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

Rika Kawakami, MD; Yoshihiko Saito, MD, PhD; Ichiro Kishimoto, MD, PhD; Masaki Harada, MD, PhD; Koichiro Kuwahara, MD, PhD; Nobuki Takahashi, MD; Yasuaki Nakagawa, MD; Michio Nakanishi, MD; Keiji Tanimoto, MD; Satoru Usami, MD; Shinji Yasuno, MD; Hideyuki Kinoshita, MD; Hideki Chusho, MD, PhD; Naohisa Tamura, MD, PhD; Yoshihiro Ogawa, MD, PhD; Kazuwa Nakao, MD, PhD

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 effect[11,12] that could prevent LV remodeling after MI. The

Received January 4, 2004; de novo received June 8, 2004; accepted July 7, 2004.

From the Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan. Dr Saito is now at the First Department of Internal Medicine, Nara Medical University, Nara, Japan.

The online-only Data Supplement, which contains an additional figure, can be found with this article at http://www.circulationaha.org.

Correspondence to Ichiro Kishimoto, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka, 565-8565, Japan. E-mail kishimot@ri.ncvc.go.jp

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000147829.78357.C5

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.[13,14] 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,[15,16] in infarcted BNP-Tg hearts.

Methods

Experimental Animals

BNP-Tg mice were developed as previously described[13] 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.[17,18] 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.[19]

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.[17]

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).

Myeloperoxidase Activity Assay

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.[20] 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.

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.[8] The probes for collagen I, collagen III, TGF-β_1, TGF-β_3, fibronectin and BNP were already available to us.[8] 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.[21,22] The gelatinolytic zones were quantified by use of NIH 1.62 image analysis software.[22]

Type IV Collagenase Activity Assay

The activity of type IV collagenases (MMP-2 and MMP-9) was assessed by use of a commercially available kit (Yagai Research Center) according to the manufacturer's instructions.[23]

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 χ^2 test. Values of $P<0.05$ were considered significant.

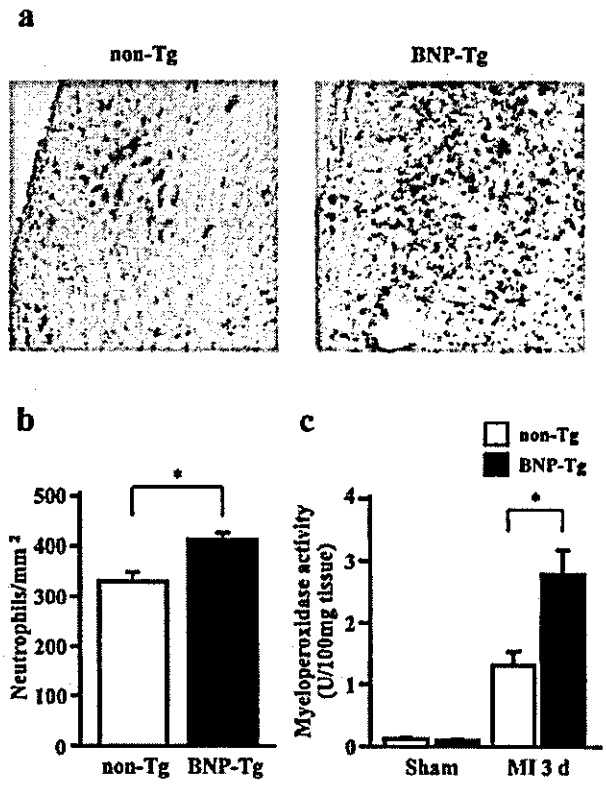

Figure 1. Accumulation of neutrophils in hearts of infarcted mice. a, Representative micrograph showing stained neutrophils within infarcted region (magnification ×200). b, Numbers of neutrophils per mm² within infarcted area 3 days after MI (n=6 for each). c, Cardiac MPO activity expressed as units/100 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.

Echocardiographic and Hemodynamic Data

	Non-Tg		BNP-Tg	
	Sham	MI	Sham	MI
Echocardiographic data				
LV EDD, mm	4.4±0.1	4.8±0.1†§	4.3±0.1	4.8±0.1*§
LV ESD, mm	3.2±0.2	3.7±0.1*§	2.9±0.1	3.6±0.1‡
FS, %	31.7±1.7	23.5±1.9*§	31.2±1.5	25.6±2.3
Wall thickness, mm				
Infarct	N/A	0.70±0.03	N/A	0.61±0.02
Noninfarct	0.55±0.03	0.73±0.01†§	0.54±0.02	0.69±0.04*‡
Hemodynamic data				
LVSP, mmHg	100±3	68±2†	82±9*	69±2†
LV EDP, mmHg	4.8±0.4	6.0±0.5	4.4±0.7	5.8±0.5
+LV dP/dt_{max}, mm Hg/s	10 936±570	6433±545†‡	9200±1119	7133±492†
−LV dP/dt_{min}, mm Hg/s	10 836±784	6416±545†	9340±1386	7116±493†

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.

*$P<0.5$, †$P<0.01$ vs sham-operated non-Tg mice.

‡$P<0.05$, §$P<0.01$ vs sham-operated BNP-Tg mice.

Results

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 (Table), which is consistent with our earlier observation that systolic blood pressure measured by use of the tail-cuff method was ≈20 mm Hg lower in BNP-Tg than non-Tg mice.[13] Conversely, there was no significant difference in LV systolic pressure, LV end-diastolic pressure, LV $+dP/dt_{max}$, or $-dP/dt_{min}$ between the 2 groups after ligation. We did, however, note a trend toward improved hemodynamic and echocardiographic parameters in BNP-Tg mice, although it did not reach statistical significance.

Infarct Infiltration by Neutrophils

Accumulation of leukocytes in the infarcted region is thought to be one step in the process of wound repair.[16,24] 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 infarcted 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

Recent evidence highlights the involvement of the plasminogen activator–metalloproteinase system in myocardial neutrophil accumulation, the repair processes, and the rupture seen after MI.[15,16] 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 2, a 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-β_1, TGF-β_3, collagen I, and collagen III, which are known to be involved in cardiac fibroblast proliferation and the biosynthesis of ECM proteins.[25–27] We found that their expression was similarly upregulated in the infarcted regions of both BNP-Tg and non-Tg hearts (Figure 2, a and b), indicating that overexpression of BNP does not affect the biosynthesis of collagen during the early phase of acute MI.

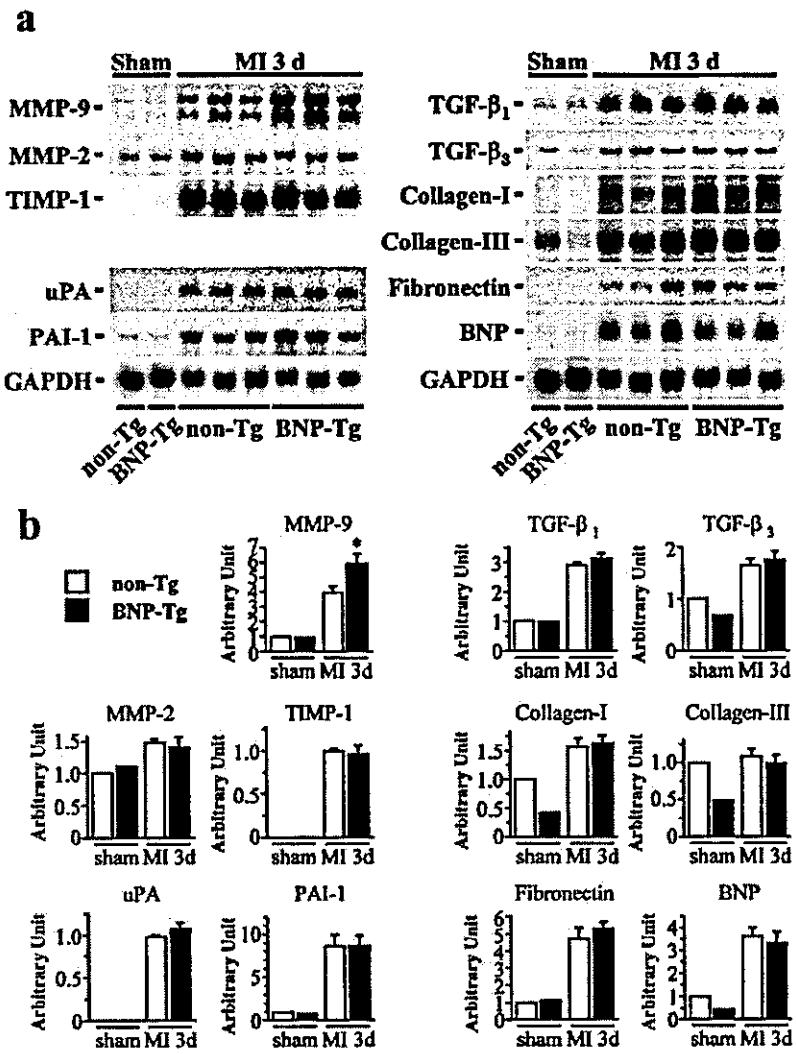

Figure 2. a, Representative autoradiograms showing Northern blot analysis in hearts harvested from sham-operated and infarcted mice 3 days after MI. b, Summary of results obtained from Northern blot analyses; mRNA levels in sham-operated non-Tg hearts were assigned a value of 1.0. Values are mean±SEM; *$P<0.05$ vs non-Tg mice with MI.

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

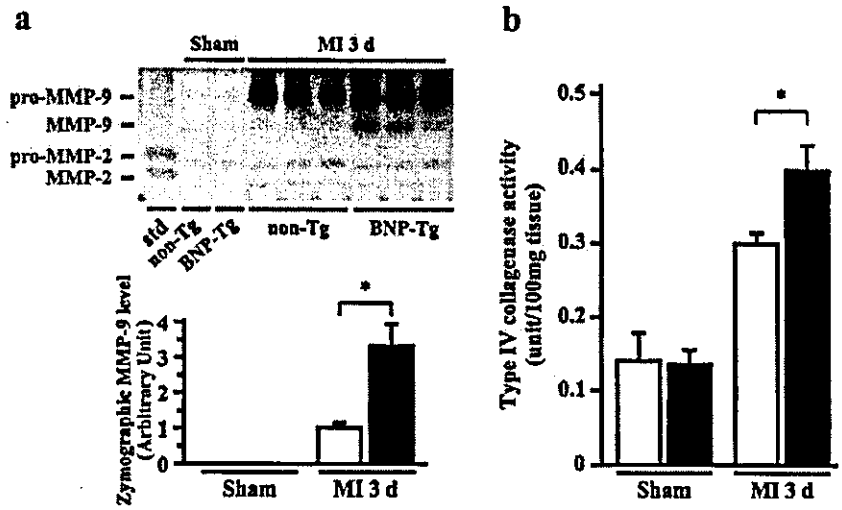

Figure 3. a, Top, Representative gelatin zymography performed 3 days after MI (n=6 for each); a mixture of human MMP-2 and pro-MMP-9 served as a standard (std). Bottom, densitometric analysis of MMP-9 abundance. b, Cardiac type IV collagenase activity expressed as units/100 mg tissue wet weight 3 days after MI (n=7 for each). Values are mean±SEM; *$P<0.05$ vs non-Tg mice with MI.

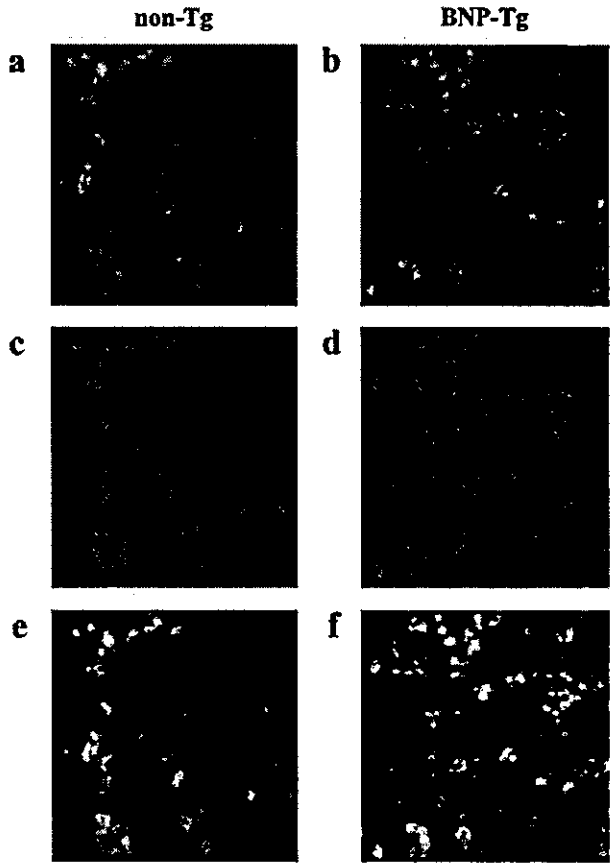

Figure 4. Confocal images of double immunostaining for MMP-9 and neutrophil 7/4. Immunostaining and cellular localization of MMP-9 within infarcted hearts of BNP-Tg (right) and non-Tg (left) mice 3 days after MI. Images show single immunostaining for MMP-9 (green) (a and b) and neutrophil 7/4 (red) (c and d). Double immunostaining (yellow) shows colocalization of MMP-9 within 7/4-stained neutrophils in infarcted region after MI (e and f). Magnification ×400.

(BNP-Tg, 0.399±0.037 U/100 mg wet wt versus non-Tg, 0.300±0.017 U/100 mg wet wt; n=7, $P<0.05$; Figure 3b). Because MMP-9 and -2 are the 2 major collagenases that degrade type IV collagen[23] 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/mm^2 versus doxycycline-treated BNP-Tg, 432.93±23.86 cells/mm^2; $P=0.90$, 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.

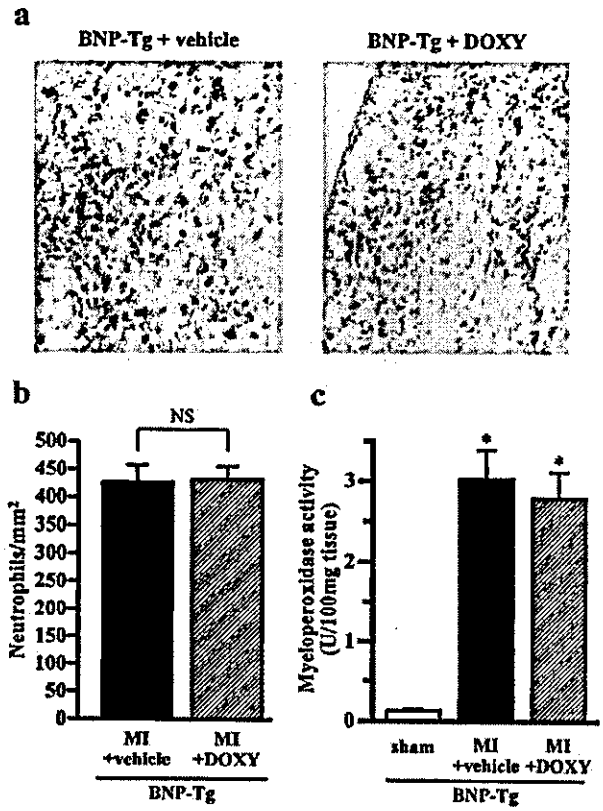

Figure 5. Accumulation of neutrophils within infarcted regions of control BNP-Tg and doxycycline-treated BNP-Tg mice. a, Representative micrograph showing stained neutrophils within infarct area (magnification ×200). b, Numbers of neutrophils per mm^2 within infarcted area 3 days after MI (n=6 for each). Values are shown as mean±SEM; NS, not significant. c, Cardiac MPO activity expressed as units/100 mg tissue wet weight in sham-operated (n=3 for each), untreated infarcted (n=18), and doxycycline-treated infarcted BNP-Tg mice (n=17). Values are mean±SEM; *$P<0.01$ vs sham values.

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.[5] 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.[28] 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-β_1, TGF-β_3, 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 4×10^{-9} to 10^{-7} mol/L.[32] The plasma BNP concentration in BNP-Tg mice was approximately 3×10^{-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 χ^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-β 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 noninfarcted 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

This work was supported in part by research grants from the Japanese Ministry of Education, Science, and Culture; the Japanese Ministry of Health and Welfare; and the Japanese Society for the Promotion of Science Research for the Future program (JSPS-RFTF96I00204 and JSPS-RFTF98L00801). Excellent secretarial work by K. Okamura is also acknowledged.

References

1. De Bold AJ. Atrial natriuretic factor: a hormone produced by the heart. *Science*. 1985;230:767–770.

2. Rosenzweig A, Seidman CE. Atrial natriuretic factor and related peptide hormones. *Annu Rev Biochem.* 1991;60:229–255.
3. Mukoyama M, Nakao K, Hosoda K, et al. Brain natriuretic peptide as a novel cardiac hormone in humans: evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. *J Clin Invest.* 1991;87:1402–1412.
4. Mukoyama M, Nakao K, Saito Y, et al. Increased human brain natriuretic peptide in congestive heart failure. *N Engl J Med.* 1990;323:757–758.
5. Morita E, Yasue H, Yoshimura M, et al. Increased plasma levels of brain natriuretic peptide in patients with acute myocardial infarction. *Circulation.* 1993;88:82–91.
6. Yamamoto K, Burnett JC Jr, Jougasaki M, et al. Superiority of brain natriuretic peptide as a hormonal marker of ventricular systolic and diastolic dysfunction and ventricular hypertrophy. *Hypertension.* 1996; 28:988–994.
7. Nagaya N, Goto Y, Nishikimi T, et al. Sustained elevation of plasma brain natriuretic peptide levels associated with progressive ventricular remodelling after acute myocardial infarction. *Clin Sci.* 1999;96: 129–136.
8. Tamura N, Ogawa Y, Chusho H, et al. Cardiac fibrosis in mice lacking brain natriuretic peptide. *Proc Natl Acad Sci U S A.* 2000;97:4239–4244.
9. Mills RM, LeJemtel TH, Horton DP, et al. Sustained hemodynamic effects of an infusion of nesiritide (human b-type natriuretic peptide) in heart failure: a randomized, double-blind, placebo-controlled clinical trial. Natrecor Study Group. *J Am Coll Cardiol.* 1999;34:155–162.
10. Colucci WS, Elkayam U, Horton DP, et al. Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group. *N Engl J Med.* 2000;343:246–253.
11. Holtwick R, van Eickels M, Skryabin BV, et al. Pressure-independent cardiac hypertrophy in mice with cardiomyocyte-restricted inactivation of the atrial natriuretic peptide receptor guanylyl cyclase-A. *J Clin Invest.* 2003;111:1399–1407.
12. Molkentin J. A friend within the heart: natriuretic peptide receptor signaling. *J Clin Invest.* 2003;111:1275–1277.
13. Ogawa Y, Itoh H, Tamura N, et al. Molecular cloning of the complementary DNA and gene that encode mouse brain natriuretic peptide and generation of transgenic mice that overexpress the brain natriuretic peptide gene. *J Clin Invest.* 1994;93:1911–1921.
14. Chusho H, Ogawa Y, Tamura N, et al. Genetic models reveal that brain natriuretic peptide can signal through different tissue-specific receptor-mediated pathways. *Endocrinology.* 2000;141:3807–3813.
15. Creemers E, Cleutjens J, Smits J, et al. Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. *Am J Pathol.* 2000;156:1865–1873.
16. Heymans S, Luttun A, Nuyens D, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med.* 1999;5: 1135–1142.
17. Michael LH, Entman ML, Hartley CJ, et al. Myocardial ischemia and reperfusion: a murine model. *Am J Physiol.* 1995;269:H2147–H2154.
18. Patten RD, Aronovitz MJ, Deras-Mejia L, et al. Ventricular remodeling in a mouse model of myocardial infarction. *Am J Physiol.* 1998;274: H1812–H1820.
19. Ichihara S, Senbonmatsu T, Price E Jr, et al. Targeted deletion of angiotensin II type 2 receptor caused cardiac rupture after acute myocardial infarction. *Circulation.* 2002;106:2244–2249.
20. Izumi T, Saito Y, Kishimoto I, et al. Blockade of the natriuretic peptide receptor guanylyl cyclase-A inhibits NF-kappaB activation and alleviates myocardial ischemia/reperfusion injury. *J Clin Invest.* 2001;108: 203–213.
21. Silvestre JS, Mallat Z, Tamarat R, et al. Regulation of matrix metalloproteinase activity in ischemic tissue by interleukin-10: role in ischemia-induced angiogenesis. *Circ Res.* 2001;89:259–264.
22. Cho A, Reidy MA. Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury. *Circ Res.* 2002;91:845–851.
23. Fujimura M, Gasche Y, Morita-Fujimura Y, et al. Early appearance of activated matrix metalloproteinase-9 and blood-brain barrier disruption in mice after focal cerebral ischemia and reperfusion. *Brain Res.* 1999;842: 92–100.
24. Jugdutt BI. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? *Circulation.* 2003;108: 1395–1403.
25. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium: fibrosis and renin- angiotensin-aldosterone system. *Circulation.* 1991;83: 1849–1865.
26. Butt RP, Laurent GJ, Bishop JE. Mechanical load and polypeptide growth factors stimulate cardiac fibroblast activity. *Ann N Y Acad Sci.* 1995;752: 387–393.
27. Cleutjens JP, Verluyten MJ, Smiths JF, et al. Collagen remodeling after myocardial infarction in the rat heart. *Am J Pathol.* 1995;147:325–338.
28. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med.* 1999; 341:738–746.
29. Etoh T, Joffs C, Deschamps A, et al. Myocardial and interstitial matrix metalloproteinase activity after acute myocardial infarction in pigs. *Am J Physiol.* 2001;281:H987–H994.
30. Tao ZY, Cavasin MA, Yang F, et al. Temporal changes in matrix metalloproteinase expression and inflammatory response associated with cardiac rupture after myocardial infarction in mice. *Life Sci.* 2004;74: 1561–1572.
31. Golub LM, Lee HM, Ryan ME, et al. Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. *Adv Dent Res.* 1998;12:12–26.
32. Elferink JG, De Koster BM. Atrial natriuretic factor stimulates migration by human neutrophils. *Eur J Pharmacol.* 1995;288:335–340.

Clin Exp Immunol 2004; 137:430–436 doi:10.1111/j.1365-2249.2004.02534.x

Osteoprotegerin (OPG) acts as an endogenous decoy receptor in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells

T. MIYASHITA*, A. KAWAKAMI*, T. NAKASHIMA†, S. YAMASAKI*, M. TAMAI*, F. TANAKA*, M. KAMACHI*, H. IDA*, K. MIGITA*, T. ORIGUCHI‡, K. NAKAO§ & K. EGUCHI* *The First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan, †Department of Hospital Pharmacy, Nagasaki University School of Medicine, Nagasaki, Japan, ‡Department of Physical Therapy, Nagasaki University School of Health Sciences, Nagasaki, Japan, and §Health Research Center, Nagasaki University, Nagasaki, Japan

(Accepted for publication 10 May 2004)

SUMMARY

We examined the role of osteoprotegerin (OPG) on tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in rheumatoid fibroblast-like synovial cells (FLS). OPG protein concentrations in synovial fluid from patients with rheumatoid arthritis (RA) correlated with those of interleukin (IL)-1β or IL-6. A similar correlation was present between IL-1β and IL-6 concentrations. Rheumatoid FLS *in vitro* expressed both death domain-containing receptors [death receptor 4 (DR4) and DR5] and decoy receptors [decoy receptor 1 (DcR1) and DcR2]. DR4 expression on FLS was weak compared with the expression of DR5, DcR1 and DcR2. Recombinant TRAIL (rTRAIL) rapidly induced apoptosis of FLS. DR5 as well as DR4 were functional with regard to TRAIL-mediated apoptosis induction in FLS; however, DR5 appeared be more efficient than DR4. In addition to soluble DR5 (sDR5) and sDR4, OPG administration significantly inhibited TRAIL-induced apoptogenic activity. OPG was identified in the culture supernatants of FLS, and its concentration increased significantly by the addition of IL-1β in a time-dependent manner. Neither IL-6 nor tumour necrosis factor (TNF)-α increased the production of OPG from FLS. TRAIL-induced apoptogenic activity towards FLS was reduced when rTRAIL was added without exchanging the culture media, and this was particularly noticeable in the IL-1β-stimulated FLS culture; however, the sensitivity of FLS to TRAIL-induced apoptosis itself was not changed by IL-1β. Interestingly, neutralization of endogenous OPG by adding anti-OPG monoclonal antibody (MoAb) to FLS culture restored TRAIL-mediated apoptosis. Our data demonstrate that OPG is an endogenous decoy receptor for TRAIL-induced apoptosis of FLS. In addition, IL-1β seems to promote the growth of rheumatoid synovial tissues through stimulation of OPG production, which interferes with TRAIL death signals in a competitive manner.

Keywords fibroblast-like synovial cells IL-1β OPG rheumatoid arthritis TRAIL

INTRODUCTION

The soluble receptor, osteoprotegerin (OPG), is a member of the tumour necrosis factor receptor (TNFR) superfamily and acts as a receptor antagonist. The decoy function of OPG towards receptor activator of nuclear factor κB ligand (RANKL) is well recognized; OPG binds RANKL, and thus prevents the interaction with, and stimulation of, RANK [1–3]. Hence, OPG inhibits osteoclast differentiation and survival as demonstrated both *in vivo* and *in vitro* [4]. OPG is also thought to be involved in inflammatory diseases based on results of experimental studies demonstrating stimulation of OPG production from endothelial cells by tumour necrosis factor (TNF)-α and interleukin (IL)-1β [5]. Furthermore, OPG has also been shown to exhibit mitogenic and/or anti-apoptotic properties for foreskin fibroblasts and/or endothelial cells [6,7].

Marked hyperplasia of synovial tissues is a characteristic feature of rheumatoid arthritis (RA), which is mediated, at least in part, by impaired apoptosis of synovial cells *in situ* [8]. The anti-apoptotic feature of rheumatoid synovial cells *in situ* may not be intrinsic, but rather develop in the inflammatory rheumatoid microenvironment. In this regard, we have demonstrated that the sensitivity of fibroblast-like synovial cells (FLS) to apoptogenic

Correspondence: Katsumi Eguchi MD, The First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852–8501, Japan.
E-mail: eguchi@net.nagasaki-u.ac.jp

stimuli is modulated by various inflammatory cytokines [9–11]. OPG acts also as a receptor antagonist for TNF-related apoptosis-inducing ligand (TRAIL) [12]. We have shown recently that FLS are sensitive toward TRAIL-mediated apoptosis [13]. TRAIL triggers the activation of caspase-8 in FLS, which induces mitochondrial perturbation [13]. As both mitochondrial perturbation and DNA fragmentation were completely inhibited by caspase-8 inhibitor, FLS are classed into type II cell death in response to TRAIL [13]. The regulatory mechanisms of inflammatory cytokines on OPG production may influence TRAIL-induced apoptosis of synovial cells, and thus defines a new functional role for OPG in the pathological process of RA.

In the present study, we demonstrate that IL-1β in rheumatoid synovial fluid is the major inflammatory cytokine responsible for stimulation of OPG synthesis from FLS. Furthermore, our data suggest that OPG produced by FLS is an endogenous receptor antagonist of TRAIL-induced apoptosis in FLS which may, in part, explain the growth-promoting activity of IL-1β for rheumatoid synovial tissues.

MATERIALS AND METHODS

Determination of OPG, IL-1β, IL-6 and TNF-α concentrations in synovial fluid from patients with RA

Synovial fluid samples were obtained from patients with RA, who fulfilled the criteria of the American Rheumatism Association for RA [14], admitted to the National Ureshino Hospital (28 synovial fluid samples from 28 RA patients). The experimental protocol was approved by the Hospital Human Ethics Review Committee and signed consent was obtained from each patient. The concentrations of OPG, IL-1β, IL-6 and TNF-α in the rheumatoid synovial fluid were examined by a sandwich enzyme-linked immunosorbent assay (ELISA) (OPG; Immunodiagnostik AC, Bensheim, Germany: IL-1β, IL-6 and TNF-α; Otsuka Pharmaceutical Co., Tokushima, Japan). In brief, the plates precoated with monoclonal antibody (MoAb) against OPG, IL-1β, IL-6 or TNF-α were incubated with the samples, incubated further with the secondary antibody, and colour was developed according to the protocol provided by the supplier.

Effect of inflammatory cytokines on OPG production from cultured FLS

FLS were isolated from 20 patients with RA at the time of orthopaedic surgery (total knee replacement) conducted at National Ureshino Hospital. Signed consent was also obtained from each patient. Briefly, the synovial tissues were trimmed of fat and minced with scissors, then added to a mixture of collagenase (Sigma Chemical Co., St Louis, MO, USA) and dispase (Godo Shusei Co., Tokyo). The tissue mixture was digested over a 45-min period during gentle stirring at 37°C, and the harvested cells were allowed to adhere to Petri dishes (Falcon 3003, Becton Dickinson Co., Oxnard, CA, USA). The adherent cells used in the present study at third to fifth passages were less than 1% reactive with various MoAbs, including CD3, CD68, CD20 and von-Willebrand factor, which define FLS. Isolated FLS were cultured in the presence or absence of recombinant IL-1β (rIL-1β, Otsuka Pharmaceutical Co., 20 IU/ml), rIL-6 plus r-soluble IL-6 receptor (sIL-6R) (R&D Systems Inc., Minneapolis, MN, USA, 100 ng/ml each) or rTNF-α (R&D Systems, 200 IU/ml) for indicated time intervals in RPMI-1640 containing 10% fetal calf serum (FCS) (1×10^5/35 mm dish). OPG protein concentration in the culture supernatants was examined by the sandwich ELISA as described above.

Expression of TRAIL receptors on cultured FLS

Expression of TRAIL receptors on cultured rheumatoid FLS was examined by flow cytometric analysis. Treated FLS were detached by addition of 0·265 mM EDTA, washed with phosphate buffered saline (PBS) and incubated with antihuman death receptor 4 (DR4; R&D Systems), antihuman DR5 (R&D Systems), antihuman decoy receptor 1 (DcR1; R&D Systems) or antihuman DcR2 (R&D Systems) at 4°C for 30 min. After incubation, the cells were washed with PBS, incubated further with phycoerythrin (PE)-conjugated antigout IgG (Sigma Chemical Co.), and the expression of DR4, DR5, DcR1 and DcR2 was examined by flowcytometer (Epics XL, Beckman Coulter, Hialeah, FL, USA).

Effect of OPG toward TRAIL-induced apoptosis of cultured FLS

We have shown recently that cultured FLS are committed to type II cell death in response to TRAIL [13], thus disruption of

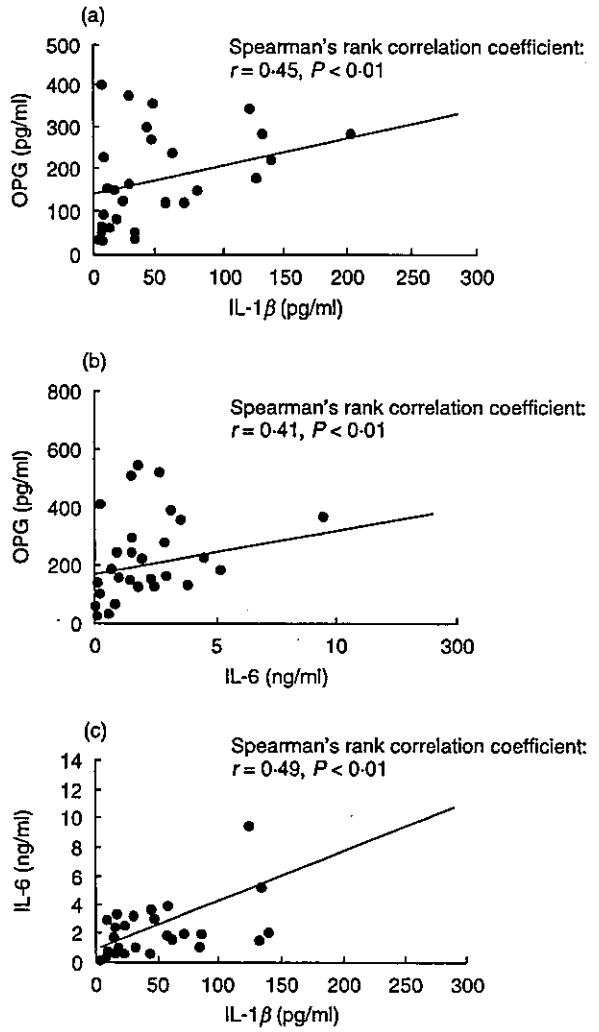

Fig. 1. Positive correlation of protein concentration between OPG and IL-1β (a), OPG and IL-6 (b) and IL-1β and IL-6 (c) found in synovial fluid samples (total 27 synovial fluids examined) of RA patients.

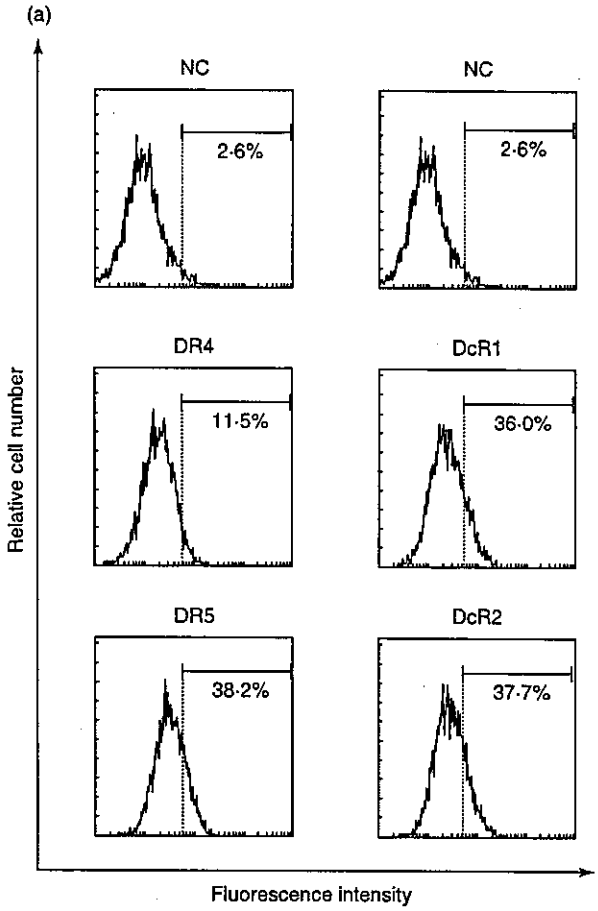

Fig. 2. TRAIL-mediated apoptosis in FLS by rTRAIL. (a) Expression of TRAIL receptors on FLS. Rheumatoid FLS were detached by adding EDTA, reacted with anti-DR4, anti-DR5, anti-DcR1 or anti-DcR2 at 4°C for 30 min, and incubated further with PE-conjugated antigout IgG at 4°C for 30 min. After incubation, surface expression of DR4, DR5, DcR1 and DcR2 on FLS was examined by flow cytometer as described in the text. Note that FLS expressed DR4, DR5, DcR1 and DcR2; however, DR4 expression was weak compared with the other three receptors. NC; negative control, stained with goat IgG instead of first antibody. Numbers are the percentage of positive cells. Results are representative data from five individual samples. (b) Quantification of TRAIL-mediated apoptosis in FLS by $\Delta\Psi m$. FLS were incubated with varying concentrations of rTRAIL for 2 h, detached by adding 0·265 mM EDTA, and $\Delta\Psi m$ was quantified as described in the text. Note that rTRAIL induced $\Delta\Psi m$ in FLS in a dose-dependent manner. Numbers are the percentage of positive cells. Results are representative data from five individual samples.

mitochondrial transmembrane potential ($\Delta\Psi m$) was used to quantify TRAIL-mediated FLS apoptosis in the present study. FLS were cultured in the presence or absence of various cytokines for indicated hours, washed, and incubated further with varying concentrations of rTRAIL (R&D Systems) with or without rOPG (R&D Systems), soluble DR4 (sDR4; R&D Systems) or sDR5 (R&D Systems) for an additional 2 h. After incubation, $\Delta\Psi m$ was examined as described recently [13]. In brief, treated FLS were detached by adding 0·265 mM EDTA, washed, and incubated further with a saturating amount of DiOC6 (3,3′-dihexyloxacarbocyamine iodide, Fluoreszenztechnologie, Grottenhofstr, Austria) at 37°C for 15 min. After incubation, the percentage of $\Delta\Psi m$ in FLS was quantified by flow cytometer (Epics XL, Beckman Coulter).In some experiments, rTRAIL was added to FLS culture without exchanging the culture media, and TRAIL-induced apoptosis in FLS was also examined by $\Delta\Psi m$. To neutralize endogenous OPG secreted from FLS in culture, anti-OPG MoAb (mouse IgG1; R&D Systems) was added, and TRAIL-mediated apoptosis in FLS was examined by $\Delta\Psi m$.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test or Spearman's rank correlation analysis. *P*-values <0·05 were selected as the level of significance.

RESULTS

Determination of OPG protein in synovial fluid of patients with RA

First, we examined whether OPG protein was present in the rheumatoid synovial fluid. As reported recently [15,16], OPG protein was detected in all samples examined, although the level varied from one sample to another (Fig. 1a). Furthermore, there was a positive correlation between the concentrations of OPG and IL-1β. In addition, a similar correlation was demonstrated between the protein concentration of OPG and IL-6, and between that of IL-1β and IL-6 (Fig. 1b,c). In contrast, TNF-α was detected in only a proportion of the samples, and the levels of this cytokine did not correlate with OPG concentration (data not shown).

TRAIL induces apoptosis in FLS through DR4 and DR5

As shown in Fig. 2a, rheumatoid FLS *in vitro* expressed DR4, DR5, DcR1 and DcR2. Expression of DR4 was not so obvious compared with DR5, DcR1 and DcR2. Figure 2B shows that rTRAIL induced apoptotic cell death in FLS in a dose-dependent manner. To clarify the functional role of DR4 and DR5 in TRAIL-mediated apoptosis in FLS, we performed the blocking experiments by the use of sDR4 and sDR5. sDR4 interferes with the interaction between TRAIL and DR4, and sDR5 interferes with that between TRAIL and DR5. As shown in Fig. 3, TRAIL-induced $\Delta\Psi$m in FLS was inhibited partially by sDR4 or sDR5, whereas it was inhibited completely by adding both sDR4 and sDR5. DR5 on FLS was suggested to be more functional to induce TRAIL-mediated apoptosis compared with DR4 (Fig. 3). In addition, OPG administration significantly suppressed TRAIL-mediated apoptosis in FLS (Fig. 3).

IL-1β-stimulated production of OPG by cultured FLS, which interferes with TRAIL-induced apoptogenic activity toward FLS

We examined the effects of inflammatory cytokines on OPG production by FLS. As shown in Fig. 4, OPG protein concentration increased time-dependently in the culture supernatants of FLS. Furthermore, such production was augmented by IL-1β at all time-points examined. Compared with IL-1β, the stimulatory effect of IL-6 (IL-6 + sIL-6 receptor) and TNF-α on OPG production from FLS was not found (data not shown). It was interesting to note that the rate of FLS apoptosis in response to TRAIL was decreased when rTRAIL was added without replacing the culture media (Fig. 5). Inhibition was more prominent in IL-1β-stimulated FLS compared with untreated FLS (Fig. 5), which was mainly restored by adding anti-OPG MoAb in culture media (Fig. 5). IL-1β treatment itself modulated neither TRAIL receptor expression (Fig. 6) nor TRAIL-mediated apoptosis (Fig. 5). These data suggest that OPG produced from FLS into the culture media is an endogenous receptor antagonist towards TRAIL-induced apoptosis of FLS.

DISCUSSION

Apoptosis occurs in a variety of physiological situations such as embryogenesis, and plays a crucial role in normal tissue homeostasis. However, a breakdown in the delicate balance between cell survival and apoptosis has been implicated in the pathogenesis of a number of rheumatic diseases, including RA [8]. The mechanisms responsible for synovial hyperplasia of RA patients may be explained by reduced synovial cell apoptosis, which cannot counteract the ongoing process of synovial cell proliferation.

TRAIL can interact potentially with five different receptors: two functional receptors DR4 and DR5, two decoy receptors DcR1 and DcR2, and a soluble decoy receptor OPG [12,17,18]. Other investigators have shown that DR5 is a sole death domain containing receptor for TRAIL on FLS [19]. We have shown here that DR5 was not sole but a prominent death domain-containing receptor for TRAIL. A previous study has shown that DR5 has the highest binding affinity towards TRAIL at 37°C [20];

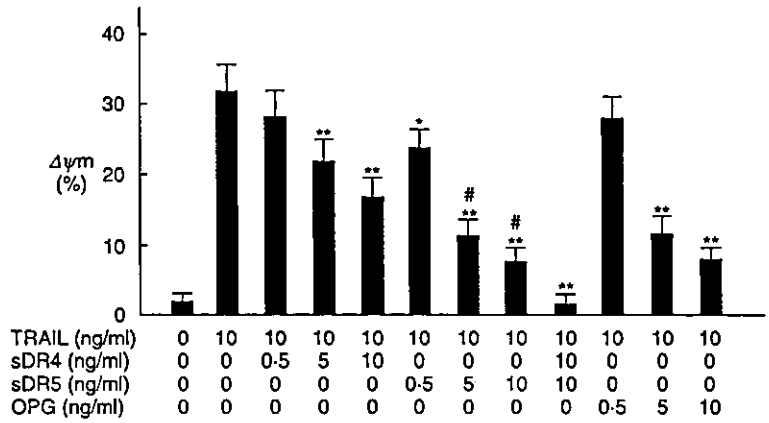

Fig. 3. Inhibition of TRAIL-mediated apoptosis in FLS by sDR4, sDR5 and OPG. FLS were cultured with 10 ng/ml of rTRAIL in the presence of varying concentrations of sDR4, SDR5 or OPG for 2 h. After incubation, apoptosis of FLS was quantified by $\Delta\Psi$m as described in the text. Note that TRAIL-mediated apoptosis in FLS was suppressed by sDR4, sDR5 and OPG; however, the inhibition was not complete. TRAIL-mediated apoptosis in FLS was inhibited completely by administration of both sDR4 and sDR5. $^*P < 0{\cdot}05$ *versus* FLS cultured with rTRAIL. $^{**}P < 0{\cdot}01$ *versus* FLS treated with rTRAIL. $^{\#}P < 0{\cdot}05$ *versus* FLS treated with rTRAIL and sDR4. Data are the mean ± s.d. of four individual samples.

however, detailed experiments such as RNA interference for each TRAIL receptor are necessary to clarify the difference.

Recent studies reported the production of OPG in culture supernatants from rheumatoid FLS [16]. Thus, we focused on the regulatory role of inflammatory cytokines on OPG production by rheumatoid FLS. Our data suggest that OPG production in the rheumatoid microenviroment is, at least in part, positively regulated by IL-1β, which is consistent with recent observations by Ziokowska *et al.* [16]. The positive correlation between IL-6 and OPG protein concentration found in the rheumatoid synovial fluid may result from the inducible effect of IL-1β on the production of IL-6 [21], as the effect of IL-6 on OPG production by synovial cells was not found compared with IL-1β. A promising inhibitory role of OPG on osteoclastogenesis in murine adjuvant arthritis has been identified, which is achieved through decoy function of OPG for RANKL; however, treatment of the mice with OPG failed to improve the severity of synovial inflammation [22]. The present study has demonstrated that OPG is a functional inhibitor of TRAIL-induced apoptosis in FLS. Therefore, we speculate that the functional role of OPG on synovial cell growth is separate from its inhibitory action on osteoclastogenesis; the former is mediated through decoy function towards TRAIL while the latter through decoy function towards RANKL. Expression of TRAIL receptors on FLS was not affected by IL-1β treatment, and the sensitivity of TRAIL-mediated apoptosis was not suppressed directly by IL-1β; however, TRAIL-mediated apoptogenic activity towards FLS was inhibited significantly in IL-1β-stimulated FLS culture. The production of OPG from cultured FLS was augmented by IL-1β, thus IL-1β may inhibit primarily TRAIL-induced apoptosis of FLS at death receptor level by OPG in a competitive manner. This is consistent with data from the blocking experiment, that neutralization of OPG produced from FLS by anti-OPG MoAb mainly restored TRAIL-mediated apoptogenic activity towards FLS. Although OPG protein concentration in culture supernatants from IL-1β-treated synovial cells was comparable to that inhibiting TRAIL-induced FLS apoptosis (compare Figs 3 and 4), the concentration was clearly higher than that in the synovial fluid of RA patients (compare Fig. 1 with Figs 3 and 4), which could not suppress TRAIL-

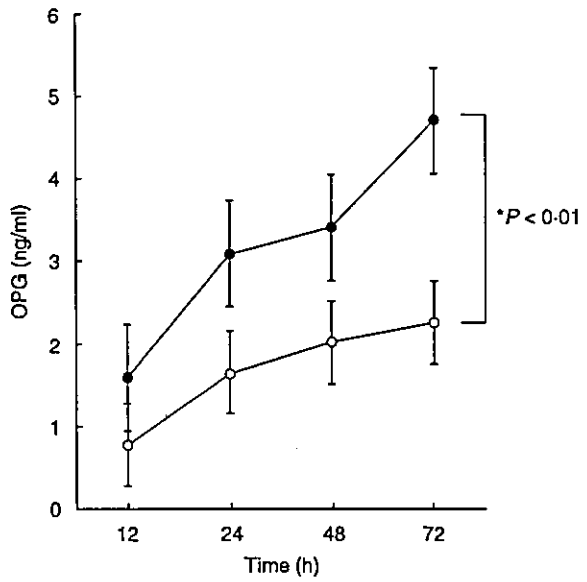

Fig. 4. IL-1β markedly stimulates OPG accumulation in culture supernatants of FLS. Rheumatoid FLS were cultured in the presence or absence of 20 IU/ml of IL-1β for indicated duration. After cultivation, OPG protein concentration in the culture supernatants was examined as described in the text. Open circles: unstimulated FLS, closed circles: IL-1β-treated FLS. *$P < 0{\cdot}01$ *versus* unstimulated FLS. Data are mean ± s.d. of seven individual samples.

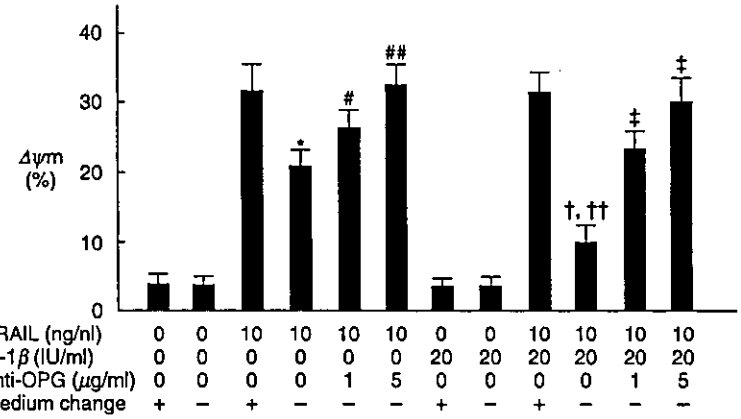

Fig. 5. OPG produced from FLS inhibits TRAIL-mediated apoptosis in FLS. FLS were cultured in the presence or absence of 20 IU/ml of IL-1β for 72 h with or without adding anti-OPG MoAb (1 μg/ml or 5 μg/ml). After cultivation, 10 ng/ml of rTRAIL was added to the culture with or without exchanging the culture media for an additional 2 h. After incubation, apoptosis of FLS was quantified by $\Delta\Psi$m as described in the text. Note that the sensitivity of FLS to TRAIL-mediated apoptosis was reduced when rTRAIL was added without exchanging the culture media, which was found significantly in IL-1β-treated FLS compared with unstimulated FLS. Furthermore, that inhibition was mainly restored by adding anti-OPG MoAb. Anti-OPG MoAb was mouse IgG1, thus 5 μg/ml of control mouse IgG1 (MBL) was used for negative control (0 μg/ml of anti-OPG MoAb means the addition of 5 μg/ml of control mouse IgG1). *$P < 0{\cdot}01$ *versus* unstimulated FLS treated with rTRAIL with exchanging culture media. #$P < 0{\cdot}05$. ##$P < 0{\cdot}01$ *versus* unstimulated FLS treated with rTRAIL without exchanging culture media. †$P < 0{\cdot}01$ *versus* IL-1β-stimulated FLS treated with rTRAIL with exchanging culture media. ††$P < 0{\cdot}05$ *versus* unstimulated FLS treated with rTRAIL without exchanging culture media. ‡$P < 0{\cdot}01$ *versus* IL-1β-stimulated FLS treated with rTRAIL without exchanging culture media. Data are the mean ± s.d. of four experiments.

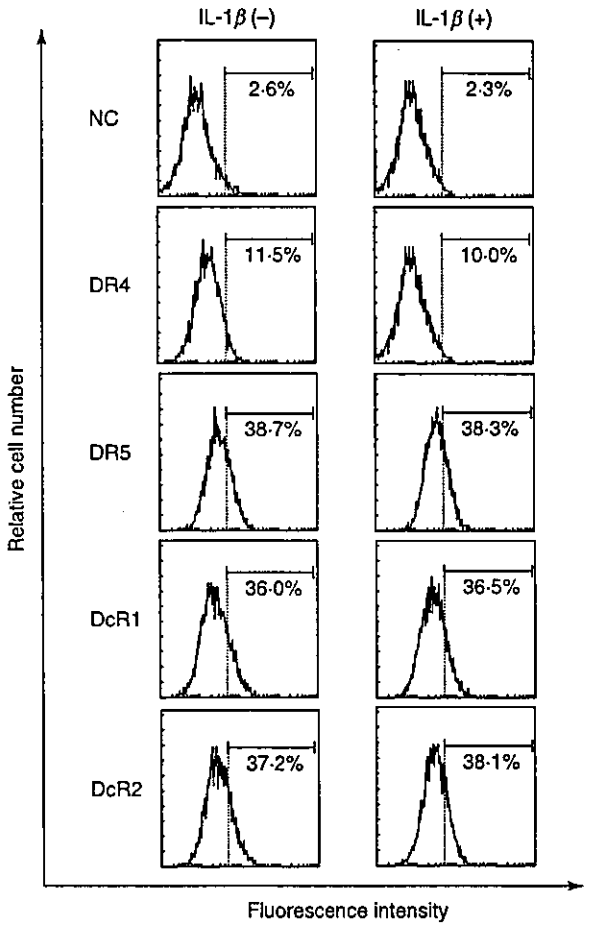

Fig. 6. IL-1β does not modulate the expression of DR4, DR5, DcR1 and DcR2 on FLS. Rheumatoid FLS, cultured in the presence or absence of rIL-1β (20 IU/ml) for 72 h, were detached by adding 0·265 mM EDTA and reacted with anti-DR4, anti-DR5, anti-DcR1 or anti-DcR2 at 4°C for 30 min. The cells were incubated further with PE-conjugated antigoat IgG at 4°C for 30 min, and surface expression of DR4, DR5, DcR1 and DcR2 on FLS was examined by flow cytometer as described in the text. Note that the expression of DR4, DR5, DcR1 and DcR2 on FLS was not changed by IL-1β treatment. NC: negative control, stained with goat IgG instead of first antibody. Numbers are the percentage of positive cells. Results are representative data from five individual samples.

induced apoptosis in FLS (see Figs 1 and 3). OPG protein concentration in rheumatoid synovial fluid reported by other groups [15,16,23] was much higher than the present data. Factors responsible for the difference are unclear; however, the variation in clinical situations of the subjects and MoAb used might result in the difference. Recent investigations have shown the elevation of OPG in rheumatoid synovial fluid, compared with serum consentration [15]; however, other investigators demonstrated a relatively low OPG protein concentration in the rheumatoid synovial fluid compared with synovial fluid from osteoarthritis patients [23]. Further investigation is necessary to clarify the functional expression of OPG in synovial tissue of RA patients.

TRAIL is thought to be an inhibitor of synovial cell hyperplasia as the blockage of TRAIL signalling by sDR5 in type II collagen-induced arthritis in mice exacerbates the proliferation of synovial cells [24]. Although we have not examined the expression of other soluble receptor antagonists such as sDR4 and sDR5 in the culture supernatants of FLS, our present study indicates that OPG produced by synovial cells acts as an endogenous decoy receptor of TRAIL-induced apoptosis, and part of the growth-promoting activity of IL-1β may be achieved by overproduction of OPG to suppress the biological function of TRAIL.

ACKNOWLEDGEMENT

This study was supported in part by grant-in-aid 13557042/13670461 from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1 Simonet WS, Lacey DL, Dunstan CR *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997; 89:309–19.
2 Tsuda E, Goto M, Mochizuki S *et al.* Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun 1997; 234:137–42.
3 Yasuda H, Shima N, Nakagawa N *et al.* Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. Endocrinology 1998; 139:1329–37.
4 Tomoyasu A, Goto M, Fujise N *et al.* Characterization of monomeric and homodimeric forms of osteoclastogenesis inhibitory factor. Biochem Biophys Res Commun 1998; 245:382–7.
5 Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-κB and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 2001; 276:20659–72.
6 Kwon BS, Wang S, Udagawa N *et al.* TR1, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferation and inhibits osteoclastogenesis and bone resorption. FASEB J 1998; 12:845–54.
7 Malyankar UM, Scatena M, Suchland KL *et al.* Osteoprotegerin is an $\alpha_v\beta_3$-induced, NF-$\acute{e}$B-dependent survival factor for endothelial cells. J Biol Chem 2000; 275:20959–62.
8 Vaishnaw AK, McNally JD, Elkon KB. Apoptosis in the rheumatic diseases. Arthritis Rheum 1997; 40:1917–27.
9 Kawakami A, Eguchi K, Matsuoka N *et al.* Inhibition of Fas antigen-mediated apoptosis of rheumatoid synovial cells *in vitro* by transforming growth factor beta 1. Arthritis Rheum 1996; 39:1267–76.
10 Tsuboi M, Eguchi K, Kawakami A *et al.* Fas antigen expression on synovial cells was down-regulated by interleukin 1 beta. Biochem Biophys Res Commun 1996; 218:280–5.
11 Kawakami A, Nakashima T, Yamasaki S *et al.* Regulation of synovial cell apoptosis by proteasome inhibitor. Arthritis Rheum 1999; 42:2440–8.
12 Emery JG, McDonnell P, Burke MB *et al.* Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem 1998; 273:14363–7.
13 Miyashita T, Kawakami A, Tamai M *et al.* Akt is an endogenous inhibitor toward tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis in rheumatoid synovial cells. Biochem Biophys Res Commun 2003; 312:397–404.
14 Arnett FC, Edworthy SM, Bloch DA *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1998; 31:315–24.
15 Feuerherm AJ, Borset M, Seidel C *et al.* Elevated levels of osteoprotegerin (OPG) and hepatocyte growth factor (HGF) in rheumatoid arthritis. Scand J Rheumatol 2001; 30:229–34.
16 Ziolkowska M, Kurowska M, Radzikowska A *et al.* High levels of osteoprotegerin and soluble receptor activator of nuclear factor kappa B ligand in serum of rheumatoid arthritis patients and their normalization after anti-tumor necrosis factor alpha treatment. Arthritis Rheum 2002; 46:1744–53.

17 Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 1997; **277**:815–8.
18 Pan G, O'Rourke K, Chinnaiyan AM *et al.* The receptor for the cytotoxic ligand TRAIL. Science 1997; **276**:111–3.
19 Ichikawa K, Liu W, Fleck M *et al.* TRAIL-R2 (DR5) mediates apoptosis of synovial fibroblasts in rheumatoid arthritis. J Immunol 2003; **171**:1061–9.
20 Truneh A, Sharma S, Doyle ML *et al.* Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. J Biol Chem 2000; **275**:23319–25.
21 Duff GW. Cytokines and acute phase proteins in rheumatoid arthritis. Scand J Rheumatol 1994; **100**:9–19.
22 Kong YY, Feige U, Sarosi I *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999; **402**:304–9.
23 Kotake S, Udagawa N, Hakoda M, Tomatsu T, Suda T, Kamatani N. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. Arthritis Rheum 2001; **44**:1003–12.
24 Song K, Chen Y, Goke R *et al.* Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. J Exp Med 2000; **191**:1095–104.

Plasma Level of B-Type Natriuretic Peptide as a Prognostic Marker After Acute Myocardial Infarction

A Long-Term Follow-Up Analysis

Satoru Suzuki, MD; Michihiro Yoshimura, MD; Masafumi Nakayama, MD; Yuji Mizuno, MD; Eisaku Harada, MD; Teruhiko Ito, MD; Shota Nakamura, MD; Koji Abe, MD; Megumi Yamamuro, MD; Tomohiro Sakamoto, MD; Yoshihiko Saito, MD; Kazuwa Nakao, MD; Hirofumi Yasue, MD; Hisao Ogawa, MD

Background—Circulating levels of B-type natriuretic peptide (BNP), a cardiac hormone, reflect the severity of cardiac dysfunction. Because the plasma BNP level changes dramatically during the period after the onset of acute myocardial infarction (AMI), identification of a suitable sampling time is problematic. There have been several reports indicating that the plasma BNP level obtained in the acute phase of AMI can be used as a prognostic marker. We examined whether the plasma BNP level measured 3 to 4 weeks after the onset of AMI represents a reliable prognostic marker for patients with AMI.

Methods and Results—We analyzed 145 consecutive patients with AMI. Plasma BNP levels were measured during the 3 to 4 weeks after onset of AMI. Of those patients, 23 experienced fatal cardiac events during this study. The mean follow-up period was 58.6 months. Log BNP, left ventricular end-diastolic pressure, and pulmonary vascular resistance were all significantly higher in the cardiac death group, and there were more men and more patients with a history of heart failure in the cardiac death group. A Cox proportional hazards model analysis showed that log BNP was an independent predictor of cardiac death. The survival rate was significantly higher in patients with log BNP <2.26 (180 pg/mL) than in those with log BNP ≥ 2.26.

Conclusions—The plasma BNP level obtained 3 to 4 weeks after the onset of AMI can be used as an independent predictor of cardiac death in patients with AMI. (***Circulation.*** **2004;110:1387-1391.)**

Key Words: natriuretic peptides ■ myocardial infarction ■ prognosis

B-type natriuretic peptide (BNP) is a cardiac hormone that is secreted mainly from the ventricles; it has many biological effects, including vasodilation, natriuresis, and inhibition of both the rennin-angiotensin and sympathetic nervous systems.[1–7] BNP is secreted from the failing heart into the systemic circulation; the plasma level of this peptide is elevated in patients with heart failure.[8–11] The plasma BNP level has been recognized as a biochemical marker of ventricular dysfunction.[9–15]

We previously reported that the plasma BNP level increases rapidly and markedly just after the onset of acute myocardial infarction (AMI).[13,16] The plasma level of BNP changes dramatically in some patients. The time course of the plasma BNP level could be divided into 2 patterns after the onset of AMI: a biphasic pattern with 2 peaks and a monophasic pattern with 1 peak. There were significantly more patients with anterior infarction, congestive heart failure, a higher maximum level of creatine kinase-MB isoenzyme, and a lower left ventricular ejection fraction in the biphasic group than in the monophasic group; this suggests that the biphasic pattern reflects the degree of left ventricular dysfunction or the size of the infarct.[13]

The mechanism for the formation of the first plasma BNP peak was shown to be due to the genetic characteristics of BNP: The DNA of BNP has an AT-rich sequence in the 3′-untranslated region, which destabilizes mRNA.[12,17] For this reason, BNP is considered to be an acute-phase reactant in response to acute tissue injuries.[12,13,17,18] Hemodynamic parameters, as well as some humoral factors such as interleukin-1β, endothelin-1, and angiotensin II, induce secretion of BNP in the early phase of AMI, thus accounting for the first peak.[13,19–22] The mechanisms for the formation of the

Received January 16, 2004; de novo received March 30, 2004; revision received May 4, 2004; accepted May 6, 2004.

From the Department of Cardiovascular Medicine (S.S., M. Yoshimura, M.N., S.N., K.A., M. Yamamuro, T.S., H.O.), Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; Division of Cardiology (Y.M., E.H., T.I., H.Y.), Kumamoto Aging Research Institute, Kumamoto, Japan; First Department of Internal Medicine (Y.S.), Nara Medical University, Nara, Japan; and Department of Medicine and Clinical Science (K.N.), Kyoto University Graduate School of Medicine, Kyoto, Japan.

Reprint requests to Michihiro Yoshimura, Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail bnp@kumamoto-u.ac.jp

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000141295.60857.30

second peak are considered to be related to infarct expansion and subsequent ventricular remodeling.[12,13]

As mentioned above, the changing pattern of the plasma BNP level varies according to the cardiac condition after AMI. This raises the possibility that the plasma BNP level could reflect or predict prognosis after the onset of AMI. The changing pattern of the plasma BNP level after the onset of AMI is dynamic during the first month; therefore, identification of a suitable time frame for blood sampling for BNP measurement is an important issue. To exploit the plasma BNP level as a clinically useful prognostic marker after AMI in the future, it would be convenient to measure it 3 to 4 weeks after the onset of AMI. It is generally difficult to target the timing of the blood sampling to the formation of the second plasma BNP peak, although this strict time point may be better as a prognostic marker than the later phase, such as 3 to 4 weeks after the onset. According to our previous study, the plasma BNP level was still significantly higher in patients with the biphasic pattern than in those with the monophasic pattern at 3 to 4 weeks after onset of AMI,[13] which suggests that sampling at this time would also be valuable for clinical use. In the present study, we examined whether the plasma BNP level measured 3 to 4 weeks after the onset of AMI represents a reliable prognostic marker after AMI by monitoring patients with AMI for a long-term period. The present study began just after the discovery of BNP, and therefore we were able to monitor the patients for ≈5 years on average and as long as 13 years.

Methods

Study Patients

This study began in January 1990, just after the discovery of BNP, and an antibody for BNP was established by our research group.[9,23,24] The end of the patient recruitment period was in March 1999. The final follow-up date was on May 31, 2003. During this time period, there were 403 admitted patients with AMI who underwent cardiac catheterization and in whom we were able to measure the plasma BNP level 3 to 4 weeks after the onset of AMI. We followed up 285 patients and were ultimately able to follow up 145 patients with highly reliable information about their prognosis from themselves, their families, and/or their affiliated hospitals. The 145 study patients consisted of 106 men and 39 women with a mean age of 65.1 years (range 31 to 90 years).

The diagnosis of AMI was made from clinical symptoms, including chest pain, ECG changes including ST elevation and ST depression, and an elevation of serum creatine kinase-MB isoenzyme to more than twice the normal upper level. In the present study, we defined the cardiac death group as patients who died of heart failure or sudden cardiac death and the non–cardiac-death group as survivors, including patients who died of causes other than cardiac events. The protocol was in agreement with the guidelines of the ethics committee at our institution, and written informed consent was obtained from each patient before they were enrolled in the study.

Cardiac Catheterization

Cardiac catheterization was performed in 145 patients at 3 to 4 weeks after AMI. A Swan-Ganz catheter was inserted into the femoral or subclavian vein, and hemodynamic measurements were obtained, including pulmonary capillary wedge pressure, pulmonary artery pressure, right atrial pressure, and cardiac output. Cardiac output was determined in triplicate by the thermodilution technique. Blood samples that included BNP were obtained from either the femoral or subclavian vein.

After the Swan-Ganz catheterization procedure, aortic pressure and left ventricular end-diastolic pressure were measured; then, coronary angiography and left ventriculography were performed. The left ventricular ejection fraction was determined by left ventriculography.

Measurement of BNP Plasma Level

The plasma BNP concentration was measured with a specific radioimmunoassay for human BNP from 1990 to 1993[9,25,26] and then a specific immunoradiometric assay for human BNP from 1993 to 1999 (Shionoria BNP; Shionogi Inc).[27] There were no significant differences in the BNP values obtained by the radioimmunoassay and immunoradiometric assay methods.[28]

Statistical Analysis

Continuous values are expressed as the mean±SD. Statistical significance was defined as a probability value <0.05. A Cox proportional hazards regression analysis was performed to identify independent predictors of cardiac death by using variables including log BNP, left ventricular ejection fraction, history of heart failure, heart rate, male gender, anterior myocardial infarction, ACE inhibitor or β-blocker use, history of renal dysfunction, age, history of left ventricular hypertrophy, and revascularization. Because the BNP plasma level was not normally distributed, we selected log BNP for analysis. A log BNP cutoff point was selected to define a large patient group with a low risk of cardiac death. A Kaplan-Meier survival curve was used for survival comparisons between patient groups stratified according to this cutoff point.

Results

Follow-Up Periods

The mean follow-up period was 58.6 months (range 1 to 158 months) for all study patients, with mean follow-up periods of 41.3 months (range 1 to 127 months) for the cardiac death group and 61.9 months (range 1 to 158 months) for the non–cardiac-death group.

Prognosis of Patients and Causes of Death

Of the 145 patients, 115 survived and 30 died during the study period. Of the 30 patients who died, 8 (27%) died of sudden death, 15 (50%) had heart failure, and 7 (23%) died of other causes (2 of pneumonia, 2 of lung cancer, 1 of renal cell carcinoma, 1 of liver dysfunction, and 1 of blood dyscrasia).

Comparisons of Clinical Characteristics, Hemodynamic Parameters, and Plasma BNP Levels Between the Cardiac Death Group and the Non–Cardiac-Death Group

The clinical characteristics and hemodynamic parameters of the study patients are shown in Tables 1 and 2. Among all the patients, 72 (50%) had anterior infarction, and 73 (50%) had inferior or posterolateral infarction. Eighty-four (60%) had a history of smoking, 48 (35%) had a history of hypertension, 37 (27%) were obese, 62 (45%) had diabetes mellitus, and 57 (41%) had dyslipidemia (Table 1).

The number of male patients and patients with a history of heart failure was significantly higher in the cardiac death group than in the non–cardiac-death group. There were no significant differences for age, anterior myocardial infarction, coronary risk factors, history of left ventricular hypertrophy, history of renal dysfunction, revascularization, or pharmacotherapy between the cardiac death group and the non–cardiac-death group (Table 1).

TABLE 1. Patients' Characteristics

	Non–Cardiac-Death (n=122)	Cardiac Death (n=23)	P
Age, y	64.7±11.1	66.7±7.9	NS
Male gender	70 (85/122)	91 (21/23)	0.03
Anterior MI	47 (57/122)	65 (15/23)	NS
Smoking	60 (71/119)	65 (13/20)	NS
History of hypertension	37 (44/119)	20 (4/20)	NS
Obesity	28 (33/119)	20 (4/20)	NS
Diabetes mellitus	46 (55/119)	35 (7/20)	NS
History of dyslipidemia	44 (52/119)	25 (5/20)	NS
History of heart failure	4 (5/117)	18 (4/22)	0.015
History of LVH	17 (20/117)	5 (1/22)	NS
History of renal dysfunction	19 (19/102)	33 (6/18)	NS
Revascularization*	53 (62/116)	52 (11/21)	NS
Pharmacotherapy			
ACEI	45 (50/111)	45 (9/20)	NS
Digitalis	3 (3/111)	5 (1/20)	NS
Furosemide	17 (19/111)	25 (5/20)	NS
Spironolactone	3 (3/111)	5 (1/20)	NS
Calcium channel blocker	80 (88/111)	75 (15/20)	NS
Nitrate	59 (65/111)	60 (12/20)	NS
β-Blocker	20 (22/111)	30 (6/20)	NS

Values are percentage (number) of patients or mean±SD. Anterior MI indicates patients with anterior myocardial infarction; LVH, left ventricular hypertrophy.

*Including percutaneous coronary intervention, thrombolytic therapy, and CABG.

Log BNP was significantly higher in the cardiac death group than in the non–cardiac-death group (2.44±0.57 versus 1.91±0.59, $P<0.0001$; Table 2).

Univariate and Multivariate Predictors of Cardiac Death

Table 3 shows the results of univariate and multivariate Cox proportional hazards model analyses for cardiac death. In the univariate analysis, log BNP, left ventricular ejection fraction, history of heart failure, heart rate, and male gender were predictive factors. In the multivariate analysis, log BNP was the only independent predictor of cardiac death.

TABLE 3. Univariate and Multivariate Relations for Prediction of Cardiac Death

	Univariate		Multivariate	
	χ^2	P	χ^2	P
Log BNP	20.06	<0.0001	7.003	0.008
LVEF (%)	7.354	0.007	...	...
History of heart failure	7.304	0.007	...	...
Heart rate, bpm	4.228	0.040	...	...
Male gender	4.096	0.043	...	...
Anterior MI	...	...	...	...
ACEI or β-blocker	...	...	...	...
History of renal dysfunction	...	...	...	...
Age, y	...	...	...	...
History of LVH	...	...	...	...
Revascularization	...	...	...	...

LVEF indicates left ventricular ejection fraction; anterior MI, patients with anterior myocardial infarction; and LVH, left ventricular hypertrophy.

*Including percutaneous coronary intervention, thrombolytic therapy, and CABG.

Kaplan-Meier Survival Analysis

We examined the sensitivity and specificity of various cutoff values of log BNP for predicting survival and created receiver operating characteristic curves. The best value of log BNP with the highest sensitivity and specificity was 2.26, equivalent to a BNP level of up to 180 pg/mL (Figure 1). Figure 2 shows that the survival rates were significantly higher in patients with log BNP <2.26 than in patients with log BNP ≥2.26.

Discussion

During the follow-up period, 23 patients died of cardiac death. The plasma BNP level 3 to 4 weeks after the onset of AMI was significantly higher in the cardiac death group (n=23) than in the non–cardiac-death group (n=122). We examined the sensitivity and specificity of various cutoff values of log BNP for predicting survival. We created

TABLE 2. Hemodynamic Parameters and BNP Levels

	Non–Cardiac-Death (n=122)	Cardiac Death (n=23)	P
Log BNP	1.91±0.59 (81±4 pg/mL)	2.44±0.57 (277±4 pg/mL)	<0.0001
Hemodynamics			
Heart rate, bpm	71±13	80±25	NS
Mean blood pressure, mm Hg	97±15	95±23	NS
SVR, dyne·s·cm^{-5}	1786±52	2209±1039	NS
PVR, dyne·s·cm^{-5}	141±76	223±127	0.0280
LVEDP, mm Hg	11±6	18±5	0.0002
LVEF, %	56±17	44±16	0.0064
CI, L·min^{-1}·m^{-2}	2.3±0.5	2.8±0.6	0.0040

Values are mean±SD. SVR indicates systemic vascular resistance; PVR, pulmonary vascular resistance; LVEDP, left ventricular end-diastolic pressure; LVEF, left ventricular ejection fraction; and CI, cardiac index.

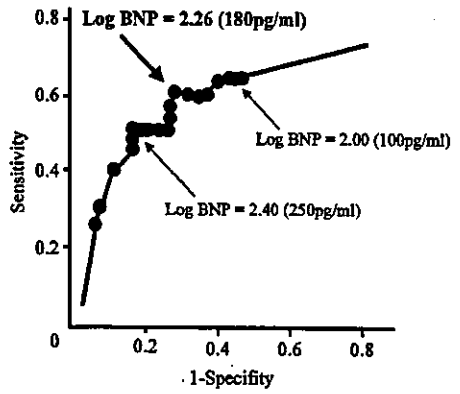

Figure 1. Receiver operating characteristic curves of BNP level for predicting cardiac death. True-positive rates (sensitivity) and false-positive rates (1-specificity) are plotted for various log BNP cutoff values for predicting cardiac death.

receiver operating characteristic curves and concluded that the best cutoff value of log BNP was 2.26, equivalent to a plasma BNP level of 180 pg/mL. The Cox proportional hazards model analysis showed that plasma BNP level was an independent predictor of cardiac death. The survival rate was significantly higher in the patients with log BNP <2.26 (equivalent to a plasma BNP level of up to 180 pg/mL) than in those with log BNP ≥2.26. This plasma BNP level for predicting cardiac events is almost the same as in previous reports.[29–32]

The present study began just after the discovery of BNP, and an antibody for BNP was created by our research group.[9,23] Thus, we were able to follow up patients for a mean period of 58.6 months for all patients, with mean follow-up periods of 41.3 months for the cardiac death group and 61.9 months for the non–cardiac-death group. We demonstrated that the plasma BNP level measured at 3 to 4 weeks after AMI onset was a significant prognostic marker of AMI in this long-term follow-up study.

There have been several reports indicating that the plasma BNP level obtained in the acute phase of AMI can be used as a prognostic marker for patients with AMI[31–37]; however, there are no reports on sampling at 3 to 4 weeks after the onset of AMI. In the present study, we performed BNP sampling at 3 to 4 weeks after AMI onset. Because the changing pattern of the plasma BNP level in patients with AMI is dynamic,[13] the timing

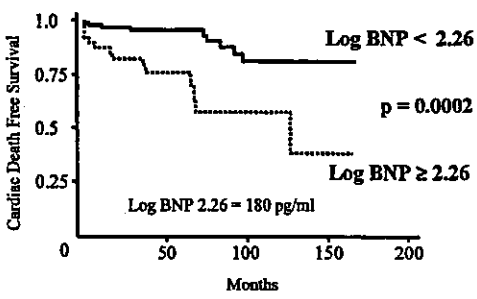

Figure 2. Kaplan-Meier survival curves of cumulative survival rates in patients with AMI divided into 2 groups according to log BNP values. Patients with log BNP ≥2.26 differed significantly from patients with log BNP values below this cutoff point.

of the blood sampling is an important matter. The first peak of plasma BNP was shown ≈20 hours after AMI onset, and the second peak was shown at approximately the fifth day after onset. Plasma BNP levels in the second peak would reflect the degree of ventricular remodeling after AMI.[13] We considered that timing the blood sampling to occur just at the second peak might be ideal to predict prognosis; however, it is quite difficult to obtain the blood sample just at the second peak, because the authentic second peak of plasma BNP varies among patients with AMI. Given this background, a sampling point of 3 to 4 weeks after AMI onset appears better, because plasma BNP levels would be quite stable during this phase, as shown in our previous report.[13] The results of the present study revealed that a sampling time at 3 to 4 weeks after the onset of AMI in addition to the acute phase would provide prognostic insights, as previously speculated.[31,38]

In the present study, the number of patients taking ACE inhibitors (ACEIs) and/or β-blockers appears to be relatively low compared with recent studies[39–43]; it seems that historical background plays a role in accounting for this. There were many patients who participated in the present study around 1995; the rate of calcium channel blocker prescriptions was very high in Japan during that time. We prescribed calcium channel blockers for many patients with AMI, in part because we expected calcium channel blockers to prevent coronary artery spasm, which commonly occurs in Japanese patients.[44] Of course, the use of ACEIs or β-blockers in addition to calcium channel blockers is increasing even now in Japan. It has been reported that ACEIs are useful in the treatment of heart failure after AMI[39–42]; thus, it would be highly recommended that an ACEI be prescribed, or that the dose of the ACEI be increased, if the plasma BNP level is elevated 3 to 4 weeks after the onset of AMI. In addition, an aldosterone antagonist would be useful in the treatment of AMI.[45] In the present study, baseline medications, such as ACEIs or β-blockers, were not related to plasma BNP levels (data not shown) or the prognosis of the study patients (Table 3).

In our previous report, we stated that the biphasic pattern of the plasma BNP level indicated poor ventricular function after AMI, whereas the monophasic pattern did not.[13] Plasma BNP levels at 3 to 4 weeks after AMI onset were higher in the biphasic pattern than in the monophasic pattern. This observation and the present data suggest that the biphasic pattern would indicate a poor prognosis after AMI.

In the present long-term follow-up study, we found that plasma levels of BNP measured at 3 to 4 weeks after AMI were a prognostic marker statistically; however, the sample size was limited. Thus, it might be necessary to perform analysis on a larger scale in another series of studies. It is also necessary to examine how BNP levels at 3 to 4 weeks after the onset of AMI affect the subsequent treatment, including medical therapies and implantation of an internal defibrillator.

In conclusion, the plasma BNP level measured 3 to 4 weeks after the onset of AMI is a significant prognostic marker of AMI, as determined by this long-term follow-up study.

Acknowledgments

This study was supported in part by grants-in-aid for the Ministry of Education, Culture, Sports, Science, and Technology, Tokyo [B

(2)-15390248 and B (2)-15390249], the Ministry of Health, Labor and Welfare, Tokyo (14C-4 and 14A-1), and the Smoking Research Foundation, Tokyo.

References

1. Laragh JH. Atrial natriuretic hormone, the renin-aldosterone axis, and blood pressure–electrolyte homeostasis. *N Engl J Med.* 1985;313: 1330–1340.
2. Needleman P, Greenwald JE. Atriopeptin: a cardiac hormone intimately involved in fluid, electrolyte and blood-pressure homeostasis. *N Engl J Med.* 1986;314:828–834.
3. Floras JS. Sympathoinhibitory effects of atrial natriuretic factor in normal humans. *Circulation.* 1990;81:1860–1873.
4. Burnett JC Jr, Kao PC, Hu DC, et al. Atrial natriuretic peptide elevation in congestive heart failure in the human. *Science.* 1986;231:1145–1147.
5. Raine AEG, Erne P, Burgisser E, et al. Atrial natriuretic peptide and atrial pressure in patients with congestive heart failure. *N Engl J Med.* 1986; 315:533–537.
6. Saito Y, Nakao K, Nishimura K, et al. Clinical application of atrial natriuretic polypeptide in patients with congestive heart failure: beneficial effects on left ventricular function. *Circulation.* 1987;76:115–124.
7. Yoshimura M, Yasue H, Morita E, et al. Hemodynamic, renal, and hormonal responses to brain natriuretic peptide infusion in patients with congestive heart failure. *Circulation.* 1991;84:1581–1588.
8. Yasue H, Obata K, Okumura K, et al. Increased secretion of atrial natriuretic polypeptide (ANP) from the left ventricle in the patients with dilated cardiomyopathy. *J Clin Invest.* 1989;83:46–51.
9. Mukoyama M, Nakao K, Hosoda K, et al. Brain natriuretic peptide as a novel cardiac hormone in humans: evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. *J Clin Invest.* 1991;91:1402–1412.
10. Yasue H, Yoshimura M, Sumida H, et al. Localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. *Circulation.* 1994;90:195–203.
11. Yoshimura M, Yasue H, Okumura K, et al. Different secretion patterns of atrial natriuretic peptide and brain natriuretic peptide in patients with congestive heart failure. *Circulation.* 1993;87:464–469.
12. Nakagawa O, Ogawa Y, Itoh H, et al. Rapid transcriptional activation and early MRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. *J Clin Invest.* 1995;96:1280–1287.
13. Morita E, Yasue H, Yoshimura M, et al. Increased plasma levels of brain natriuretic peptide in patients with acute myocardial infarction. *Circulation.* 1993;88:82–91.
14. de Lemos JA, Morrow DA, Bentley JH, et al. The prognostic value of B-type natriuretic peptide in patients with acute coronary syndromes. *N Engl J Med.* 2001;345:1014–1021.
15. Sumida H, Yasue H, Yoshimura M, et al. Comparison of secretion pattern between A-type and B-type natriuretic peptides in patients with old myocardial infarction. *J Am Coll Cardiol.* 1995;25:1105–1110.
16. Mukoyama M, Nakao K, Obata K, et al. Augmented secretion of brain natriuretic peptide in acute myocardial infarction. *Biochem Biophys Res Commun.* 1991;180:431–436.
17. Kojima M, Minamoto N, Kangawa K, et al. Cloning and sequence analysis of cDNA encoding a precursor for rat brain natriuretic peptide. *Biochem Biophys Res Commun.* 1989;159:1420–1426.
18. Kushner I. The phenomenon of the acute phase response. *Ann N Y Acad Sci.* 1982;389:39–48.
19. Harada E, Nakagawa O, Yoshimura M, et al. Effect of interleukin-1 beta on cardiac hypertrophy and production of natriuretic peptides in rat cardiocyte culture. *J Mol Cell Cardiol.* 1999;31:1997–2006.
20. Shubeita HE, McDonough PM, Harris AN, et al. Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes: a paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem.* 1990;265:20555–20562.
21. Baker KM, Aceto JF. Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells. *Am J Physiol Heart Circ Physiol.* 1990;259:610–618.
22. Conti E, Andreotti F, Sciahbasi A, et al. Markedly reduced insulin-like growth factor-1 in the acute phase of myocardial infarction. *J Am Coll Cardiol.* 2001;38:26–32.
23. Saito Y, Nakao K, Itoh H, et al. Brain natriuretic peptide is a novel cardiac hormone. *Biochem Biophys Res Commun.* 1989;158:360–368.
24. Sudoh T, Kangawa K, Minamino N, et al. A new natriuretic peptide in porcine brain. *Nature.* 1988;332:78–81.
25. Mukoyama M, Nakao K, Saito Y, et al. Human brain natriuretic peptide, a novel cardiac hormone. *Lancet.* 1990;335:801–802.
26. Mukoyama M, Nakao K, Saito Y, et al. Increased human brain natriuretic peptide in congestive heart failure. *N Engl J Med.* 1990;323:757–758.
27. Kono M, Yamaguchi A, Tsuji T, et al. An immunoradiometric assay for brain natriuretic peptide in human plasma. *Kaku Igaku.* 1993;13:2–7.
28. Yasue H, Yoshimura M, Jougasaki M, et al. Plasma levels of brain natriuretic peptide in normal subjects and patients with chronic congestive heart failure: measurement by immunoradiometric assay (IRMA). *Horm Clin.* 1993;41:397–403.
29. Anand IS, Fisher LD, Chiang YT, et al, for the Val-HeFT Investigators. Changes in brain natriuretic peptide and norepinephrine over time and mortality and morbidity in the Valsartan Heart Failure Trial (Val-HeFT). *Circulation.* 2003;107:1278–1283.
30. Berger R, Huelsman M, Strecker K, et al. B-type natriuretic peptide predicts sudden death in patients with chronic heart failure. *Circulation.* 2002;105:2392–2397.
31. Omland T, Aakvaag A, Bonarjee VV, et al. Plasma brain natriuretic peptide as an indicator of left ventricular systolic function and long-term survival after acute myocardial infarction: comparison with plasma atrial natriuretic peptide and N-terminal proatrial natriuretic peptide. *Circulation.* 1996;93:1963–1969.
32. Richards AM, Nicholls MG, Espiner EA, et al. B-type natriuretic peptides and ejection fraction for prognosis after myocardial infarction. *Circulation.* 2003;107:2786–2792.
33. Arakawa N, Nakamura M, Aoki H, et al. Plasma brain natriuretic peptide concentrations predict survival after acute myocardial infarction. *J Am Coll Cardiol.* 1996;27:1656–1661.
34. Darbar D, Davidson NC, Gillespie N, et al. Diagnostic value of B-type natriuretic peptide concentrations in patients with acute myocardial infarction. *Am J Cardiol.* 1996;78:284–287.
35. Palmer BR, Pilbrow AP, Yandle TG, et al. Angiotensin-converting enzyme gene polymorphism interacts with left ventricular ejection fraction and brain natriuretic peptide levels to predict mortality after myocardial infarction. *J Am Coll Cardiol.* 2003;41:729–736.
36. James SK, Lindahl B, Siegbahn A, et al. N-terminal pro-brain natriuretic peptide and other risk markers for the separate prediction of mortality and subsequent myocardial infarction in patients with unstable coronary artery disease. *Circulation.* 2003;108:275–281.
37. Omland T, Persson A, Ng L, et al. N-terminal pro-B-type natriuretic peptide and long-term mortality in acute coronary syndromes. *Circulation.* 2002;106:2913–2918.
38. Bonow RO. New insights into the cardiac natriuretic peptides. *Circulation.* 1996;93:1946–1950.
39. Pfeffer MA, Braunwald E, Moye LA, et al. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction: results of the Survival And Ventricular Enlargement trial: the SAVE Investigators. *N Engl J Med.* 1992;327: 669–677.
40. Køber L, Torp-Pedersen C, Carlsen JE, et al. A clinical trial of the angiotensin-converting-enzyme inhibitor trandolapril in patients with left ventricular dysfunction after myocardial infarction: Trandolapril Cardiac Evaluation (TRACE) Study Group. *N Engl J Med.* 1995;333:1670–1676.
41. Hall AS, Murray GD, Ball SG. Follow-up study of patients randomly allocated ramipril or placebo for heart failure after acute myocardial infarction: AIRE Extension (AIREX) Study: Acute Infarction Ramipril Efficacy. *Lancet.* 1997;349:1493–1497.
42. Yusuf S, Sleight P, Pogue J, et al. Effects of an angiotensin-converting–enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients: the Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med.* 2000;342:145–153.
43. ISIS-1 (First International Study of Infarct Survival) Collaborative Group. Randomised trial of intravenous atenolol among 16027 cases of suspected acute myocardial infarction: ISIS-1. *Lancet.* 1986;2:57–66.
44. Yasue H, Kugiyama K. Coronary spasm: clinical features and pathogenesis. *J Intern Med.* 1997;36:760–765.
45. Pitt B, Remme W, Zannad F, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med.* 2003;348:1309–1321.

Serum leptin level is a regulator of bone mass

F. Elefteriou*†‡, S. Takeda*†‡§, K. Ebihara¶, J. Magre‖, N. Patano*, C. Ae Kim**, Y. Ogawa§††, X. Liu*, S. M. Ware*, W. J. Craigen*, J. J. Robert‡‡, C. Vinson§§, K. Nakao¶, J. Capeau‖, and G. Karsenty*†¶¶

*Department of Molecular and Human Genetics, †Bone Disease Program of Texas, Baylor College of Medicine, Houston, TX 77030; ¶Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; ‖Institut National de la Santé et de la Recherche Médicale U.402, Faculté de Médecine St-Antoine, 75012 Paris, France; **Genetica Instituto da Criança, CEP 05403-900, São Paulo, Brazil; §Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo 101-0062, Japan; ††Department of Molecular Medicine and Metabolism, Medical Research Institute, ‡‡Department of Pediatric Diabetology, Necker Hospital, 75730 Paris, France; and §§Laboratory of Biochemistry, National Cancer Institute, Bethesda, MD 20892

Communicated by Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France, January 5, 2004 (received for review October 29, 2003)

Leptin is a powerful inhibitor of bone formation *in vivo*. This antiosteogenic function involves leptin binding to its receptors on ventromedial hypothalamic neurons, the autonomous nervous system and β-adrenergic receptors on osteoblasts. However, the mechanisms whereby leptin controls the function of ventromedial hypothalamic antiosteogenic neurons remain unclear. In this study, we compared the ability of leptin to regulate body weight and bone mass and show that leptin antiosteogenic and anorexigenic functions are affected by similar amounts of leptin. Using a knock-in of *LacZ* in the *leptin* locus, we failed to detect any leptin synthesis in the central nervous system. However, increasing serum leptin level, even dramatically, reduced bone mass. Conversely, reducing serum-free leptin level by overexpressing a soluble receptor for leptin increased bone mass. Congruent with these results, the high bone mass of lipodystrophic mice could be corrected by restoring serum leptin level, suggesting that leptin is an adipocyte product both necessary and sufficient to control bone mass. Consistent with the high bone mass phenotype of lipodystrophic mice, we observed an advanced bone age, an indirect reflection of premature bone formation, in lipodystrophic patients. Taken together, these results indicate that adipocyte-derived circulating leptin is a determinant of bone formation and suggests that leptin antiosteogenic function is conserved in vertebrates.

A growing body of work has established the central role of the hypothalamus in the regulation of bone formation by osteoblasts (1–4). The thrust of this influence is exerted by neurons located in the ventromedial hypothalamus. These neurons are themselves the target of leptin, which was demonstrated to be a powerful antiosteogenic hormone (1). Based on chemical lesioning, genetic manipulations, and pharmacological experiments, hypothalamic neurons mediating leptin antiosteogenic and anorexigenic functions could be distinguished. Moreover, genetic evidence indicates that, peripherally, the sympathetic nervous system mediates preferentially leptin antiosteogenic function (2). Thus leptin uses distinct pathways to regulate bone mass and body weight.

The discovery of leptin antiosteogenic function was a surprise for at least two reasons. First, it was not the function for which leptin was molecularly cloned, and this implied a more pleiotropic role for this hormone than originally anticipated. Second, and more important physiologically, the high bone mass of leptin signaling-deficient mice existed despite the presence of an increase in bone resorption caused by the hypogonadism of these mice. This coexistence of hypogonadism and high bone mass is pivotal to appreciate the physiological importance of leptin antiosteogenic function. Indeed, it established genetically that leptin signaling plays a dominant role over gonadal function in the regulation of bone mass and thereby implied that this is a major function of leptin.

In the present study we addressed several questions raised by the identification of leptin as an antiosteogenic hormone. Specifically, can we assess *in vivo* the relative importance of leptin antiosteogenic and anorexigenic functions, can we determine whether the blood circulation is the main, if not the only, mean whereby leptin regulates bone mass, and, lastly, can we provide evidence suggesting a conservation of this function during evolution?

Materials and Methods

Animals. *Ob/+* mice were obtained from The Jackson Laboratory. Generation of *A-ZIP/F-1* and *SAP-Leptin* transgenic mice has been reported (5–7). *ApoE-leptin* transgenic mice were generated by cloning the mouse *Leptin* cDNA 3′ of the liver specific Apolipoprotein E promoter and 5′ of the liver-specific enhancer sequence contained in the pLiv7 construct. *ApoE-ObRe* mice were generated by inserting the *ObRe* cDNA of the *ObR* gene into pLiv7. *ApoE-leptin* and *ApoE-ObRe* mice were generated on a FVB and C57BL6J background, respectively, and two independent strains were analyzed for each strain. The knock-in of nuclear *LacZ* gene into the *Leptin* locus was generated by replacing exon 3 of the *leptin* coding sequence by the nuclear *lacZ* gene fused to the *neomycin*-resistant gene. Electroporation of embryonic stem (ES) cells, subsequent selection, injection of positive clones into blastocysts, and implantation into pseudopregnant females were done according to standard protocols (8).

Intracerebroventricular (ICV) Infusions. A 28-gauge cannula (Brain infusion kit II, Alza) infusing human leptin (Sigma) for 28 days was implanted into the third ventricle of 2-month-old C57BL6J female mice, as described (1). The cannula was connected to an osmotic pump (Alza) placed in the dorsal s.c. space of the animal.

5-Bromo-4-chloro-3-indolyl β-D-Galactoside (X-Gal) Staining. Animals were killed at 2 months of age, and the tissues were dissected in cold PBS. X-Gal staining was done according to standard protocols (9). Specimens were postfixed in phosphate buffered formalin for 5 h, dehydrated, cleared, and embedded in paraffin. Specimens were cut at 7 μm, and nuclei were stained with Hoechst reagent.

Leptin-Induced STAT3-luc Reporter Analysis. Recombinant histidine-tagged rObRe was produced *in vitro* in 293EBNA cells and purified on a nickel-nitrilotriacetic affinity column (Qiagen, Valencia, CA) according to the manufacturer's recommendations (a detailed procedure is available in *Supporting Text*, which is published as supporting information on the PNAS web site). HEK293 cells stably expressing *ObRb* were transfected by Fugene reagent (Roche Diagnostics) with a STAT3-Luc leptin-

Abbreviations: ICV, Intracerebroventricular; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

‡F.E. and S.T. contributed equally to this work.

¶¶To whom correspondence should be addressed. E-mail: karsenty@bcm.tmc.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0308744101