Overexpression of Brain Natriuretic Peptide Facilitates Neutrophil Infiltration and Cardiac Matrix Metalloproteinase-9 Expression After Acute Myocardial Infarction

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Background—Recent clinical trials have shown that systemic infusion of nesiritide, a recombinant human brain natriuretic peptide (BNP), improves hemodynamic parameters in acutely decompensated hearts. This suggests that BNP exerts a direct cardioprotective effect and might thus be a useful therapeutic agent with which to treat acute myocardial infarction (MI). In the present study, we used BNP-transgenic (BNP-Tg) mice with elevated plasma BNP to determine whether and how BNP contributes to left ventricular remodeling and healing after MI.

Methods and Results—We examined the accumulation of neutrophils and the expression and activation of matrix metalloproteinase (MMP)-9 in the ventricles of male BNP-Tg mice and their nontransgenic (non-Tg) littermates during the early phase after acute MI. The numbers of neutrophils infiltrating the infarcted area were significantly increased in BNP-Tg mice 3 days after MI. In addition, both the gene expression and zymographic activity of MMP-9, but not MMP-2, were significantly higher in BNP-Tg than non-Tg mice. Double immunostaining revealed that neutrophils are the main source of the MMP-9, although doxycycline, an MMP inhibitor, had no effect on neutrophil infiltration of the infarcted area in BNP-Tg mice.

Conclusions—These results demonstrate that elevated plasma BNP facilitates neutrophil infiltration of the infarcted area after MI and increases the activity of the MMP-9 they produce. This suggests that BNP plays a key role in the processes of extracellular matrix remodeling and wound-healing during the early phase after acute MI. (Circulation. 2004;110:3306-3312.)

Key Words: metalloproteinases ▪ myocardial infarction ▪ natriuretic peptides ▪ remodeling ▪ neutrophils

By secreting both atrial and brain natriuretic peptides (ANP and BNP, respectively), which act via natriuretic peptide receptor A (NPRA) to induce natriuresis, diuresis, and vasodilation and to inhibit the renin-angiotensin-aldosterone and sympathetic nervous systems, the heart serves as an important endocrine organ involved in the regulation of blood pressure and fluid-electrolyte balance.1,2 ANP is synthesized and secreted primarily from the atria in adult mammals, whereas BNP is secreted primarily from the ventricle.3 Synthesis and secretion of both ANP and BNP are markedly increased in patients with congestive heart failure.4 Plasma BNP levels are also strongly increased during the early phase of acute myocardial infarction (MI), although plasma ANP levels are increased only slightly.5 Such sustained increases in plasma BNP are correlated with enlargement of the left ventricle (LV), decreased ventricular contractility, and increased ventricular stiffness,6,7 which suggests that BNP might play a significant role in ventricular remodeling. In fact, using BNP-deficient mice, we previously showed that endogenous BNP is a local regulator of ventricular fibrosis.8 Intravenous infusion of nesiritide, a recombinant human BNP, was recently reported to have beneficial hemodynamic effects in patients with decompensated congestive heart failure.9,10 In addition to alleviating cardiac preload and afterloads, BNP might exert a direct cardioprotective effect11,12 that could prevent LV remodeling after MI. The
effects of continuously high levels of BNP on the infarcted myocardium are unknown, however. We therefore used BNP-transgenic (BNP-Tg) mice to investigate the effects of sustained increases in plasma BNP on cardiac repair pathways and remodeling after MI. These mice overexpress the BNP in their livers and show a >100-fold increase in plasma BNP levels throughout their lives. In the present study, we focused on leukocyte infiltration, the genetic regulation of myocardial collagen synthesis including transforming growth factor (TGF)-β, and the activity of matrix metalloproteinase (MMP)-9, an important regulatory enzyme involved in extracellular matrix (ECM) degradation and cell migration during cardiac wound healing. In infarcted BNP-Tg hearts.

Methods

Experimental Animals
BNP-Tg mice were developed as previously described by use of the liver-specific human serum amyloid P component promoter. These mice show plasma BNP concentrations that are at least 2 orders of magnitude higher than those of their wild-type littermates, C57BL/6J nontransgenic (non-Tg) mice. Acute MI was induced in male BNP-Tg (n = 51) and non-Tg (n = 43) mice (age, 8 to 12 weeks; weight, 25 to 30 g) by ligation of the left coronary artery. The same procedure without coronary artery ligation served as the sham operation. The experimental animals were monitored for 7 days after MI had been induced.

Echocardiography
Echocardiography was performed under light anesthesia with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg) and spontaneous respiration.

Hemodynamic and Infarct Size Measurements
After 3 days, a 2F Millar Micro-Tip catheter transducer (Millar Instruments) was inserted into the right carotid artery and then advanced into the LV for recording of LV systolic pressure, LV end-diastolic pressure, and LV maximum and minimum rates of pressure development (dP/dt). The ventricles were excised after evaluation of hemodynamic parameters. Infarct size was expressed as the ratio of the infarct to total LV mass as previously described.

Immunohistochemistry and Quantitative Analysis of Histology
In a subset of animals (6 BNP-Tg and 6 non-Tg), the LV was cut into 3 transverse sections (apex, middle ring, and base) 3 days after MI. Immunostaining was then performed on frozen tissue specimens (6 μm) with rat anti-mouse 7/4 antibody (Serotec), which recognizes a polymorphic 40-kDa antigen expressed by neutrophils, and goat anti-mouse MMP-9 antibody (Santa Cruz Biotechnology). For each section, neutrophil 7/4-positive cells were counted in the infarcted area in at least 8 to 10 randomly selected high-power fields by use of a computer program (KS400 Version 3.00; Carl Zeiss). The activity of type IV collagenases (MMP-2 and MMP-9) was assayed by use of a commercially available kit (Yagai Research Center) according to the manufacturer’s instructions.

Type IV Collagenase Activity Assay
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Treatment With Doxycycline
In the doxycycline study, mice receiving 60 mg/kg doxycycline per day by gavage were compared with an untreated control group. Administration of doxycycline was started 3 days before induction of experimental MI and continued for 7 days after MI.

Data Analysis
All results are reported as mean ± SEM. Two-way ANOVA followed by Tukey-Kramer tests was used to evaluate the effects of MI and genotype. The mortality data (deaths during the 7-day protocol, including causes of death) were analyzed by use of the χ² test. Values of P < 0.05 were considered significant.

Northern Blot Analysis
Northern blots were made using 20 μg of total RNA isolated from frozen LV tissue by use of a technique described in detail elsewhere. The probes for collagen I, collagen III, TGF-β, fibronectin, and BNP were already available to us. The other cDNA probes were prepared using reverse transcription–polymerase chain reaction with primers based on the published sequences.

Zymographic Measurement of Gelatinase Activity
MMP activity in 30 μg of myocardial extract was measured by gelatin zymography as previously described. The gelatinolytic zones were quantified by use of NIH 1.62 image analysis software.

Myeloperoxidase (MPO) Activity
Myeloperoxidase (MPO) activity was measured spectrophotometrically at 460 nm in 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine hydrochloride (Sigma) and 0.0005% hydrogen peroxide as described previously. One unit of MPO was defined as the quantity of enzyme needed to hydrolyze peroxide at a rate of 1 mmol/min at 25°C.

Figure 1. Accumulation of neutrophils in hearts of infarcted mice. a, Representative micrograph showing stained neutrophils in infarcted region (magnification ×200). b, Numbers of neutrophils per mm² within infarcted area 3 days after MI (n = 8 for each). c, Cardiac MPO activity expressed as units/100 mg tissue wet wt in sham-operated and infarcted mice 3 days after MI (n = 8 for each). Values are mean ± SEM; P < 0.01 vs non-Tg mice with MI.
Infarct Size, Echocardiography, and Hemodynamics

Three days after left coronary artery ligation, the sizes of the resultant infarcts were similar in BNP-Tg and non-Tg mice (BNP-Tg, 42.2±3.7% versus non-Tg, 40.4±3.8%; \( P = 0.75, n = 7 \)). To evaluate the effect of a high plasma BNP concentration on the performance of the infarcted heart, we assessed cardiac function and LV geometry by use of echocardiography. The Table shows that the increase in LV chamber size and the noninfarcted wall thickness were the same in BNP-Tg and non-Tg mice 3 days after MI. LV systolic pressure measured by use of a Millar catheter was lower in sham-operated BNP-Tg mice than in sham-operated non-Tg mice. In addition, expression of MMP-9 mRNA was significantly higher in BNP-Tg than non-Tg mice after MI operated animals. In contrast, expression of MMP-2, TIMP-1, urokinase-type plasminogen activator–metalloproteinase system in myocardial neutrophil accumulation, the repair processes, and the rupture seen after MI. When we examined gene expression of plasminogen activators and MMPs 3 days after MI, we found that, with the exception of GAPDH, transcription of all the genes examined was upregulated compared with sham-operated animals. In addition, expression of MMP-9 mRNA was significantly higher in BNP-Tg than non-Tg mice after ligation (Figure 2a and b), whereas there was no difference in the expression of MMP-2, TIMP-1, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 mRNA in the 2 MI groups.

Cardiac Gene Expression in Infarcted Hearts

Recent evidence highlights the involvement of the plasminogen activator–metalloproteinase system in myocardial neutrophil accumulation, the repair processes, and the rupture seen after MI. When we examined gene expression of plasminogen activators and MMPs 3 days after MI, we found that, with the exception of GAPDH, transcription of all the genes examined was upregulated compared with sham-operated animals. In addition, expression of MMP-9 mRNA was significantly higher in BNP-Tg than non-Tg mice after ligation (Figure 2a and b), whereas there was no difference in the expression of MMP-2, TIMP-1, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 mRNA in the 2 MI groups.

We also focused on the synthetic processes involved in collagen turnover by examining the expression of mRNAs encoding TGF-\( \beta \), TGF-\( \beta \), collagen I, and collagen III, which are known to be involved in cardiac fibroblast proliferation and the biosynthesis of ECM proteins. We found that their expression was similarly upregulated in the infarcted regions of both BNP-Tg and non-Tg hearts (Figure 2a and b), indicating that overexpression of BNP does not affect the biosynthesis of collagen during the early phase of acute MI.

Results

Infarct Infiltration by Neutrophils

Accumulation of leukocytes in the infarcted region is thought to be one step in the process of wound repair. We therefore counted the leukocytes infiltrating the infarcted region after MI by use of a neutrophil-specific antibody. Neutrophils were identified throughout the infarcted segments after MI (Figure 1a). Moreover, although quantitative analysis of images of the infarcted region obtained 3 days after MI showed that their numbers increased in both BNP-Tg and non-Tg mice, there were significantly greater numbers of neutrophils in BNP-Tg than non-Tg hearts (BNP-Tg, 415.41±12.90 cells/mm\(^2\) versus non-Tg, 330.70±16.82 cells/mm\(^2\); \( P < 0.01, n = 6 \); Figure 1b).

To further assess neutrophil accumulation in the infarced areas, we also measured MPO activity. As shown in Figure 1c, cardiac MPO activity was significantly higher in BNP-Tg than non-Tg mice 3 days after MI (BNP-Tg, 2.80±0.40 U/100 mg tissue versus non-Tg, 1.33±0.23 U/100 mg tissue; \( n = 8 \) to 10, \( P < 0.01 \)), whereas there was no difference between the sham-operated groups.

Taken together, the data presented in this section clearly indicate that within 3 days after MI, neutrophils accumulate to a significantly greater degree in the infarcted regions of BNP-Tg hearts than non-Tg hearts.

### Cardiac Gene Expression in Infarcted Hearts

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### Echocardiographic and Hemodynamic Data

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<thead>
<tr>
<th></th>
<th>Non-Tg</th>
<th>BNP-Tg</th>
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<tr>
<td><strong>Echocardiographic data</strong></td>
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<tr>
<td>LV EDD, mm</td>
<td>4.4±0.1</td>
<td>4.8±0.1(\dagger)$</td>
</tr>
<tr>
<td>LV ESD, mm</td>
<td>3.2±0.2</td>
<td>3.7±0.1(\dagger)$</td>
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<tr>
<td>FS, %</td>
<td>31.7±1.7</td>
<td>23.5±1.9(\dagger)$</td>
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<tr>
<td><strong>Hemodynamic data</strong></td>
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<tr>
<td>LVSP, mmHg</td>
<td>100±3</td>
<td>68±2†</td>
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<tr>
<td>LVEDP, mmHg</td>
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<td>6.0±0.5</td>
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<tr>
<td>+LV dP/dt(_{max}), mm Hg/s</td>
<td>10 936±570</td>
<td>6433±545††</td>
</tr>
<tr>
<td>−LV dP/dt(_{min}), mm Hg/s</td>
<td>10 836±784</td>
<td>6416±545††</td>
</tr>
</tbody>
</table>

Values are shown as mean±SEM. EDD indicates end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; N/A, not applicable; SP, systolic pressures; EDP, end-diastolic pressure; and dP/dt, maximum and minimal rate of pressure development. 

*\( P < 0.05 \), †\( P < 0.01 \) vs sham-operated non-Tg mice. 

†\( P < 0.05 \), ‡\( P < 0.01 \) vs sham-operated BNP-Tg mice.
Increased MMP Activity in Infarcted Hearts

We next used gelatin zymography to evaluate the extent to which overexpression of BNP affects MMP-9 enzymatic activity. As shown in Figure 3a, the gelatinase activity of MMP-9, but not MMP-2, was significantly (P<0.05) elevated in infarcted BNP-Tg hearts compared with infarcted non-Tg hearts. Likewise, type IV collagenase activity was significantly higher in infarcted BNP-Tg than non-Tg hearts.
Because MMP-9 and -2 are the 2 major collagenases that degrade type IV collagen and zymography showed that there was no difference in MMP-2 activity within the infarcts of BNP-Tg and non-Tg mice, the increased digestion of type IV collagen in BNP-Tg hearts is attributable to the increase in MMP-9 activity.

Neutrophils Are the Predominant Source of MMP-9

We then evaluated the distribution of the MMP-9 by using confocal fluorescence microscopy to visualize the double immunostaining of MMP-9 (green) and neutrophils (red) in thin sections of frozen mouse LV. Three days after MI, immunoreactive MMP-9 and neutrophils were detected within the infarcted myocardium and the border regions in both BNP-Tg and non-Tg hearts (Figure 4, a–d). Moreover, the double labeling revealed that the distribution of immunoreactive neutrophils overlapped that of MMP-9 (Figure 4, e and f), indicating that the major source of MMP-9 is the neutrophils infiltrating the infarcted region. By contrast, MMP-9 levels were negligible in the sham-operated mice and the noninfarcted regions of the infarcted mice (data not shown).

MMP-9 Inhibition Did Not Affect Neutrophil Infiltration in BNP-Tg

Finally, we assessed the functional significance of MMPs in BNP-Tg mice subjected to experimental MI by treating the mice with doxycycline, a nonselective MMP inhibitor. We found that the numbers of neutrophils detected by use of anti-mouse neutrophil 7/4 antibody were similarly increased in control BNP-Tg and doxycycline-treated BNP-Tg mice (control BNP-Tg, 428.24±29.84 cells/mm² versus doxycycline-treated BNP-Tg, 432.93±23.86 cells/mm²; P=0.90, n=6; Figure 5, a and b). Likewise, there were no significant differences in the cardiac MPO activity in control BNP-Tg and doxycycline-treated BNP-Tg mice (control BNP-Tg, 3.03±0.36 U/100 mg tissue versus doxycycline-treated BNP-Tg, 2.80±0.32 U/100 mg tissue; P=0.63; Figure 5c). Thus, the increased infiltration of neutrophils into the infarcted area was not dependent on increased MMP-9 activity in the neutrophils themselves.
Discussion

We previously showed that plasma BNP levels are greatly elevated in patients with acute MI; they reach a peak within 24 hours after onset, then decline and increase again to a second peak over the next 3 to 7 days.\(^3\) Thus, high plasma BNP levels persist during the period when neutrophils and other inflammatory cells infiltrate the infarcted area. In the present study, we used BNP-Tg mice to assess the effects of a pharmacological dose of BNP on the myocardium early after acute MI. We found that (1) there is a greater accumulation of neutrophils in BNP-Tg hearts; (2) gene expression and enzyme activity of MMP-9 are higher in BNP-Tg hearts; (3) the major source of MMP-9 is the neutrophils infiltrating the infarcted region of BNP-Tg hearts; and (4) doxycycline, a potent MMP inhibitor, has no effect on the increased infiltration of neutrophils into the infarcted area in BNP-Tg mice.

The wound repair process involves temporally overlapping phases that include inflammation, new tissue formation, and tissue remodeling.\(^2\) During the inflammatory phase, collagen and other ECM components may be degraded as a result of increased MMP activity.\(^29,30\) In the present study, we found that early after MI, neutrophil infiltration of the infarcted area is augmented in BNP-Tg mice, and that there are corresponding increases in MMP-9 expression and activity associated with the infiltrating neutrophils. By contrast, there were no significant changes in the levels of TGF-\(\beta_1\), TGF-\(\beta_2\), collagen I, collagen III, or fibronectin mRNA, which suggests that overexpression of BNP leads to exaggerated collagen degradation by MMP-9 produced by neutrophils without an apparent increase in synthesis. Moreover, the fact that the increase in zymographic MMP-9 activity in BNP-Tg mice appeared to be more pronounced than the increase in neutrophil number suggests that BNP may have a direct effect on the amount of MMP-9 activity produced by each activated neutrophil. This idea is supported by the results of supplemental experiments showing that in the presence of the neutrophil-activating factor formyl-methionyl-leucyl-phenylalanine (fMLP; 10\(^{-7}\) mol/L), ANP (10\(^{-8}\) mol/L), which shares its receptor (NPRA) with BNP and is equally potent, elicited a 2.1-fold increase (\(P<0.01\)) in the transcription and activation of MMP-9 in human neutrophils.

We also tested whether upregulation of MMP-9 contributes to the accumulation of neutrophils in BNP-Tg mice. On the basis of evidence that it suppresses the activity of such MMPs as collagenase, gelatinase, and stromelysin both directly and indirectly,\(^31\) we used doxycycline to evaluate the extent to which elevated MMP production is involved in neutrophil accumulation within the infarcted regions of BNP-Tg hearts. That we found no difference in the neutrophil accumulation in control BNP-Tg and doxycycline-treated BNP-Tg mice suggests that the increased MMP-9 activity is most likely not responsible for the neutrophil accumulation in BNP-Tg animals. Conversely, one possible explanation for the increased leukocyte infiltration is that BNP exerts a direct effect on neutrophil chemotaxis. In fact, a recent study has shown that ANP affects human neutrophil migration at concentrations ranging from 4\(\times\)10\(^{-9}\) to 10\(^{-7}\) mol/L.\(^32\) The plasma BNP concentration in BNP-Tg mice was approximately 3\(\times\)10\(^{-9}\) mol/L, which is comparable to the effective ANP concentration reported earlier, because ANP and BNP act via NPRA with equal potency. Another possible explanation is an indirect effect via endothelial adhesion molecules. We previously showed that the diminished neutrophil accumulation seen during ischemia/reperfusion in NPRA-deficient mice is probably a result of suppressed expression of P-selectin in coronary endothelial cells and that ANP upregulates P-selectin expression in cultured endothelial cells exposed to oxidative stress.\(^20\) Thus, BNP might increase neutrophil accumulation by upregulating one or more of the endothelial adhesion molecules that tether circulating neutrophils to the endothelium.

Heymans et al\(^{16}\) recently showed that MMP-9 deficiency retards the wound healing process after MI in mice, which increases the size of residual necrotic areas. In the same study, these investigators also showed that the lack of MMP-9 proteolytic activity results in almost complete protection against infarct rupture. These results suggest that MMP-9 is a key regulator of infarct healing and rupture, acting via degradation of ECM early after acute MI. Indeed, BNP-Tg mice tended to die of cardiac rupture more frequently than non-Tg mice; among the dead mice (26 BNP-Tg and 9 non-Tg), 47.1% (\(n=24\)) of the BNP-Tg mice died of cardiac rupture after MI, whereas 18.6% (\(n=2\)) of non-Tg mice died of the same cause (\(P=0.75\) by \(\chi^2\) analysis). Moreover, although the effect did not reach statistical significance, doxycycline tended to attenuate cardiac rupture in BNP-Tg mice, suggesting that elevated MMP-9 activity may be involved. However, because the level of collagen and TGF-\(\beta\) expression is lower in sham-operated BNP-Tg hearts than in sham-operated non-Tg hearts (Figure 2), the apparent high frequency of cardiac rupture in BNP-Tg mice might be attributable to a reduction in collagen matrix in BNP-Tg mice. More importantly, the transient activation of MMP-9 induced by BNP may speed up infarct healing and modulate the overall late remodeling process. In fact, at 6 weeks after ligation, LV dilatation and hypertrophy of the noninfarct zone seen in the non-Tg mice are attenuated in BNP-Tg mice (our unpublished data). These observations suggest that transient MMP-9 expression induced by the elevation in BNP during the earliest phase after MI is a cardioprotective mechanism affecting late LV remodeling.

In summary, overexpression of BNP in mice led to neutrophil infiltration and MMP-9 expression in the infarcted region after MI, an effect that could lead to exaggerated degradation of ECM components. This suggests that BNP plays a novel role in the process of cardiac repair during the acute phase of MI.

Acknowledgments

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References


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