# Chronic Administration of Adrenomedullin Attenuates Transition From Left Ventricular Hypertrophy to Heart Failure in Rats

Toshio Nishikimi, Fumiki Yoshihara, Shigeo Horinaka, Naohiko Kobayashi, Yosuke Mori, Kazuyoshi Tadokoro, Kazumi Akimoto, Naoto Minamino, Kenji Kangawa, Hiroaki Matsuoka

*Abstract*—Acute administration of adrenomedullin (AM) exerts beneficial hemodynamic, renal, and neurohormonal effects in heart failure (HF). However, chronic effects of AM administration on HF remain unknown. This study sought to examine the effect of chronic infusion of AM on progression of HF in rat. Human recombinant AM was administered by osmotic minipump for 7 weeks in the HF model of Dahl salt-sensitive rats. The effect was compared with vehicle and diuretic treatment group. Chronic AM infusion significantly decreased left ventricular end-diastolic pressure, right ventricular systolic pressure, right atrial pressure, and left ventricular weight/body weight ($P<0.01$ for all). AM significantly attenuated the increase in circulating renin-aldosterone, endogenous rat AM, and atrial natriuretic peptide levels ($P<0.01$ for all). AM also inhibited the myocardial tissue levels of angiotensin II and atrial and brain natriuretic peptide ($P<0.01$ for all). These changes were associated with the improvement of cardiac output and systemic vascular resistance (both $P<0.05$). Furthermore, AM improved left ventricular end-systolic elastance ($P<0.01$). These improvements were greater in the AM than in the diuretic group, although both drugs similarly decreased systolic blood pressure and increased urinary sodium excretion. Kaplan-Meier survival analysis showed that AM significantly prolonged survival time compared with diuretic ($P<0.05$) and vehicle ($P<0.01$) treatment groups. These results suggest that endogenous AM plays a compensatory role in HF and that chronic AM infusion attenuates progression of left ventricular dysfunction and improves survival, at least in part, through inhibition of circulating and myocardial neurohormonal activation. (***Hypertension.* 2003;42:1034-1041.**)

**Key Words:** adrenomedullin ■ heart failure ■ renin ■ natriuretic peptides ■ angiotensin

Adrenomedullin (AM) is a 52–amino acid peptide that was originally discovered in human pheochromocytoma tissue.[1] Subsequent studies demonstrated that AM infusion causes vasodilation, diuresis, and natriuresis and inhibits aldosterone secretion in normal animals.[2] In addition, previous studies have shown that plasma AM levels are increased in patients with heart failure in proportion to its severity[3] and lungs of rats with heart failure.[4] These findings suggest that AM may play an important role as a defense mechanism in volume and pressure homeostasis in the heart failure. Indeed, acute administration of AM improved central hemodynamics and renal function in animal models of heart failure.[5,6] Furthermore, we and other groups recently demonstrated that acute intravenous infusion of AM has beneficial hemodynamic, hormonal, and renal effects in patients with heart failure.[7,8] However, the effects of chronic infusion of AM on progression of heart failure remains unknown. To address this question, we used Dahl salt-sensitive (DS) rats. In these rats under a high-salt diet, systemic hypertension induces compensated concentric left ventricular (LV) hypertrophy at the age of 11 weeks, followed by marked LV dilation and global hypokinesis at the age of 16 to 19 weeks.[9] Therefore, we started chronic AM infusion in DS rats 11 weeks of age and examined the effect on progression of heart failure by measuring central hemodynamics, intrinsic LV function, plasma neurohormonal levels, myocardial biochemical and molecular markers, and survival. Furthermore, we compared the effects of AM with those of a diuretic to investigate whether the beneficial effects of AM are mediated by natriuretic and diuretic effects.

## Methods

All procedures were in accordance with institutional guidelines for animal research.

### Experimental Animals and Protocols

Male inbred DS rats (Eisai Co, Ltd) were fed a diet containing 8% NaCl (high salt) after the age of 6 weeks and were randomly divided

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From the Department of Hypertension and Cardiorenal Medicine (T.N., S.H., N.K., Y.M., K.T., H.M.) and the Laboratory of Molecular and Cellular Biology (K.A.), Dokkyo University School of Medicine, Tochigi, Japan; and the Research Institute (F.Y., N.M., K.K.), National Cardiovascular Center, Osaka, Japan.

Correspondence to Toshio Nishikimi, MD, Department of Hypertension and Cardiorenal Medicine, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan. E-mail nishikim@dokkyomed.ac.jp



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into 3 groups: (1) AM treatment group, (2) diuretic treatment group, and (3) vehicle treatment group. The rats in groups 1 and 3 were subcutaneously implanted with an osmotic minipump (model 2ML4, Alza) filled with recombinant human AM dissolved in 0.9% saline in the AM treatment group (500 ng/h) and 0.9% saline in the vehicle group, as previously reported.[10] Diuretic (trichlormethiazide; 0.133 mg/dL) was given in drinking water. Age-matched male Dahl salt-resistant (DR) rats fed the same diet served as a control group. All rats had their systolic blood pressure measured by the tail-cuff method before feeding with the high salt diet and at 2-week intervals thereafter.

### Recombinant Human AM

Human recombinant AM was kindly provided by Shionogi & Co, Ltd (Osaka, Japan). The method of production of human recombinant AM has been described previously.[10]

### Urine Collection

Twenty-four-hour urine samples were collected from rats in metabolic cages 7 weeks after treatment with AM, diuretic, or vehicle, as previously reported.[11] Urine electrolytes were analyzed by standard methods.

### Central Hemodynamic Measurements

After approximately 7 weeks of treatment of AM, diuretic, or vehicle, mean arterial pressure (MAP), LV end-diastolic pressure (LVEDP), right atrial pressure (RAP), and right ventricular systolic pressure (RVSP) were measured under anesthesia, as previously reported.[6,12]

### Plasma Neurohormonal Analysis

After central hemodynamic measurements, 3 mL of blood was obtained from the carotid artery. Human AM, rat endogenous AM-mature and total AM, plasma renin concentration (PRC), plasma aldosterone, and atrial natriuretic peptide (ANP) concentrations were measured by radioimmunoassay, as previously reported.[10–12]

### Myocardial Neurohormonal Analysis

For the measurements of myocardial peptides levels, other rat groups were used (each n=6). After the heart was excised, it was perfused with cold phosphate-buffered saline (pH 7.4), as previously reported.[13] The radioimmunoassays for ANP, brain natriuretic peptide (BNP), and angiotensin II in myocardial tissue were performed as reported previously.[13–15]

### RNA Preparation and Northern Blot Analysis

All procedures were performed as described in detail in our previous reports.[4,11]

### Serial Measurement of Cardiac Output and Systemic Vascular Resistance

For the serial measurement of cardiac output and systemic vascular resistance (SVR), other rat groups were used (each n=3 to 6). Cardiac output was measured in 11-, 14-, and 17-week-old treated DS rats and DR by thermodilution methods with the use of a computerized cardiac output monitor (Cardiotherm-500, Columbus Instruments), as previously reported.[6] MAP, LVEDP, and RVSP were also measured. SVR was calculated by standard formula.

### LV End-Systolic Pressure-Volume Relation

We obtained the slope of the LV end-systolic pressure-volume relation (Ees) in another rat group (each n=5 to 8) by a gradual inferior vena cava occlusion with the use of the conductance catheter technique, as previously reported.[16–18]

### Effect on Survival Rate

To examine the effect of treatment on survival, DS rats were randomly divided into 3 groups: AM, trichlormethiazide, and vehicle treatment group, as described above. A DR group was also produced. Animals were carefully monitored, and deaths were recorded every day. Survival rates were compared among the groups at 19 weeks after the start of drug treatment.

### Statistical Analysis

All values are expressed as mean±SD. The data on blood pressure were analyzed by 2-way ANOVA, and the differences between each group at each time point were determined by the least-squares mean test. Statistical comparisons between more than 2 groups were carried out by ANOVA followed by the Bonferroni test for multiple comparisons. Comparisons between 2 groups were performed by an unpaired Student $t$ test. Survival was analyzed by the standard Kaplan-Meier analysis with a log-rank test. A probability value $<0.05$ was considered statistically significant.

## Results

We performed 4 series of experiments. In experiment 1, DR and DS rats at 11 weeks and DR, AM-, diuretic-, and vehicle-treated DS rats at 18 weeks were killed for the determination of central hemodynamics, neurohormonal factors, and myocardial biochemical and molecular markers. In experiment 2, serial changes of cardiac output, SVR, MAP, RVSP, and LVEDP were determined in DR and DS rats at 11 weeks and DR, AM-, diuretic-, and vehicle-treated DS rats at 14 and 18 weeks. In experiment 3, the LV pressure-volume relation was measured in DR and AM-, diuretic-, and vehicle-treated DS rats at 18 weeks. In experiment 4, survival was determined in AM-, diuretic-, and vehicle-treated DS rats.

### Experiment 1

#### *Cardiac Weight, Lung Weight, Urine Volume, Urinary Excretion of Sodium and Potassium, and Blood Pressure*

The physiological profiles of the 6 experimental groups are summarized in the Table. Right and left ventricular weight/body weight (RVW/BW and LVW/BW, respectively) was higher in the DS than in the DR rats at both 11 and 18 weeks. LVW/BW were further elevated in DS rats at 18 weeks compared with that in DS rats at 11 weeks. Lung weight, as an index of pulmonary congestion, was increased only in DS rats at 18 weeks compared with the other 3 treatment groups. AM and diuretic treatment reduced LVW/BW and lung weight/BW (all $P<0.01$) compared with DS at 18 weeks.

AM and diuretic treatment significantly increased daily sodium excretion without changing potassium excretion compared with the vehicle treatment group.

As shown in Figure 1, DS rats fed a high-salt diet from 6 weeks of age had progressive development of hypertension. AM and diuretic treatment reduced systolic blood pressure in DS rats at 13, 15, and 17 weeks to a comparable degree.

#### *Central Hemodynamic Responses to Chronic AM Therapy*

As shown in Figure 2, DS rats at 11 weeks had higher MAP, but they had normal RAP and RVSP with a slight increase in LVEDP compared with DR at 11 weeks, indicating a compensated hypertrophy against increased afterload. DS rats at 18 weeks were characterized by obviously higher MAP, LVEDP, RAP, and RVSP compared with DR. Chronic AM infusion and diuretic therapy significantly reduced the LVEDP, RAP, and RVSP without changing the MAP. Chronic AM infusion therapy was more effective in reducing LVEDP than was diuretic therapy.

**Physiological Profiles of the 6 Experimental Groups**

| Parameter | DR 11W | DR 18W | DS 11W | DS 18W | DS AM | DS Diu |
|---|---|---|---|---|---|---|
| n | 10 | 10 | 10 | 14 | 12 | 10 |
| BW, g | 391±16 | 452±25† | 356±19†§ | 356±34†§ | 395±23§#†† | 403±20§#†† |
| LVW, g | 0.75±0.07 | 0.82±0.08 | 1.03±0.15†§ | 1.25±0.11†§# | 1.17±0.12†§ | 1.20±0.20†§# |
| LV/BW, g/kg BW | 1.91±0.14 | 1.81±0.10 | 2.89±0.37†§ | 3.54±0.49†§# | 2.98±0.32†§†† | 2.99±0.61†§†† |
| RVW, g | 0.19±0.02 | 0.24±0.03† | 0.23±0.02† | 0.23±0.05† | 0.23±0.04† | 0.23±0.03† |
| RV/BW, g/kg BW | 0.48±0.05 | 0.52±0.05 | 0.65±0.03†§ | 0.67±0.21†§ | 0.60±0.09* | 0.57±0.09‡** |
| Lung weight, g | 1.75±0.11 | 1.67±0.13 | 1.44±0.14 | 2.56±0.70†§# | 1.64±0.37†† | 1.72±0.33†† |
| Lung/BW, g/kg BW | 4.48±0.19 | 3.61±0.24 | 4.04±0.30 | 7.50±2.92†§# | 4.17±0.93†† | 4.28±0.97†† |
| UV, mL/d | 42.2±14.9 | 32.3±11.2 | 47.0±20.6 | 68.1±25.6†§# | 70.2±14.6†§# | 53.4±25‡** |
| UNaV, mEq/d | 10.7±6.3 | 10.7±5.8 | 13.3±7.5 | 15.6±7.6‡ | 21.2±4.6†§#** | 20.7±8.9†§‖** |
| UKV, mEq/d | 3.1±0.9 | 3.0±1.0 | 3.1±1.0 | 2.9±0.8 | 3.5±0.5 | 3.2±1.1 |

BW indicates body weight; LVW, left ventricle weight; RVW, right ventricle weight; DR 11W, DR at 11 weeks; DR 18W, DR at 18 weeks; DS 11W, DS at 11 weeks; DS 18W, DS treated with vehicle at 18 weeks; DS AM, DS treated with AM 500 ng/h at 18 weeks; DS Diu, DS treated by methylchrolothiazide 0.133 mg/mL at 18 weeks.

*$P$<0.05 vs DR 11W; †$P$<0.01 vs DR 11W; ‡$P$<0.05 vs DR 18W; §$P$<0.01 vs DR 18W; ‖$P$<0.05 vs DS 11W; #$P$<0.01 vs DS 11W; **$P$<0.05 vs DS 18W; ††$P$<0.01 vs DS 18W.

### *Plasma Neurohormonal Responses to Chronic AM Therapy*

As shown in Figure 3, DS rats at 11 weeks had higher plasma total AM, AM-mature, and ANP levels than the other two DR groups, and these indexes were further elevated in DS rats at 18 weeks. In contrast, DS rats at 11 weeks had normal plasma aldosterone and PRC; however, these indexes were increased in DS rats at 18 weeks. Chronic AM infusion significantly inhibited the activation of circulating renin-aldosterone system and further increase of AM and ANP. In contrast, chronic diuretic treatment significantly reduced PRC and total AM compared with vehicle-treated DS rats at 18 weeks; however, it did not significantly reduce ANP, AM-mature, or aldosterone levels. Human AM immunoreactivity was only detected in AM-treated DS rats (2.3±1.5 pmol/L), which was comparable to AM-mature levels of DS rats at 11 weeks.

### *Myocardial Neurohormonal Responses to Chronic AM Therapy*

As shown in Figure 4, the LV tissue level and gene expression of ANP was markedly increased and LV tissue levels of angiotensin II and BNP were moderately increased in DS rats at 18 weeks compared with DS rats at 11 weeks or the other two DR. Chronic AM infusion significantly decreased the LV tissue level and gene expression of ANP and myocardial levels of BNP and angiotensin II. Chronic diuretic treatment significantly reduced LV tissue level and gene expression of ANP but not LV tissue levels of angiotensin II or BNP.

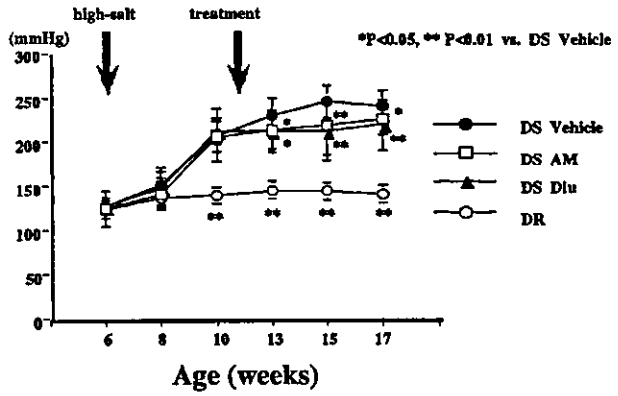


Figure 1. Time course of systolic blood pressure of DS and DR rats. DS and DR (n=15) rats were fed a high-salt diet from 6 weeks of age (left arrow). DS rats were treated with vehicle (DS vehicle, n=15), adrenomedullin (DS AM, 500 ng/h, n=13), and trichlormethiazide (DS Diu, 0.133 mg/dL, n=11) from 11 weeks of age.

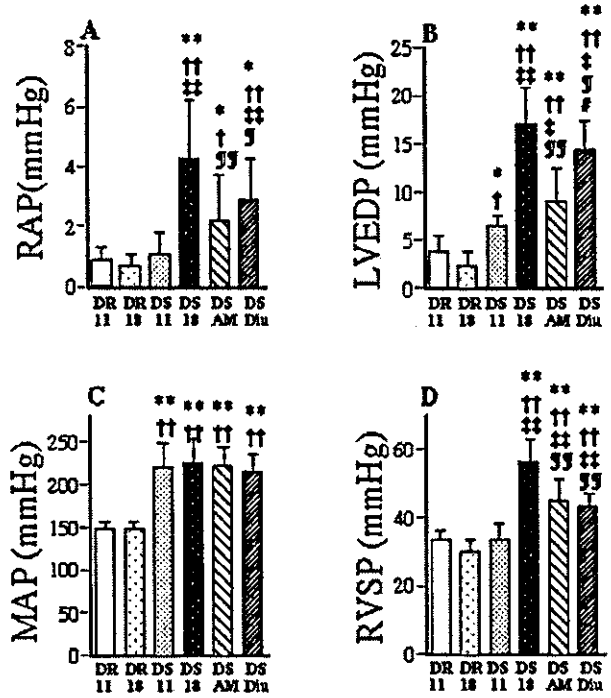


Figure 2. Central hemodynamics in DR and DS rats at 11 and 18 weeks. Right atrial pressure (RAP) (A), left ventricular end-diastolic pressure (LVEDP) (B), mean arterial pressure (MAP) (C), and right ventricular systolic pressure (RVSP) (D). Abbreviations and number of rats in each group as in the Table. *$P$<0.05 vs DR 11; **$P$<0.01 vs DR 11; †$P$<0.05 vs DR 18; ††$P$<0.01 vs DR 18, $P$<0.05 vs DS 11; ‡‡$P$<0.01 vs DS 11; ¶$P$<0.05 vs DS 18; ¶¶$P$<0.01 vs DS 18; #$P$<0.05 vs DS AM.

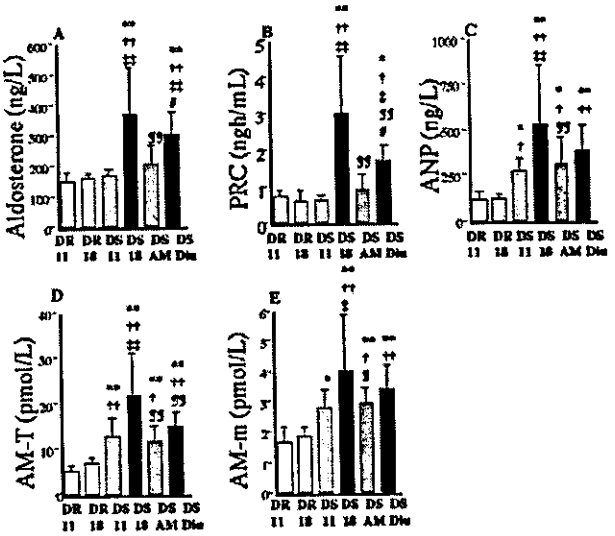


**Figure 3.** Plasma neurohormonal factors in DR and DS rats at 11 and 18 weeks. Plasma aldosterone level (A), plasma renin concentration (PRC) (B), plasma atrial natriuretic peptide (ANP) level (C), plasma rat total AM level (AM-T) (D), and plasma rat AM-mature level (AM-m) (E). Abbreviations and number of rats as in the Table. *$P<0.05$ vs DR 11; **$P<0.01$ vs DR 11; †$P<0.05$ vs DR 18; ††$P<0.01$ vs DR 18, $P<0.05$ vs DS 11; ‡‡$P<0.01$ vs DS 11; ¶¶$P<0.01$ vs DS 18; #$P<0.05$ vs DS AM.

## Experiment 2

### *Serial Measurement of Cardiac Output and SVR*

The serial changes in cardiac output, SVR, MAP, LVEDP, RVSP, and LVW/BW were assessed in other experiment groups (Figure 5). At 11 weeks, DS rats had higher MAP as the result of increased SVR than DR; however, there was no difference in cardiac output between DS rats and DR. After 14 weeks, DS rats had lower cardiac output than DR. Chronic AM infusion treatment significantly increased cardiac output and reduced SVR without changing MAP. In contrast, diuretic did not change them significantly.

## Experiment 3

### *LV End-Systolic Elastance*

Representative results of LV pressure-volume relations in DR and DS rats treated with vehicle, AM, and diuretic and the results of Ees in four groups are shown in Figure 6. Ees was significantly lower in vehicle-treated DS rats than in DR. Chronic AM and diuretic treatment significantly prevented the fall in Ees seen in DS rats; however, its effect was greater in AM than in diuretic.

## Experiment 4

### *Survival Rate*

As shown in Figure 7, survival rate was analyzed at 8 weeks after the start of treatment. All DS rats treated with vehicle died of congestive heart failure between 12 and 19 weeks of age. Kaplan-Meier survival analysis showed that long-term AM infusion and diuretic treatment significantly prolonged the survival of DS rats ($P<0.01$ and $P<0.05$, respectively); however, the effect was greater in the AM than in the diuretic group ($P<0.05$).

## Discussion

The circulating renin-angiotensin-aldosterone system is known to be excessively activated in patients with heart failure, leading to adverse effects. In this study, plasma renin was not activated at 11 weeks; however, DS rats at 18 weeks had higher plasma renin and aldosterone concentrations than DR, indicating activation of the circulating renin-angiotensin-aldosterone system in the transition from LV hypertrophy to heart failure in DS rats. Interestingly, chronic AM infusion significantly inhibited the activation of circulating renin in DS rats. In previous studies, the acute effects of AM on plasma renin activity were controversial in vivo[19,20] because excessive reduction of blood pressure induced by AM may induce renin secretion. However, Khan et al[21] reported that chronic administration of AM significantly reduced plasma renin activity despite the reduction of blood pressure in renovascular hypertensive rats, suggesting an inhibitory effect of chronic AM infusion on renin secretion. Furthermore, AM significantly inhibited the activation of aldosterone in the transition from LVH to heart failure in this study. AM has been shown to inhibit production of angiotensin II–induced aldosterone by dispersed rat adrenal zona glomerulosa cells.[22] Acute inhibitory effects of AM on aldosterone secretion was also reported in human and experimental heart failure.[5,7] Thus, beneficial effects of AM may be partly mediated by an inhibitory effect of the activation of the circulating renin-angiotensin-aldosterone system.

In the present study, DS rats with heart failure exhibited not only increased LV weight but also myocyte phenotypic modulation, as shown by the upregulation of ANP genes and increased myocardial ANP and BNP levels. Interestingly, long-term AM infusion was effective in reduction of ANP gene expression and ANP and BNP myocardial levels. In addition, previous studies have shown that the cardiac renin-angiotensin system plays a critical role in progression of heart failure in DS rats fed a high-salt diet.[23] Furthermore, myocardial angiotensin II is also involved in the development of heart failure induced by myocardial infarction or hemodynamic overload.[13,24] In the present study, the myocardial angiotensin II level was significantly higher in DS rats with heart failure than in DR. Importantly, this increase in myocardial angiotensin II level was significantly attenuated by chronic AM administration. These findings suggest that not only the reduction of circulating renin-angiotensin levels but also inhibition of the intracardiac renin-angiotensin system may be involved in the amelioration of heart failure by chronic AM therapy.

We compared the effect of AM with a diuretic to determine whether the beneficial effects of AM are mediated solely through its natriuretic and antihypertensive actions. Although AM and diuretic treatment similarly decreased systolic blood pressure and increased sodium excretion in DS rats, the beneficial effects were greater in AM than in the diuretic group. This may be explained in part by the direct effects of AM on the cardiovascular and endocrine systems. Previous studies have shown that AM inhibits angiotensin II–induced cardiac hypertrophy in cardiac myocytes as well as angiotensin II–induced collagen synthesis and proliferation in cardiac fibroblasts,[25,26] suggesting an inhibitory effect of AM in

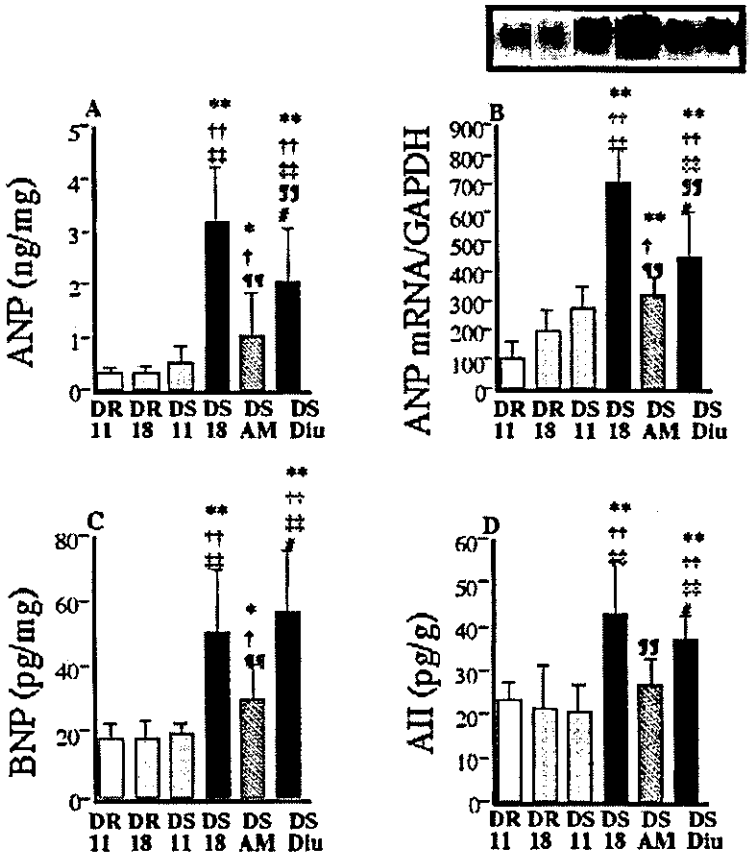


**Figure 4.** Myocardial neurohormonal factors and gene expression in DR and DS rats at 11 and 18 weeks. Left ventricular tissue ANP level (A), ANP mRNA level (B), tissue brain natriuretic peptide (BNP) level (C), and tissue angiotensin II (AII) level (D). Top of B, Representative autoradiograms of LV ANP mRNA bands. Number of rats was 6 in all groups. *$P<0.05$ vs DR 11; **$P<0.01$ vs DR 11; †$P<0.05$ vs DR 18; ††$P<0.01$ vs DR 18, $P<0.05$ vs DS 11; ‡‡$P<0.01$ vs DS 11; ¶$P<0.05$ vs DS 18; ¶¶$P<0.01$ vs DS 18; #$P<0.05$ vs DS AM.

cardiac remodeling. In fact, AM gene therapy significantly reduced cardiac hypertrophy and fibrosis in a model of hypertension.[27,28] In addition, antiproliferative effects of AM in vascular smooth muscle cells and mesangial cells has also been reported.[2] Indeed, AM(+/−) mice, the plasma and organ AM concentrations of which were almost half of those in AM(+/+) mice, have marked coronary arterial perivascular fibrosis and intimal hyperplasia in angiotenisn II and salt-loaded conditions, suggesting a protective action of endogenous AM against cardiovascular damage.[29] Thus, direct anticardiac remodeling, antiatherosclerotic, and/or renoprotective effects of AM may have been associated with the beneficial effects of chronic administration of AM.

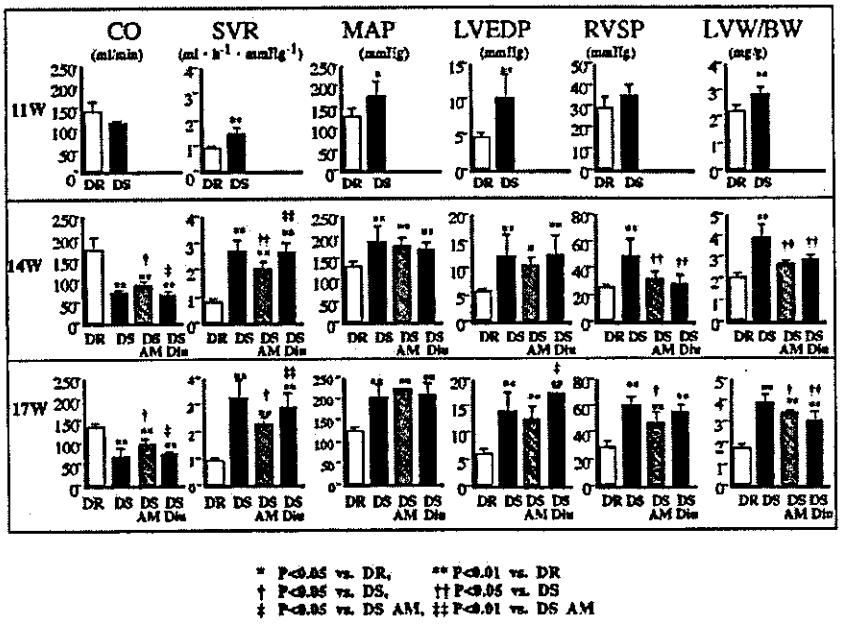


**Figure 5.** Serial changes of cardiac output (CO), systemic vascular resistance (SVR), MAP, LVEDP, RVSP, and LVW/BW. Abbreviations as in Figure 1. Number of rats in each group is DR (n=6), DS (n=6) at 11 weeks; DR (n=5), DS (n=5), DS AM (n=6), and DS Diu (n=6) at 14 weeks; and DR (n=4), DS (n=3), DS AM (n=3), and DS Diu (n=3) at 18 weeks.

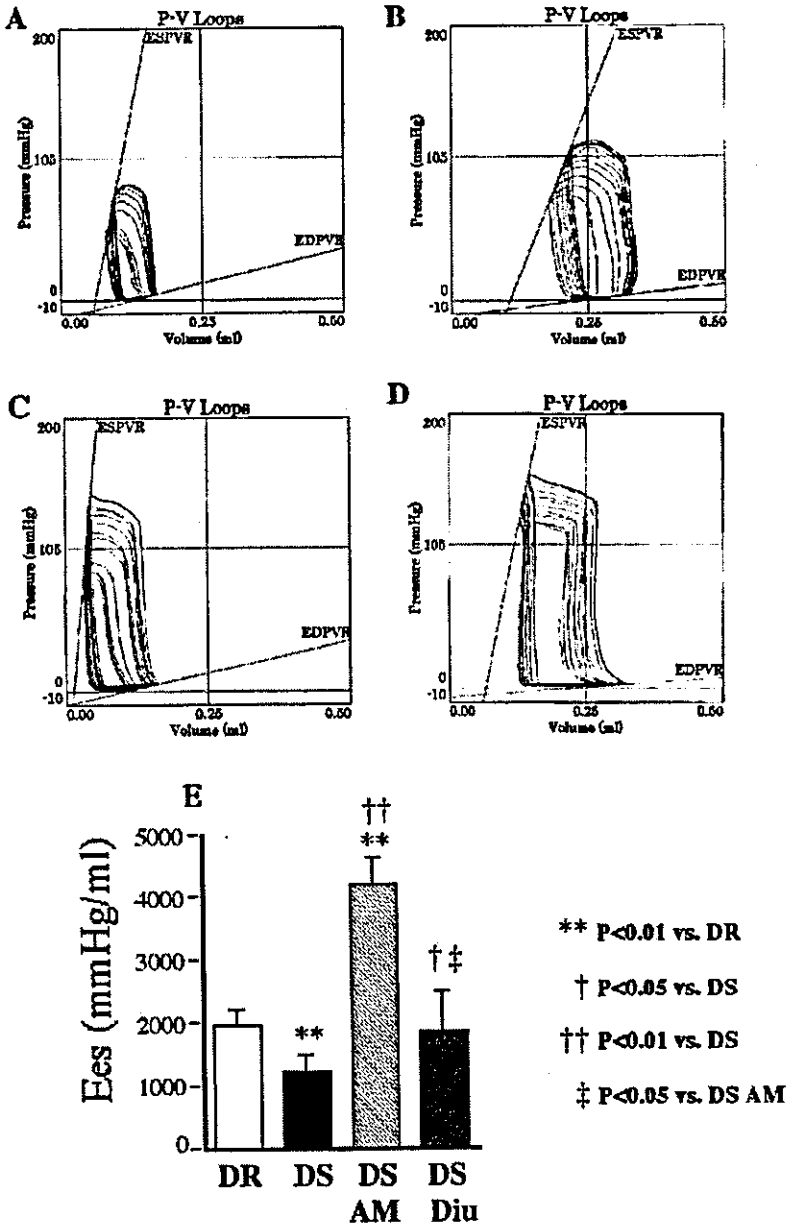


**Figure 6.** LV end-systolic pressure-volume relations by gradual occlusion of inferior vena cava and Ees. Data show typical results in DR 18 (A), DS 18 (B), DS AM (C), and DS Diu (D). Abbreviations as in Figure 1. Number of rats in each group is DR (n=8), DS (n=8), DS AM (n=5), and DS Diu (n=5).

Rademaker et al[30] very recently reported that intravenous infusion of AM for 4 days increases cardiac output with a decrease of SVR in sheep with pacing-induced heart failure. They showed the improvement of cardiac function after 4 days of AM treatment; however, whether chronic treatment of AM has beneficial effect on cardiac function remains unknown. In the present study, an increase in cardiac output and decrease in SVR were still observed after 7 weeks of treatment with AM. Furthermore, from the analysis of LV pressure-volume relations, DS rats treated with AM exhibited an increase in Ees, suggesting the improvement of LV contractility by AM. Thus, long-term administration of AM improves cardiac output not only by the reduction of afterload but also by the preservation of LV contractility.

Interestingly, chronic human recombinant AM infusion significantly decreased the elevated plasma endogenous rat AM levels. Although the plasma level of human AM was low, the beneficial effects of chronic AM infusion therapy on progression of heart failure were significant. Previous studies have shown that chronic AM infusion significantly reduced plasma renin activity in renovascular hypertensive rats,[21] attenuated progression of pulmonary hypertension in rats,[31] attenuated cardiac remodeling in myocardial infarct rats,[32] and had a renoprotective effect in severe hypertensive rats,[10] with an increase of plasma AM levels in the pathophysiological range. These findings were consistent with the present results. The fact that this slight increase in plasma AM was effective may be attributable to an increase of AM-mature, an active form of AM. Recent studies have shown that major molecular form of plasma AM is AM-glycine, an inactive molecular form of AM, and that AM-mature only constitutes ≈10% of plasma AM.[33,34] In the present study, we also demonstrated that AM-mature is not the

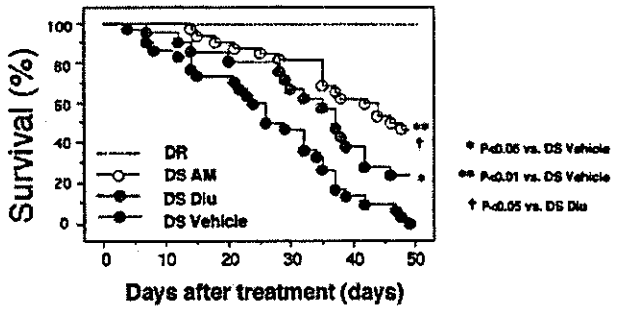


**Figure 7.** Kaplan-Meier survival curves. DS fed a high-salt diet were treated with vehicle (DS, n=20), AM (DS AM, n=23), and trichlormethiazide (DS Diu, n=17).

major molecular form of rat plasma AM and that increased human AM was comparable to rat AM-mature levels. Thus, the finding that exogenous AM administration reduces endogenous AM levels may support the hypothesis that increased plasma AM may play a compensatory role in the pathophysiology of heart failure.

In conclusion, chronic administration of AM had beneficial hemodynamic, plasma neurohormonal, myocardial biochemical, and cardiac functional effects and thereby improved survival in DS rats with heart failure. Chronic AM administration may be a new therapeutic approach for the treatment of heart failure.

## Perspectives

The current study provides insight into the role of increased plasma AM in heart failure. Our results suggest that AM behaves as a beneficial endogenous peptide for hemodynamic and cardiac function in heart failure, partly through inhibition of circulating and tissue renin-angiotensin-aldosterone system. Furthermore, this study suggests that long-term infusion of AM may be a new therapeutic approach to the treatment of heart failure. Currently, infusion of ANP or brain natriuretic peptide is clinically used for the treatment of heart failure. Because AM also has the advantage as an endogenous peptide, a clinical trial is necessary to confirm a potential therapeutic benefit of AM in patients with heart failure.

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# Over expression of bone morphogenetic protein-3b (BMP-3b) using an adenoviral vector promote the osteoblastic differentiation in C2C12 Cells and augment the bone formation induced by bone morphogenetic protein-2 (BMP-2) in rats

Shinji Kaihara[a], Kazuhisa Bessho[a,*], Yasunori Okubo[a], Junya Sonobe[a], Yasato Komatsu[b], Masako Miura[b], Shin-Ichi Miyatake[c], Kazuwa Nakao[b], Tadahiko Iizuka[a]

[a]*Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo, Kyoto 606-8507, Japan*
[b]*Department of Medicine and Clinical Science, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo, Kyoto 606-8507, Japan*
[c]*Department of Neurosurgery, Faculty of Medicine, Osaka Medical College, 2-7 Daigaku-cho, Takatsuki, Osaka 569-8686, Japan*

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**Abstract**

BMP-3b is a novel BMP-3-related protein and its biological functions are unknown. In order to investigate the biological actions of BMP-3b, we constructed a BMP-3b-expressing recombinant adenoviral vector (AxCAKBMP-3b). We show that over expression of BMP-3b stimulated the induction of differentiation and the osteoinduction activity of a human BMP-2-expressing recombinant adenoviral vector (AxCAOBMP-2). C2C12 cells were infected in vitro with AxCAKBMP-3b, AxCAOBMP-2 and a control vector containing no foreign genes (AxCAwt). Cells infected with AxCAOBMP-2 and AxCAKBMP-3b produced more alkaline phosphatase and secreted more osteocalcin into the culture medium than cells infected with AxCAOBMP-2 and AxCAwt. When AxCAOBMP-2, AxCAKBMP-3b, and AxCAwt were injected into the calf muscles of

* Corresponding author. Tel.: +81-75-751-3405; fax: +81-75-761-9732.
*E-mail address:* bes@kuhp.kyoto-u.ac.jp (K. Bessho).

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nude rats (F 344/N Jcl-rnu), the osteoinduction seen with AxCAOBMP-2 and AxCAKBMP-3b was greater than that seen with AxCAOBMP-2 and AxCAwt.


*Keywords:* Bone morphogenetic protein-3b (BMP-3b); Bone morphogenetic protein-2 (BMP-2); Adenoviral vector; C2C12; Nude rat (F 344/N Jcl-rnu); Osteoinduction

## Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor − β superfamily and play an important role in both osteogenesis and bone metabolism [1,2]. Recently, Takao et al. [3] cloned the cDNA of rat BMP-3b, which is closely related to BMP-3. The mature region of rat BMP-3b shows a high degree of amino acid homology with the comparable region of rat BMP-3 (81%) [3], but the biological functions of BMP-3b are still unknown. To clarify BMP-3b gene functions, we constructed a rat BMP-3b-expressing replication-deficient recombinant adenoviral vector (AxCAKBMP-3b). Since this vector system can achieve high-level expression of target gene both in vitro and in vivo. In the present study, we examined the differentiation effect and the osteoinduction activity of BMP-3b. We transfected the BMP-3b gene into mouse myoblastic cell line C2C12. These cells were converted to osteoblast lineage cells with some BMPs [4–6]. Moreover, we transfected the BMP-3b gene into intramuscular site in rats. BMPs especially BMP-2 remarkably convert C2C12 myoblasts in vitro to osteoblast lineage cells [4] and induce bone matrix in vivo. We also examined the interaction of BMP-3b and BMP-2 both in vitro and in vivo.

## Materials and methods

### *Construction of the BMP-3b-expressing recombinant adenoviral vector*

A replication-deficient recombinant adenoviral vector carrying the BMP-3b gene (AxCAKBMP-3b) was constructed by the cosmid cassette and adenoviral DNA terminal protein complex method (COS/TPC; Adenovirus expression vector kit, Takara Shuzo Co., Ltd., Shiga, Japan), as described previously [7]. The cDNA digested with EcoRV (1.43 kbp) containing full-length rat BMP-3b was subcloned into pBluescript II SA (a gift from K. Kangawa and J. Hino). The DNA fragment containing the coding region of rat BMP-3b was excised from the plasmid and purified by gel electrophoresis. Then the fragment was ligated into the SwaI site of cosmid pAxCAwt, a cassette cosmid bearing an expression unit and the full-length adenoviral vector type 5 genome with deletion of the E1 and E3 regions [8]. This expression unit consists of a chicken β-actin promoter and a cytomegalovirus enhancer, as well as DNA cloning site and a rabbit β-globin poly A tail. Next, the constructed cosmid was cotransfected into 293 embryonic cells (American Type Culture Collection, Rockville, MD) together with an EcoT221-digested adenoviral DNA-terminal protein complex [9], using the lipofection method (Lipofect AMINE PLUS TM Reagent, Gibco, Grand Island, NY). The viruses generated through homologous recombination were purified by the limiting dilution technique, and the target clone was then propagated in 293 cells. Next, viruses were purified by centrifugation through a CsCl cushion, titrated, and stored at − 80 °C in phosphate-buffered saline (PBS) containing 10% glycerol [10].

A BMP-2-expressing recombinant adenoviral vector (AxCAOBMP-2) was also used, which carried the full-length human BMP-2 gene in the same expression unit as AxCAKBMP-3b [11,12].

The control vector was AxCAwt which contained no foreign genes and had the same expression unit as AxCAKBMP-3b and AxCAOBMP-2. The titers of AxCAKBMP-3b, AxCAOBMP-2, and AxCAwt were determined by the method described previously [10], and were $2.3 \times 10^9$ plaque-forming units (pfu/ml), $6.6 \times 10^8$ pfu/ml, and $2.3 \times 10^9$ pfu/ml, respectively.

*In vitro study*

*Cell culture*

C2C12 myoblasts (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (FBS; Gibco), penicillin G (100 IU/ml), and streptomycin (100 μg/ml). These cells were cultured in an incubator at 37 °C in a humidified atmosphere containing 5% $CO_2$ and 95% air.

*Western blot analysis*

C2C12 cells were grown to subconfluence in 6-well plates, rinsed with PBS twice, and then infected with AxCAKBMP-3b or with AxCAwt at a multiplicities of infection (MOI) of 100 for 1 h. The infected cells were subsequently washed twice with PBS, and placed into fresh medium. After 72 h, the conditioned medium (1.4 ml) from cultures of infected C2C12 cells was obtained and incubated with heparin sepharose CL-6B (Amersham, Sweden) for 24 h. Bound proteins were eluted by heating at 70 °C for 10 min and subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to a nylon membrane (Immobilon-P; Millipore Co., MA). Recombinant rat BMP-3b was also analyzed as a positive control (a gift from K. Kangawa and J. Hino). The membrane was blocked (Block Ace; Dainippon Pharmaceutical Co., Osaka, Japan) and then was incubated with a polyclonal antibody (a gift from K. Kangawa and J. Hino) directed against BMP-3b (diluted 1:200) in Block Ace for 5 h. The secondary antibody (a horseradish peroxidase-labeled anti-rabbit antibody; Vector Laboratories, CA) was used at a dilution of 1:3000. Then the membrane was processed and detection was performed using an enhanced chemiluminescence detection kit (ECL; Amersham, IL).

*ALP activity and osteocalcin production*

Cells were seeded at $1.5 \times 10^5$ per well in 6-well plates in DMEM containing 5% FBS. After 48 h, the cells were exposed to AxCAOBMP-2, AxCAKBMP-3b, and AxCAwt for 1 h. We also used AxCAwt as the correction vector for total MOI. The following groups were examined (6 wells per group); Group I (AxCAKBMP-3b at an MOI of 25.5), Group II (MOCK: uninfected), Group III (AxCAOBMP-2 at an MOI of 1.5 and AxCAwt at an MOI of 24, control group), Group IV (AxCAOBMP-2 at an MOI of 1.5, AxCAwt at an MOI of 1.5, and AxCAKBMP-3b at an MOI of 22.5), Group V (AxCAOBMP-2 at an MOI of 1.5, AxCAwt at an MOI of 6, and AxCAKBMP-3b at an MOI of 18), Group VI (AxCAOBMP-2 at an MOI of 1.5 and AxCAKBMP-3b at an MOI of 24). Then the cells were rinsed with PBS and the medium was replaced with fresh medium. Two days after infection, the cells were rinsed with PBS and the medium was replaced again. On day 5 after infection, the medium was removed and the cells were washed with PBS and lysed in 50 mM Tris-HCl with 0.5% Nonidet P-40 (ICN Biomedicals Inc, OH), pH 7.5. The ALP activity and the total protein content of the cell lysates were determined by the 4-nitrophenylphosphate method [13]. The amount of osteocalcin secreted into the conditioned medium collected on day 5 after

infection was determined by radioimmunoassay using a mouse osteocalcin assay kit (Biomedical Technologies Inc., MA).

*In vivo study*

*Animals*

Thirty nude rats (F 344/N Jcl-rnu; male; 10 weeks old; weight 180–200 g, obtain from CLEA JAPAN Inc., Tokyo, Japan) were randomly divided into the six groups (5 rats per group) and infected with AxCAOBMP-2, AxCAKBMP-3b, and AxCAwt as follows; Group A (AxCAKBMP-3b; $5.61 \times 10^7$ pfu), Group B (AxCAwt; $5.61 \times 10^7$ pfu), Group C (AxCAOBMP-2; $3.3 \times 10^6$ pfu and AxCAwt; $5.28 \times 10^7$ pfu: control group), Group D (AxCAOBMP-2; $3.3 \times 10^6$ pfu, AxCAwt; $4.95 \times 10^7$ pfu, and AxCAKBMP-3b; $3.3 \times 10^6$ pfu), Group E (AxCAOBMP-2; $3.3 \times 10^6$ pfu, AxCAwt; $3.96 \times 10^7$ pfu, and AxCAKBMP-3b; $1.32 \times 10^7$ pfu), Group F (AxCAOBMP-2; $3.3 \times 10^6$ pfu, and AxCAKBMP-3b; $5.28 \times 10^7$ pfu). They were allowed free access to rodent chow (Certified diet MF; Oriental Koubo Inc., Tokyo, Japan) preoperatively and postoperatively. All procedures and virus inoculation were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

*Surgical procedure*

Rats were anaesthetized with diethyl ether. Following disinfection of the target region, AxCAOBMP-2, AxCAKBMP-3b, and/or AxCAwt were injected into the right calf muscle using a microsyringe.

*Radiographic evaluation*

Twenty-one days after vector injection, the rats were sacrificed with an overdose of sodium pentobarbital. Then the injected region was excised together with the surrounding tissue and subjected to the soft X-ray analysis (SOFRON; SRO-M50, Sofron Inc., Tokyo, Japan). Each resected specimen was cut into halves, with one half being used for histological examination and the other for biochemical analysis.

*Histological examination*

The specimens were fixed in 10% neutral buffed formalin (pH 7.4), demineralized in ethylenediamine tetraacetic acid, and embedded in paraffin. Then 4 μm thick sections were cut and stained with hematoxylin and eosin.

*Biochemical examination*

Samples were weighed and then were homogenized in 0.25 M sucrose in a Polytron homogenizer (Bio-Mixer; type ABM, Nissei Inc., Osaka, Japan). The sediment was demineralized in 0.5 N HC1, and the calcium (Ca) content of the soluble fraction was determined by the orthocresol-phthalein complex method [14]. The alkaline phosphatase (ALP) activity and total protein in the resultant supernatant were determined by the 4-nitrophenylphosphate method [11]. The Ca content (μg/mg tissue) and the ALP activity (IU/mg protein) were used as indices of bone formation [12].

*Statistical analysis*

Results are presented as the mean ± standard deviation(SD). Statistical analysis of differences in the ALP activity and Ca content was performed by analysis of variance (ANOVA), followed by Fisher's test.


Fig. 1. Detection of BMP-3b protein in C2C12 cells by western blotting. Lane 1, medium from AxCAKBMP-3b-infected cells (MOI of 100); lane 2, medium from AxCAwt-infected cells (MOI of 100); lane 3, BMP-3b solution.

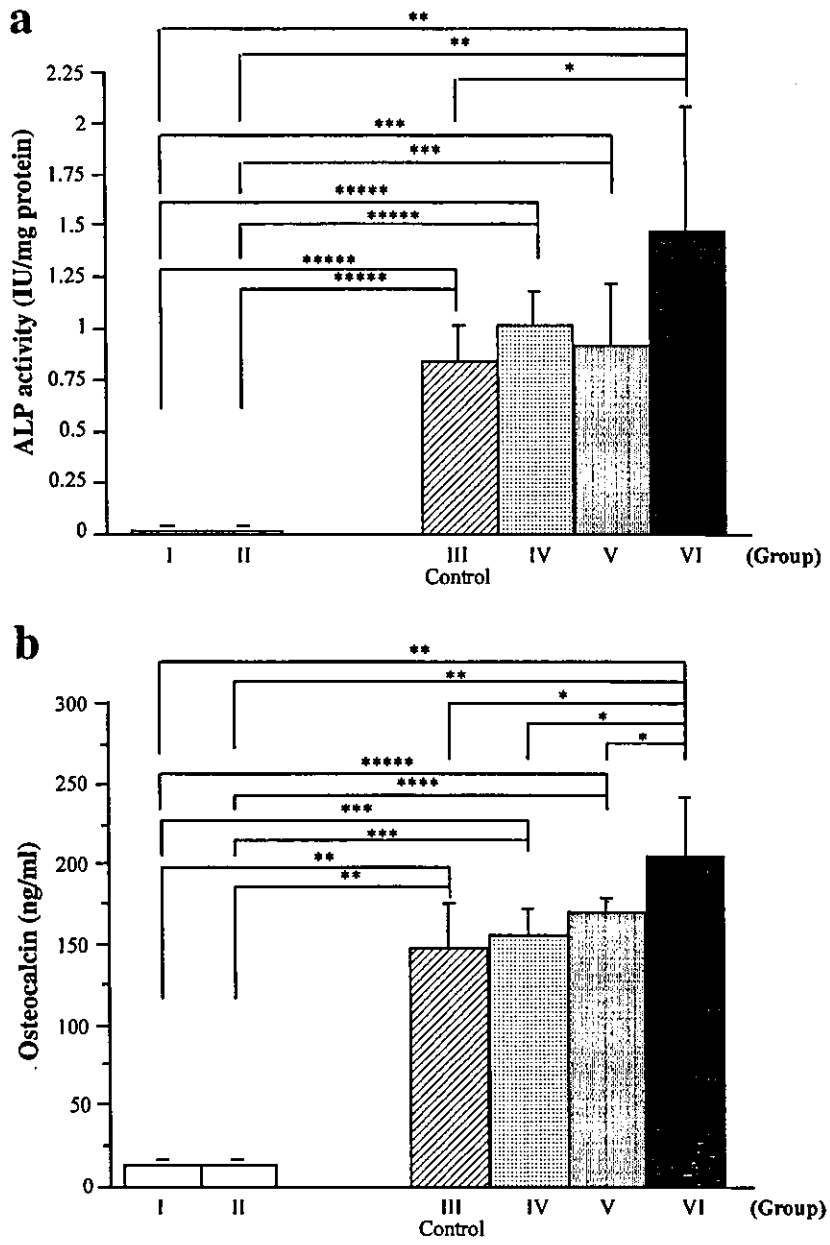


Fig. 2. The ALP activity of cell lysates (a) and the osteocalcin level in culture medium (b) on day 5 after infection. Data are the mean ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, ****$P < 0.0005$, *****$P < 0.0001$.

## Results

### *In vitro study*

*Western blot analysis*

To examine the secretion BMP-3b protein into the culture medium by C2C12 cells infected with AxCAKBMP-3b, western blot analysis was performed (Fig. 1). A single band was detected in culture medium of AxCAKBMP-3b-infected C2C12 cells corresponding to the recombinant BMP-3b protein. In contrasts, no definite band was detected in conditioned medium from cells infected with AxCAwt under the same assay conditions.

*ALP activity and osteocalcin production in vitro*

The ALP activity and osteocalcin production by C2C12 cells infected with AxCAOBMP-2, AxCAKBMP-3b, and AxCAwt at total MOIs of 25.5 and 0 (mock: uninfected) are shown in Fig. 2a and b on day 5 after treatment. In the uninfected cells and the cells infected only with AxCAKBMP-3b (Group I), ALP activity and osteocalcin production were hardly changed on day 5. In contrast, cells infected with AxCAOBMP-2 showed considerable level of ALP activity and osteocalcin production. The ALP activity in cells infected with AxCAOBMP-2 at an MOI of 1.5 and AxCAKBMP-3b at an MOI of 24 (Group VI) were 1.7-fold higher than those in cells treated with

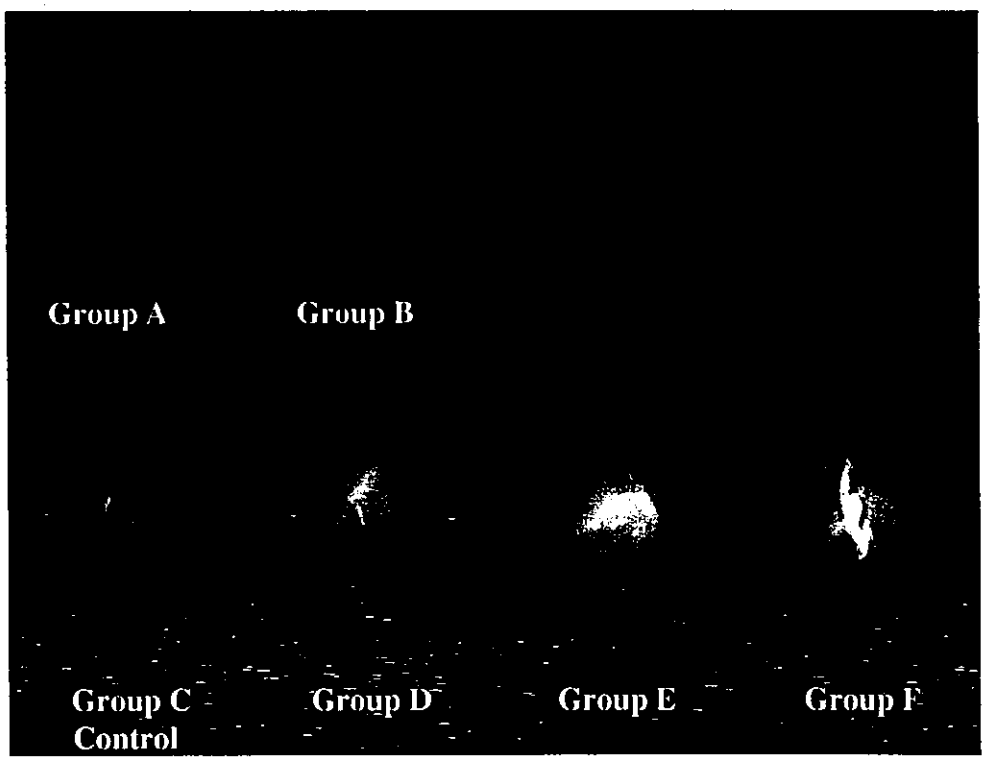


Fig. 3. Soft X-ray images of calf muscles at 21 days after treatment.

AxCAOBMP-2 at an MOI of 1.5 and AxCAwt at an MOI of 24 (Group III). Osteocalcin production in cells infected with AxCAOBMP-2 at an MOI of 1.5 and AxCAKBMP-3b at an MOI of 24 (Group VI) were 1.4-fold higher than those in cells treated with AxCAOBMP-2 at an MOI of 1.5 and AxCAwt at an MOI of 24 (Group III).

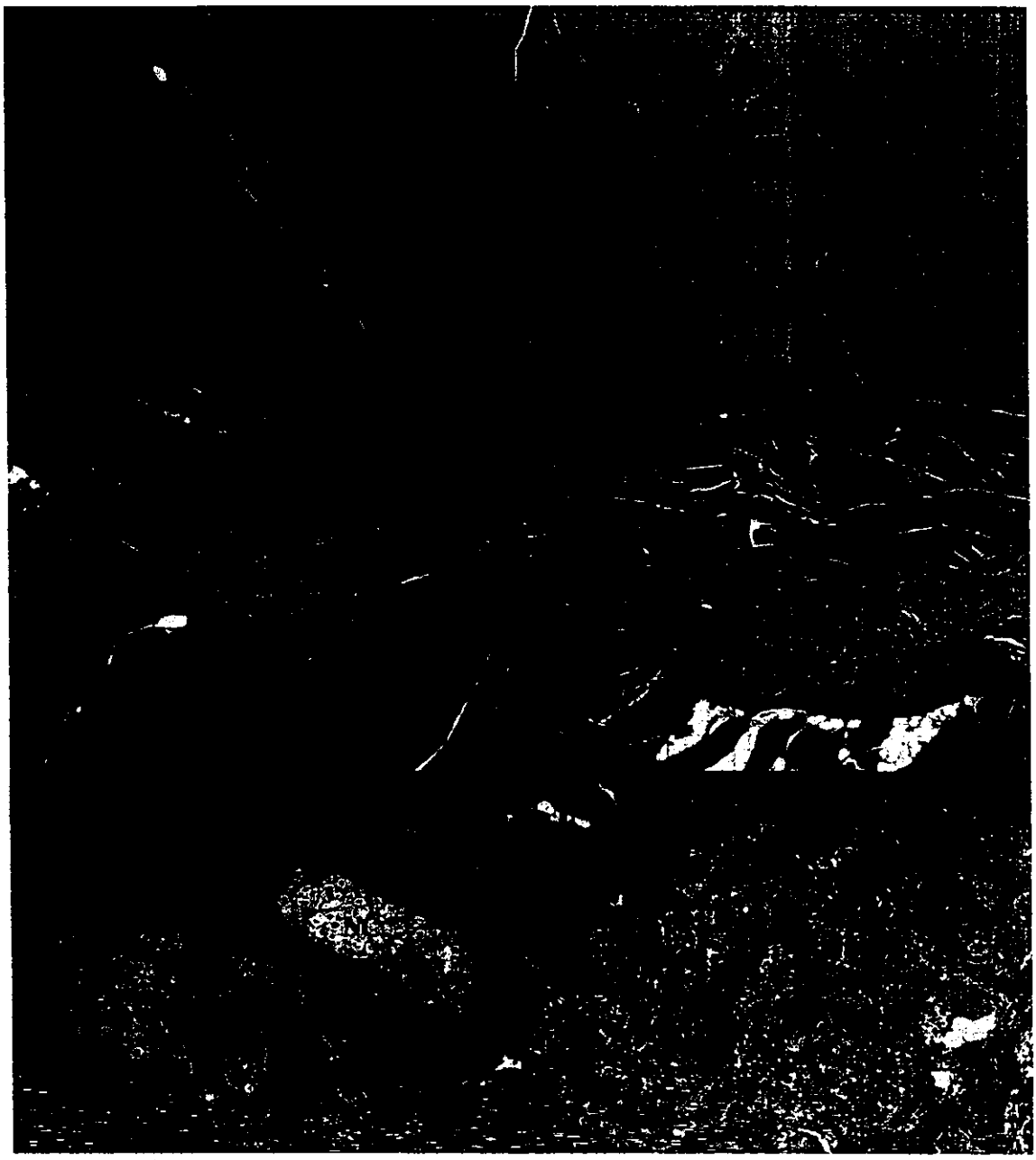

Fig. 4. Histological features of new bone formation at 21 days after treatment (M. host calf muscle; NC, newly induced cartilage; NB, newly induced bone; BM, bone marrow; hematoxylin-eosin staining. Original magnification, × 25). (A) Group A, (B) Group B, (C) Group C, (D) Group D, (E) Group E, (F) Group F.

*In vivo study*

*Radiographic Findings*

All rats survived until the scheduled date of sacrifice with no apparent complications. On day 21, soft X-rays revealed opacities in Groups C, D, E, and F (Fig. 3). The area of opacity on the X-ray film increased in the order of Group F, E, and D. The area did not differ between Groups C and D. However, opacities were not observed, in Groups A and B.

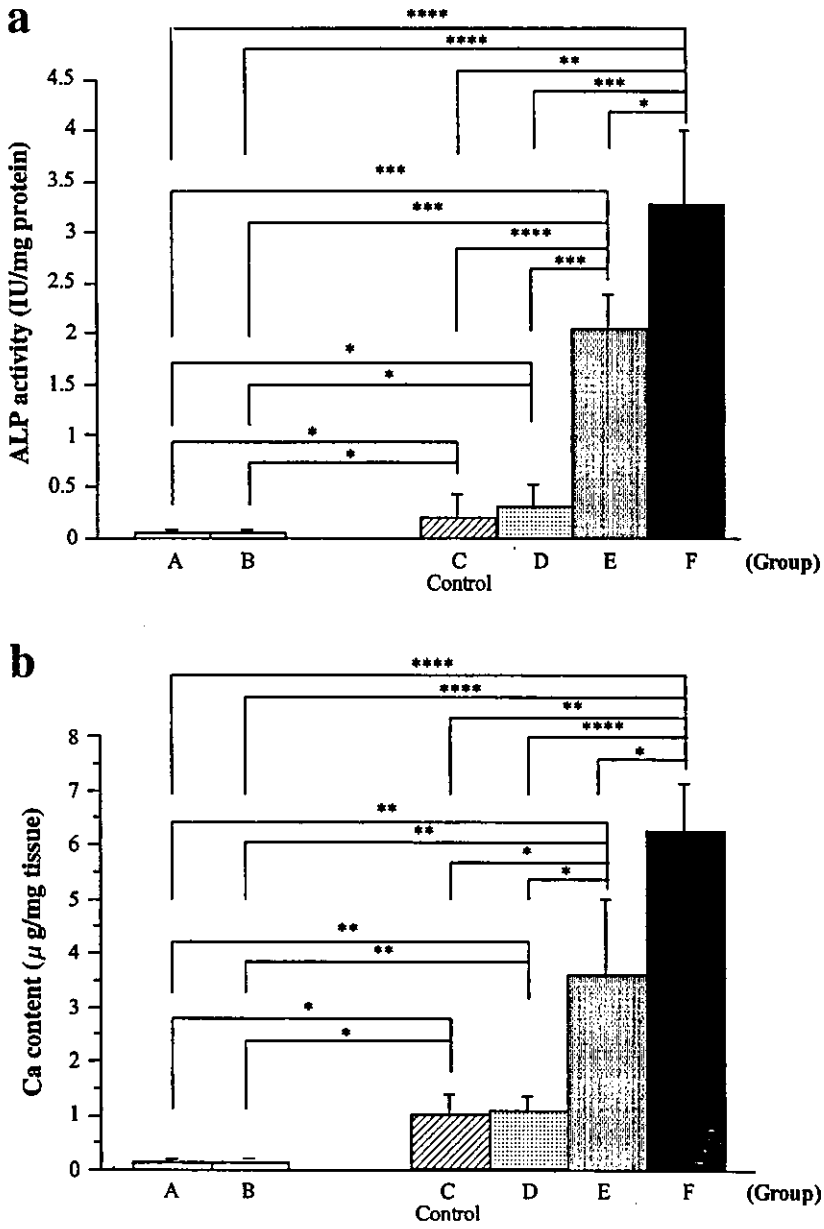


Fig. 5. The ALP activity (a) and Ca content (b) at 21 days after treatment in vivo. Data are the mean ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, ****$P < 0.0005$.

*Histological Findings*

Light-microscopic examination disclosed new bone formation in Groups C, D, E, and F (Fig. 4C–F). However, there was no evidence of osteoinduction in Groups A and B (Fig. 4A and B). Osteoclasts and lining osteoblasts were observed around the trabecular bone in Groups C, D, E, and F. The trabecular bone area increased in the order of Group F, E, D, and C. The bone marrow area including fatty marrow was increased in the order of Group F, E, D, and C.

*Biochemical Indices*

The ALP activity and Ca content on day 21 after the infection are shown in Fig. 5a and b, respectively. In Groups A and B, the ALP activity and Ca content showed little elevation. Both the ALP activity and Ca content were significantly higher in Groups F and D than in Groups C. There was no significant difference between Groups C and D.

## Discussion

Takao et al. [3] previously reported that recombinant BMP-3b expressed in CHO cells did not induce ectopic bone formation, but rat BMP-3b mRNA expression induced bone tissue and cartilage. Hino et al. [15] reported that BMP-3b gene expression was closely associated with the differentiation of primary osteoblastic cells. They suggested that BMP-3b plays an important role in bone formation, remodeling and differentiation of osteoblasts. In the present study, we constructed a rat BMP-3b-expressing recombinant adenoviral vector (AxCAKBMP-3b). We have previously reported on the effectiveness of gene transfer by a BMP-2-expressing recombinant adenoviral vector (AxCAOBMP-2) into C2C12 cells and the usefulness of this vector for osteoinduction in vivo [11,12]. In the present study, we recognized that AxCAKBMP-3b had the same gene transfer capacity as AxCAOBMP-2. However, AxCAKBMP-3b itself could not convert C2C12 cells into the osteoblast lineage cells and produce bone matrix. These findings suggested that the BMP-3b gene has no osteogenic activity. Accordingly, we examined the effect of double infection with AxCAOBMP-2 and AxCAKBMP-3b in these cells and intramuscular infection in nude rats. As shown in Fig. 2, C2C12 cells infected with AxCAOBMP-2 and AxCAKBMP-3b at an MOI of 16-fold that of AxCAOBMP-2 produced more ALP and secreted more osteocalcin into the culture medium than cells infected with AxCAOBMP-2 and AxCAwt at the same MOI as AxCAKBMP-3b. In our preliminary experiments, we examined the ALP activity and osteocalcin production in C2C12 cells infected with AxCAOBMP-2 at MOIs of 0.5 and 1.0 and with AxCAKBMP-3b and AxCAwt at 16-fold the MOI of AxCAOBMP-2 alone. The ALP activity and osteocalcin production in cells infected with AxCAOBMP-2 and AxCAKBMP-3b were significantly higher than in cells treated with AxCAOBMP-2 and AxCAwt (data not shown). These results suggest that the BMP-3b gene stimulated the ability of converting these cells to the osteoblast lineage by BMP-2. Moreover, as shown in Figs. 3–5, bone formation at the intramuscular site injected with AxCAOBMP-2 and AxCAKBMP-3b at titers of 4- and 16-fold that of AxCAOBMP-2 was significantly greater than in the control group (AxCAOBMP-2 and AxCAwt at a titer of 16-fold that of AxCAOBMP-2). These findings also suggest that the BMP-3b gene stimulate the osteoinductive activity of BMP-2 in vivo.

Recombinant adenovirus has certain advantages as a vector system for gene transfer. However, the adenovirus vector often stimulates an immune response to the infected cells and results in loss of therapeutic gene expression by 1–2 weeks after infection [16,17]. To prevent immune response to the

infected virus, we used nude rats in this study. In addition, the infected genes are not integrated into the host chromosomes, so the expression of gene transferred via an adenovirus vector is only transient. We could only detect a transient effect of BMP-3b. However, Okubo et al. [11,12] previously reported that C2C12 cells were converted into cells of the osteoblast lineage on day 5 after treatment with AxCAOBMP-2 and that ectopic bone formation was seen on day 21 after intramuscular injection in Wistar rats using immunosuppressive drug, cyclophosphamide [12]. These results suggested that the effect of BMP-3b on osteoinduction mediated by AxCAOBMP-2 was demonstrated in the present study. However, the mechanism is still unknown as to why BMP-3b lacking osteoinductive activity can assist osteoinduction by BMP-2. Moreover, BMP-3b is closely related to BMP-3 and it is speculated that the biological activities of BMP-3b are similar to those of BMP-3. Daluiski et al. [18] reported that BMP-3 is an antagonist of osteogenic BMPs, so it seems possible that BMP-3b may not exert its effects by activating BMP-2 signaling but rather via other pathways.

In summary, our in vitro and in vivo studies demonstrated that an appropriate amount of BMP-3b could increase the bone-inducing activity of BMP-2. The amount of required BMP-2 for induction of bone is much greater than the amounts of other cytokines and growth factors that are needed to express their activities [19]. The present findings raise the possibility that BMP-3b may be able to compensate for this deficiency of BMP-2.

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# A Case of Parathyroid Carcinoma with Suppression of Serum Parathyroid Hormone (PTH) Level After Vitamin $D_3$ Pulse Therapy

Yasato Komatsu[1)]　Ran Nakajima[1)]　Akihiro Yasoda[1)]
Ami Ozasa[1)]　Yuko Nakatsuru[1)]　Hiroshi Arai[1)]
Kazuwa Nakao[1)]　Chohei Shigeno[2)]

[1)]Department of Endocrinology and Metabolism, Kyoto University Graduate School of Medicine
[2)]Department of Radiology, JT Kyoto Hospital

Parathyroid carcinoma is one cause of primary hyperparathyroidism, a condition in which there is hypercalcemia and deregulation of secretion of PTH. As in normal parathyroid cells, PTH secretion is down-regulated by 1, $25(OH)_2$ vitamin $D_3$, the vitamin $D_3$ pulse therapy is commonly applied to the treatment of the patients with secondary hyperparathyroidism related to chronic renal failure. We report the case of parathyroid carcinoma treated with vitamin $D_3$ pulse therapy with suppression of serum parathyroid hormone level. A 52-year old woman was admitted with hypercalcemia, marked elevation of PTH, and general fatigue. At the age of 45 and 50, neck operations were done for the treatment of parathyroid carcinoma. After the second operation, serum calcium was decreased from 18.3mg/dL to 9.8mg/dL, and intact PTH (iPTH) was decreased 1230pg/mL to 359pg/mL. As 7 months after the second operation, serum calcium was elevated to 13.2mg/dL, and iPTH was elevated to 872pg/mL, we performed the vitamin $D_3$ pulse therapy, oral administration of calcitriol 4 $\mu$g twice a week. To control the serum calcium level, intravenous administration of alendronate was done once a month. After one month of treatment, iPTH was suppressed to 337pg/mL. During one year of treatment, serum calcium has been 12-14mg/dL, and iPTH has been 400-600pg/mL. No adverse clinical effects have been observed. No apparent recurrence of parathyroid carcinoma has been detected with MRI, CT, MIBI scintigraphy, or FDG-PET. Vitamin $D_3$ pulse therapy appears to have been effective at controlling parathyroid carcinoma for this patient.