang II) stimulates osteopontin expression by cardiac fibroblasts14 and angiotensin-converting enzyme (ACE) inhibitor can prevent many of the features of myocar-
dial failure in SHR,1,2 we also examined the effect of treatment with the ACE inhibitor captopril on the expression of osteopontin.

Methods

Animal Models

Male SHR and nonhypertensive control WKY were purchased (Taconic, Germantown, NY) at the age of 6 to 9 months and boarded until the time of study. Beginning at 18 months of age, all animals were observed twice per week for tachypnea and labored respiration.2,5,7,8 SHR were studied within 7 days of the observation of respiratory difficulties. Age-matched WKY and SHR without these signs were studied at the same time.

The aortic-banded rats and sham-operated WKY were purchased from Taconic, Germantown, NY (surgeries were performed at Taconic). A loop of 3.0 surgical silk was placed around the aortic arch between the brachiocephalic and left carotid artery. A blunted 18.5-gauge needle was placed over the aorta, and the silk was tied around the needle and aorta.16 The needle was then carefully removed, resulting in a nonconstricting band. The development of heart failure was suggested by the observation of respiratory difficulties and documented by pathological examination, as described previously.2,17 In 4 animals, it was added before the onset of failure and continued for 2 to 4 months before euthanasia at the age of 24 months.

After the rats were killed, the hearts were quickly removed and placed in Krebs-Henseleit buffer. The RV and LV were carefully dissected, weighed, and immediately frozen in liquid nitrogen for RNA isolation. For in situ hybridization and immunohistochemical studies, hearts were perfusion-fixed with freshly prepared 4% paraformaldehyde.6

RNA Isolation

Total RNA was isolated from the LV according to the method of Chomczynski and Sacchi.18 Briefly, samples (100 to 200 mg) were homogenized in guanidinium thiocyanate solution (4 mol/L guanidinium thiocyanate, 25 mmol/L Na citrate [pH 7.0], 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol) with a tissue homogenizer. After the RNA was extracted with phenol-chloroform, the RNA was precipitated with ethanol at −20°C. For differential display RT-PCR, RNA was treated with RNase-free DNase for 30 minutes at 37°C. The RNA was extracted again with phenol-chloroform and precipitated with ethanol.

Differential Display and Sequence Analysis

Differential display and sequence analysis were performed as described earlier.9 With the use of anchored primers 5’-AAGCTTTTTTTTTTTCG-3’, 5’-AAGCTTTTTTTTTTTC-3’, or 5’-AAGCTTTTTTTTTTATA-3’ and Moloney murine leukemia virus reverse transcriptase (GeneHunter Co), the cDNAs were prepared from Taconic. A loop of 3.0 surgical silk was placed around the aortic arch between the brachiocephalic and left carotid artery. A blunted 18.5-gauge needle was placed over the aorta, and the silk was tied around the needle and aorta.16 The needle was then carefully removed, resulting in a nonconstricting band. The development of heart failure was suggested by the observation of respiratory difficulties and documented by pathological examination, as described previously.2,17 In 4 animals, it was added before the onset of failure and continued for 2 to 4 months before euthanasia at the age of 24 months.

Captopril was added to the drinking water (2.0 g/L) of 9 SHR, as previously described.2,17 In 4 animals, it was added before the onset of failure at either 12 (n=1), 18 (n=1), or 21 (n=2) months of age and continued until the age of 24 months. In 5 rats, captopril was administered after the onset of failure and continued for 2 to 4 months before euthanasia at the age of 24 months.

After the rats were killed, the hearts were quickly removed and placed in Krebs-Henseleit buffer. The RV and LV were carefully dissected, weighed, and immediately frozen in liquid nitrogen for RNA isolation. For in situ hybridization and immunohistochemical studies, hearts were perfusion-fixed with freshly prepared 4% paraformaldehyde.6

Figure 1. Representative autoradiograph showing differential display RT-PCR of RNA samples isolated from the LV of age-matched WKY and SHR-F or SHR-NF. The oligonucleotides consisted of an anchor primer 5’-AAGCTTTTTTTTTTTTTC-3’ and an upstream primer 5’-AAGCTTTCGACTGT. The arrow indicates a band that is increased in SHR-F compared with SHR-NF or WKY. The band from the SHR-F was excised from the gel, cloned, and sequenced.
from mRNA of total RNA isolated from the LV. The reverse transcription mixture was then amplified by random priming PCR containing $[^{35}S]dATP$ with the appropriate anchored primer and 24 different arbitrary 13-mer primers (GeneHunter Co). The products were separated on a polyacrylamide sequencing gel containing 6 mol/L urea. Autoradiographs were evaluated by visual comparison of the intensities of individual bands run side by side. The differentially expressed cDNAs were excised from the dried gel and extracted with hot water. The recovered DNA was reamplified and cloned in a pCR 2.1 vector (Invitrogen). Purified plasmid DNA from *Escherichia coli* was then used for sequencing according to the dideoxy chain termination method with Sequanase 2.0 (Amersham). Sequences were compared with a sequence database with the use of the Geneworks program (Intelligenetics).

**Northern Blot Analysis**

Total RNA was size fractionated on 1.0% formaldehyde agarose gels containing 2.2 mol/L formaldehyde and transferred to nylon membranes (Gene Screen Plus; NEN DuPont) with a transblot apparatus (Bio-Rad). Blots were hybridized overnight with the radiolabeled osteopontin cDNA probe, as described previously. The blots were then deprobed and hybridized with radiolabeled ANP cDNA (courtesy of Dr. C. Seidman) probe. To normalize for loading differences, the blots were then probed with an 18S oligonucleotide (30-mer) end-labeled by T4 polynucleotide kinase. Differences in mRNA signal intensity were determined with the use of a phosphoimager (PhosphoImager, Bio-Rad).

**In Situ Hybridization**

In situ hybridization was performed as previously described. Hearts were perfusion fixed with 4% paraformaldehyde/phosphate-buffered saline. Parallel slices 1 to 2 mm thick, encompassing both RV and LV, were dehydrated and embedded in Paraplast Plus embedding medium (Oxford). Sections 4 μm thick (from WKY, SHR-NF, and SHR-F) were hybridized with single-stranded sense or antisense RNA probes transcribed from a linearized full-length osteopontin cDNA using $[^{35}S]UTP$. Probes were extracted with phenol-chloroform and precipitated with ethanol.

**Immunohistochemistry**

Sections (4 μm thick) from WKY, SHR-NF, and SHR-F were deparaffinized and stained with the use of monoclonal anti-osteopontin antibodies (MPIIIB10; development studies, hybridoma bank) and Vectastain avidin-biotin peroxidase kit (Vector Laboratories). Briefly, nonspecific binding was minimized by incubation for 20 minutes with 1.5% normal horse serum. The sections were then incubated with biotinylated secondary antibodies. Detection was performed with the use of Vectastain ABC-AP reagent and Vector Red alkaline substrate kit (Vector Laboratories). The sections were visualized and photographed under epifluorescence microscopy with the use of rhodamine excitation and emission filters.

**Statistical Analysis**

All data are expressed as mean±SEM. Two-tailed Student’s *t* test was used to compare the group means. Probability values of <0.05 were considered significant.

**Pathophysiologic Data**

The body, LV, and RV weights for each experimental group are shown in the Table. SHR is well documented to develop cardiac decompensation between 18 and 24 months. The LV/body weight ratio was increased to a similar degree (50% to 60%) in the SHR-F and SHR-NF groups. The RV/body weight ratio was higher in SHR-F than in SHR-NF, consistent with prior findings in these animals. Among the
SHR-F, all animals also showed pleuropericardial effusions and atrial thrombi, whereas these findings were absent among the SHR-NF and WKY groups.

In aortic-banded animals, the LV/body weight ratio was increased to a similar degree (40% to 60%) in both the nonfailing and failing banded animals (versus nonbanded control animals), whereas the RV/body weight ratio was increased only in failing banded animals, similar to the findings in SHR.

Differential Display RT-PCR
To identify genes with differential expression in LV myocardium from SHR-F (versus SHR-NF and age-matched WKY), 3 hearts from each group were subjected to differential display RT-PCR. An autoradiograph of a differential display gel showed the increased expression of a cDNA in SHR-F versus SHR-NF and WKY (Figure 1). This cDNA (~500 bp) was isolated and cloned, and 129 bp were sequenced, revealing 98% homology to the rat osteopontin cDNA sequence (Figure 2).

Northern Hybridization
The differential expression of osteopontin mRNA in SHR-F was confirmed by performing Northern hybridization with total RNA isolated from the LV of age-matched SHR-F, SHR-NF, and WKY. Hybridization with a 1.5-kb osteopontin cDNA identified a 1.6-kb transcript similar in size to that reported for osteopontin. Osteopontin mRNA was expressed at similar low levels in WKY and SHR-NF (Figure 3). In striking contrast, osteopontin mRNA was increased 10.2 ± 2.57-fold (P < 0.05; n = 8) in LV from SHR-F versus
Compared with age-matched WKY controls, osteopontin expression in aortic-banded rats of failure (Figure 3B). In SHR-F, ANP mRNA was further increased to >70-fold versus WKY and ~7-fold versus SHR-NF (Figure 3).

**In Situ Hybridization**
In sections from SHR-NF hearts, in situ hybridization with the antisense probe for osteopontin showed scant expression, which was limited to blood vessels, possibly in endothelial and/or smooth muscle cells (Figure 4A and 4C). Similarly, WKY hearts showed scant expression of osteopontin mRNA (not shown). In striking contrast, sections from SHR-F hearts revealed intense expression of osteopontin, primarily in the interstitial and perivascular space (Figure 4B and 4D). Hematoxylin and eosin staining of adjacent sections confirmed that osteopontin expression in SHR-F was associated primarily with nonmuscle cells infiltrating between the myocytes (Figure 4E through 4H). In some areas, the expression of osteopontin was diffuse within the interstitial space (eg, Figure 4F and 4H). In other areas, the expression was more focal and appeared to be associated with degenerating myocytes (eg, Figure 4E and 4G). Increased expression of osteopontin mRNA was also detected in the RV of SHR-F, in a distribution similar to that observed in the LV. No grains were visible with the sense osteopontin probe (not shown).

**Immunohistochemistry**
In sections from SHR-NF hearts, immunohistochemical analysis demonstrated low levels of immunoreactivity for osteopontin in the interstitial cells of the midwall region of LV (Figure 5A). Similar staining for osteopontin was observed in sections from WKY (not shown). In sections from SHR-F, intense staining was observed in the midwall region of LV, primarily in the interstitial space (Figure 5B). Most of the staining was observed in the areas of fibrosis and seemed to be present around the myocytes (Figure 5C). The papillary muscles from the hearts of WKY, SHR-NF, and SHR-F all showed intense fibrosis associated with marked staining for osteopontin.

**Effect of Captopril on Osteopontin Expression in SHR**
The effect of treatment with the ACE inhibitor captopril on LV osteopontin mRNA expression was assessed by Northern hybridization in 9 SHR at 24 months of age. Captopril prevented the increase in osteopontin expression in 4 of 4 hearts treated before failure, while it reduced osteopontin expression in 4 of 5 hearts from animals treated after the onset of failure (Figure 3B).

**Osteopontin Expression in Aortic-Banded Rats**
Compared with age-matched WKY controls, osteopontin mRNA was increased 1.9±0.6-fold (P=0.1 versus WKY; n=5) in banded animals without failure and 7.7±2.9-fold (P<0.05 versus nonfailing banded; n=5) in banded animals with signs of failure (Figure 6). ANP was increased 3.4±0.9-fold and 5.2±1.1-fold in nonfailing and failing banded animals, respectively.

**Discussion**
Using differential display RT-PCR and Northern analysis, we found that the expression of osteopontin mRNA was markedly increased coincident with appearance of pathophysiological evidence of cardiac decompensation in 2 models of chronic pressure overload, the SHR and the aortic-banded rat. These results, confirmed by in situ hybridization and immunohistochemistry, indicated that (1) there was only low basal expression of osteopontin in control WKY rats; (2) in the absence of failure, basal expression of osteopontin was unchanged or only slightly increased in SHR or aortic-banded rats with well-established LV hypertrophy; and (3) in both models there was a marked increase in osteopontin expression in age-matched animals that had manifestations of cardiac decompensation. In situ hybridization and immunohistochemistry demonstrated that in failing animals the primary source of osteopontin mRNA was nonmyocytes and that the protein is mainly localized in the interstitium.

Osteopontin is an arginine-glycine-aspartate–containing adhesive glycoprotein. Although first isolated from mineralized bone matrix, osteopontin can be synthesized by several cell types, including cardiac myocytes, microvascular endothelial cells, and fibroblasts. Osteopontin is expressed in several tissues in response to injury, suggesting a role in the reparative process, and appears capable of mediating diverse biological functions, including cell adhesion, chemotaxis, and signaling.

Several groups have recently demonstrated that osteopontin can be expressed in the myocardium. Murry et al showed the expression of osteopontin by macrophages in response to myocardial necrosis caused by transdiaphragmatic freezing. Likewise, in the cardiomyopathic Syrian hamster, Williams et al found expression of osteopontin in areas of necrosis associated with inflammation, also apparently in tissue macrophages. Recently, Graf et al demonstrated that osteopontin mRNA was increased approximately 2-fold and 3-fold, respectively, in LV from rats with 2-kidney, 1 clip hypertension or aortic banding. In these 2 models, cardiac myocytes were identified as the predominant source of osteopontin by immunohistochemistry and in situ hybridization.

In contrast to Graf et al, we did not find osteopontin mRNA to be increased in the LV of SHR-NF as assessed by differential display PCR, Northern hybridization, or in situ hybridization, despite clear evidence of LV hypertrophy and the expression of ANP mRNA. Likewise, we found only a modest (~1.9-fold) increase in osteopontin in nonfailing aortic-banded rats, despite marked LV hypertrophy and increased ANP mRNA expression. A possible explanation for differences in findings between our studies and those of Graf et al may relate to the markedly different time periods studied. Whereas Graf et al examined animals relatively soon (~4 to 6 weeks) after surgery, our studies were performed in animals that had been exposed to LV pressure overload for many months (18 to 24 months for SHR; >9 months for aortic-banded rats).
Consistent with the in situ hybridization, immunohistochemistry revealed increased expression of osteopontin in the midwall region of SHR-F (compared with SHR-NF and WKY). Similar to the in situ hybridization, most of the staining was observed in the interstitial space. In contrast to in situ hybridization, which showed increased expression of osteopontin mRNA in the papillary muscles of only SHR-F, immunoreactivity for osteopontin protein was increased in the papillary muscles of all 3 groups (ie, WKY, SHR-NF, and SHR-F). This may reflect (1) the extensive fibrosis in age-related papillary muscles from all 3 groups and/or (2) that secretory osteopontin, once incorporated into extracellular matrix, has a lower turnover rate and/or higher stability.

In SHR with cardiac decompensation, the major source of osteopontin expression appears to be nonmyocytes in the interstitium and perivascular space. Thus, our data differ from those of Graf et al., who found that myocytes were the major source of osteopontin in LV from animals with relatively short-term hypertension. However, our findings are consistent with those of Murry et al. and Williams et al., who found interstitial expression of osteopontin by macrophages in rats with thermal injury or cardiomyopathic hamsters, respectively. Murry et al. suggested that osteopontin is synthesized in response to injury. Our findings suggest that, at least with regard to osteopontin, the appearance of cardiac decompensation and the transition to failure in these rat models is associated with an “injury response.” Taken together with the data of Graf et al., it appears reasonable to suggest that the source of osteopontin mRNA may be myocytes early in LV hypertrophy but shifts to interstitial cells and focal areas of myocyte injury in late hypertrophy with the development of heart failure.

Ang II has been shown to cause focal myocyte necrosis in rats infused with Ang II or in models of renovascular hypertension. Ang II also induces osteopontin in cardiac fibroblasts in vitro, and infusion of Ang II increased osteopontin expression in kidney. In our study, focal expression of osteopontin mRNA appeared to be associated with degenerating myocytes (Figure 4E and 4G). We also found that captopril treatment of SHR reduced osteopontin mRNA levels. These findings are thus consistent with a role for Ang II in the induction of osteopontin in areas of myocyte necrosis and fibrosis during the transition to failure. However, it is also possible that rats treated with captopril before the development of heart failure remained compensated and therefore showed no increase in osteopontin, while captopril treatment after the development of heart failure might have prevented the additional necrosis and healing of lesions, thereby preventing osteopontin expression. Another possibility is that captopril suppressed the expression of osteopontin by increasing bradykinin, which might act through nitric oxide to attenuate the expression of growth-related genes.

The role of osteopontin during the transition to heart failure is not known. Indeed, relatively little is known about the role of osteopontin in the myocardium. Osteopontin can act as an adhesion molecule, as a chemotactic factor, and as a substrate for the migration of macrophages, smooth muscle cells, and endothelial cells. Osteopontin can also act as a cytokine to stimulate lymphocyte immunoglobulin production. Activities are consistent with a role for osteopontin in fibrosis or healing in response to injury.

We have also shown that osteopontin can suppress the cytokine-induced expression of inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells isolated from adult rat hearts. It is therefore of interest that high levels of nitric oxide produced by inducible nitric oxide synthase can impair myocyte function and may be toxic to cardiac myocytes. Since it appears that myocardial inducible nitric oxide synthase expression is increased with failure, osteopontin has the potential to modulate the effects of inflammatory cytokines on the myocardium by attenuating the expression of inducible nitric oxide synthase.

Thus, osteopontin expression is markedly increased in the myocardium coincident with the development of evidence of cardiac decompensation in 2 models of chronic pressure overload. Given the importance of the extracellular matrix in the pathophysiology of myocardial failure and the known biological activities of osteopontin, it is possible that this interstitial matrix protein plays an important role in the pathogenesis of heart failure.

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References


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