further investigations are needed to confirm these findings.

AM has been shown to be widely distributed in gastrointestinal tissues including the stomach, duodenum, jejunum, ileum, colon, and rectum [16,17]. AM receptor expression has also been shown to be widely distributed in many of these organs and tissues [18,19]. It is therefore believed that AM can act directly on these tissues.

Indeed, we are currently working on clarifying the mechanisms by which AM protects from colitis. In the present study, we demonstrated a protective effect of AM treatment against colitis induced by TNBS and DSS in rats. These findings indicate that the anti-inflammatory properties of AM make it an effective candidate therapeutic agent for the treatment of IBD. Further studies of AM treatment will expand on these findings.

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Gender-related alterations in plasma adrenomedullin level and its correlation with body weight gain

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Abstract

Plasma levels of adrenomedullin (AM), a bioactive peptide produced in adipose tissue, have been shown to be higher in obese patients than in non-obese patients, but little is known about gender differences in plasma AM levels. The aims of this study were to clarify gender-related alterations in plasma AM levels and to examine the body weight (BW) gain–plasma AM relationship in the general population. We measured plasma AM levels of 346 local residents (62.0±8.9 years, mean±S.D.) in the Kiyotake area, Japan, who underwent a regular health check-up, by a specific fluorescence immunoassay. Plasma AM levels in the female residents were lower than that in the males, and multiple regression analysis revealed a possible gender difference in plasma AM. The AM levels were significantly correlated with BMI or waist circumference in women, but such a relationship was not seen in men. When the subjects were divided into two groups by results of a questionnaire about BW gain of 10 kg or more since the age of 20 years, the plasma AM level of women with BW gain ≧10 kg was significantly higher than that in those without BW gain, although no difference was noted between the men with and without BW gain. In conclusion, possible gender differences were noted in the plasma AM levels and in the BW gain–plasma AM relationship in the general population. The plasma AM levels in the female residents without BW gain seem partly attributable to the lower AM of women.

Key Words
- adrenomedullin
- plasma level
- gender difference
- body weight gain

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Introduction

Adrenomedullin (AM) is a potent vasodilator peptide first isolated from human pheochromocytoma tissue, although this peptide has also been shown to be present in a wide range of human tissues or organs: the adrenal medulla, heart, blood vessels, kidneys, and adipose tissues (1, 2, 3). A number of functional analysis studies have been carried out since its discovery, revealing that AM has pleiotropic actions, including blood pressure-lowering effects, natriuresis, cardiovascular protection, and alleviation of insulin resistance (4, 5, 6, 7). On the other hand, AM was shown to circulate in human blood, and its plasma levels are elevated in diseases such as hypertension, heart failure, and sepsis (2, 8). Plasma AM levels have also been found to be higher in patients with obesity than in control subjects, suggesting the active production and secretion of AM from human adipose tissue (6, 9).

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Consistent with this, Nambu *et al.* (10) reported that mice fed a high-fat diet showed augmented AM expression in the fat tissues and elevated plasma levels concomitant with increased body weight (BW). As inflammatory cytokines, including tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL1β), were reported to increase AM production, low-grade inflammation in fat tissues associated with obesity is assumed to be involved in the increased expression of AM (6, 8, 11). When evaluating plasma levels of endogenous bioactive substances, we sometimes need to take gender-related alterations into account. For instance, plasma levels of brain natriuretic peptide (BNP), a bioactive peptide exerting vasodilatory and blood pressure-lowering effects, are higher in women than in men (12). Another example is the renin–angiotensin–aldosterone system: estrogen, a sex steroid hormone, has been shown to modulate this system in various manners (13). Meanwhile, as indicated by a search of the literature, little is known about gender-related alterations in plasma AM levels or AM production. The aims of this study are thus to clarify whether there are gender-related alterations in plasma AM level and to determine the relationship between BW gain and plasma AM levels in human subjects by examining local residents in a Japanese community using a newly developed, automated, AM-specific fluorescence immunoassay. In addition to AM, we measured plasma levels of BNP and an N-terminal fragment of the BNP precursor (NT-proBNP) in order to compare gender- or BW gain-related alterations in AM with those of the BNP peptides.

Materials and methods

Study subjects and protocol

Local residents of the Kiyotake area, Miyazaki, Japan, who underwent an annual regular health check-up as part of a specific health program of the Japanese government from 2008 to 2013, were randomly selected for this study (172 males and 174 females; 62.0±8.9 years, mean±S.D.). Upon visiting the community center of Kiyotake Town, the medical history of the residents, which included the questionnaire of whether or not they have had BW gain of 10 kg or more since 20 years of age, was taken by nurses, and blood pressure was measured with an oscillometric automatic device (BP-103iII, Colin, Nagoya, Japan) in a sitting position. The history taken was confirmed by physicians, who then carried out physical examination including auscultation. Exclusion criteria were as follows: first, residents with medical history, symptoms, or signs indicative of any heart disease were excluded because the plasma levels of AM, BNP, and NT-proBNP were shown to be elevated in patients with ischemic heart disease or heart failure (8, 14). Second, renal function is an important determinant of those peptide levels (14, 15), therefore we excluded residents with estimated glomerular filtration rate (eGFR) of 30 ml/min per 1.73 m^2 or lower. eGFR was calculated with the following formula of the Japanese Society of Nephrology (16): $194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287}$ ml/min per 1.73 m^2, further multiplied by 0.739 for women. Lastly, because elevated blood glucose was shown to increase plasma AM levels (17, 18), we excluded residents whose HbA1c was 6.5% or higher, so as not to select those with uncontrolled diabetes mellitus.

This study was approved by the Review Committee for Cooperative and Commissioned Research and the Ethics Committee of the University of Miyazaki Faculty of Medicine. All subjects examined gave their written informed consent before participating in this study.

Measurements of bioactive peptides in plasma

To measure the plasma levels of AM, BNP and NT-proBNP after overnight fasting, blood from an antecubital vein was collected into tubes with 1.0 mg/ml EDTA-2Na and 500 kallikrein inhibitory units (KIU)/ml of aprotinin. Plasma was obtained by centrifugation at 1710 ***g*** for 10 min at 4 °C and stored at −30 °C until the assay. Plasma levels of AM were measured by a specific fluorescence immunoassay (Tosoh Corporation, Tokyo, Japan) with two independent antibodies: one binds to the ringed structure and the other to the middle region between the ring and C-terminal portions of the peptide, as previously described (9, 19). The AM assay reagent prepared by an immediate freeze-dry procedure was composed of in-house magnetic beads coated with the anti-ringed structure antibody and alkaline phosphatase-labeled anti-C-terminal portion antibody. This assay reagent can be used with a commercially available, automated immunoassay analyzer (AIA-System, Tosoh Corporation). The limits of detection and quantitation of this assay were determined to be 0.133 and 0.085 pmol/l, respectively, according to the Clinical and Laboratory Standards Institute (CLSI) protocols. Intra- and inter-assay coefficients of variation of this assay were 1.8% (n=10) and 5.1% (n=11) respectively. BNP and NT-proBNP levels were determined by chemiluminescent immunoassay (Shionogi & Co. Ltd, Osaka, Japan) and electro-chemiluminescence assay (Roche Diagnostics) respectively (20, 21).

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Statistical analysis

All the data were analyzed using IBM SPSS Software, version 22.0 (IBM, Armonk, NY, USA). Two groups were compared by the unpaired *t*-test or χ^2 test, while multiple comparisons were made by ANOVA followed by Scheffe's test. Simple regression analysis was used to examine the relationships between plasma levels of the peptides and the other parameters, and these relationships were further tested by Spearman's rank correlation coefficient. A multiple linear regression analysis with a stepwise method was used to extract factors significantly associated with the plasma AM levels. All data are expressed as the means±S.D. and $P<0.05$ was considered to be statistically significant.

Results

The basal profiles and peptide measurements of the residents examined in this study are given in Table 1. The plasma level of AM in the female residents was significantly ($P<0.01$) lower than that in the males, while in contrast, the BNP and NT-proBNP levels were slightly higher in women than in men. When men and women were analyzed together by simple regression analysis, the AM levels were significantly correlated with BMI ($r=0.153$, $P<0.01$) and waist circumference (WC; $r=0.132$, $P<0.05$). As there were substantial differences in the basal profiles between the two genders (Table 1), we further analyzed the data to examine whether gender is independently associated with the plasma AM levels by multiple regression analysis with a stepwise method. The parameters included as explanatory covariates in this analysis were the BMI, mean blood pressure, and fasting blood glucose level, because these parameters, in addition to prevalence of hypertension, significantly differed between men and women (Table 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). Also those included were age and eGFR, which have been reported to be the factors influencing plasma AM levels (15, 22). As given in Table 2, although marginally significant ($P=0.043$), gender was extracted as an independent determinant of the plasma AM levels, in addition to BMI and eGFR, in the study subjects.

Table 1 Basal profiles and plasma levels of the peptides of the male and female residents examined in this study. Means±S.D.

	Men	Women
n	172	174
Age (years)	62.1±9.1	61.9±8.6
BMI (kg/m^2)	23.5±2.5	22.0±3.0*
Waist circumference (cm)	85.1±7.1	82.0±9.1*
Mean blood pressure (mmHg)	96±12	90±12*
Fasting blood glucose (mg/dl)	96±13	92±9*
HbA1c (%)	5.5±0.3	5.5±0.3
eGFR (ml/min per 1.73 m^2)	74±14	76±14
AM (pmol/l)	7.14±1.29	6.77±1.18*
BNP (pg/ml)	19.1±23.4	21.7±15.5
NT-proBNP (pg/ml)	60.5±101.8	68.1±48.2

eGFR, estimated glomerular filtration rate; AM, adrenomedullin; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP. *$P<0.01$ vs male residents.

Table 2 Identification of significant factors for plasma AM levels by multiple regression analysis with a stepwise method.

Independent variables	β	P
BMI	0.129	0.022
eGFR	−0.119	0.028
Gender (male=1 and female=2)	−0.114	0.043

eGFR, estimated glomerular filtration rate.

We then analyzed the data of the male and female residents separately. The relationships between plasma levels of the peptides and the BMI or WC are given in Table 3 as Pearson's correlation coefficients (*r*). The plasma levels of AM were found to be correlated with the BMI and WC in women, but such a relationship was not detected in men, as also shown in Fig. 1A and B. These results were confirmed by Spearman's rank correlation coefficient, which showed significant relationships between the plasma AM and BMI or WC in women but not in men (data not shown). In contrast to AM, as given in Table 3, inverse correlations were found between the plasma levels of BNP or NT-proBNP and the BMI or WC in women.

Basal profiles of the study subjects with or without BW gain of 10 kg or more since 20 years old are given in Table 4. Compared with the residents without BW gain, significantly higher ($P<0.01$) values were noted in the BW, BMI, and WC in those with BW gain, although there were no significant differences in the other clinical parameters, including age, blood pressure, blood glucose, HbA1c, and renal function, between the two groups. Prevalence of hypertension in the male residents was higher than that in the females (Supplementary Table 1), but when compared within the same gender, no differences were noted for hypertension, dyslipidemia, or diabetes mellitus (Supplementary Table 2, see section on supplementary data given at the end of this article). As given in Table 5, when comparing the residents with and without BW gain, we failed to detect a difference in the plasma AM levels of the males, but found a significantly

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Table 3 Correlation coefficients (*r*) of simple regression analysis for relationships between BMI or WC and AM, BNP, or NT-proBNP.

	Men		Women	
r	BMI	WC	BMI	WC
AM	0.002	0.008	0.231†	0.195*
BNP	−0.009	0.027	−0.151*	−0.156*
NT-proBNP	0.012	0.057	−0.155*	−0.108

AM, adrenomedullin; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP; WC, waist circumference. *$P<0.05$ and †$P<0.01$, Pearson's correlation.

higher level of plasma AM in the female residents with BW gain ($P<0.01$). In comparison between the two genders without BW gain, the plasma AM level in women was significantly lower than that in men ($P<0.01$). The plasma levels of BNP and NT-proBNP in the male and female residents with BW gain were slightly lower than that in those without, but the differences did not reach statistically significant levels. As expected, the plasma levels of two BNP peptides were slightly, but not significantly, higher in the female residents than that in the males, irrespective of BW gain.

Discussion

Plasma levels of AM, a bioactive peptide with pleiotropic actions, are increased in various human diseases including hypertension, heart failure, and obesity (3, 8), but little is known about gender-related differences in plasma AM. According to an animal study, BW gain via a high-fat diet resulted in augmented AM expression in adipose tissue with concomitant elevation of plasma AM levels in rats (10), but BW gain-induced elevation of plasma AM level has not been proven in humans yet. Examining the general population in this study, we revealed that i) plasma AM levels in women might be lower than that in men; ii) AM levels in women are associated with BW gain and a possible gender-related alteration is noted in the plasma AM–BW gain relationship; and iii) the lower AM levels in women are likely due to those without BW gain.

An important issue that arises in this study is the mechanism for the closer relationship between BW gain and the plasma AM levels in the female residents compared with that in the males. Currently, there is no clear explanation for this, but we can discuss some possibilities based on previous reports. It has been shown that the factors affecting plasma AM levels in humans without overt cardiovascular or renal diseases are age, BMI, blood pressure, and renal function (6, 22). In this study, no differences were noted in those parameters between the subjects with or without BW gain in both genders, except for BW, BMI, and WC.

As fat tissue appears to be an organ contributing to AM circulation in human blood (10, 23), it is possible that a gender difference in BW gain-induced production of AM in the adipose tissue accounts for the present phenomenon. According to a report by Paulmyer-Lacroix *et al.* (23), expression of AM is augmented in the omental adipose tissue of obese women compared with that in the non-obese. In this study, the BW gain-induced elevation of plasma AM may have resulted from increased expression of AM in the visceral fat of the female residents. This is unlikely to be the case in male residents because there was no difference in the plasma AM levels in those with or without BW gain, despite the substantial differences in BW, BMI, and WC; however, there have been no reported studies comparing AM expression in adipose tissue between non-obese and obese men.

Low-grade inflammation in adipose tissues associated with obesity seems involved in the mechanism of the increased plasma AM level in obese subjects, because the AM production is up-regulated by inflammatory cytokines such as TNFα or IL1β (6, 8, 9, 11). It was reported that body fat distribution differs from between two genders: ratios of the visceral fat to the subcutaneous or lower body fat mass were higher in men than in women (24). According to an epidemiological study by Pou *et al.* (25), increased volumes of the visceral and subcutaneous fat were associated with elevation of inflammatory markers, while the former was more closely related to these markers than the latter. In this context, the intimate relationship between the plasma AM and BW gain in women of this study is somehow contradictory. Although there are no data available about menopausal state in this study, it seems

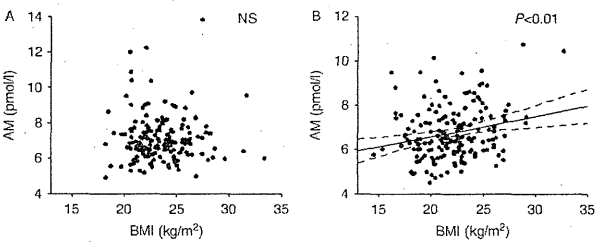

Figure 1
Relationships between BMI and plasma AM levels in the male (A) and female (B) residents. The regression line and the 95% confidence limits are shown by solid and broken lines respectively. NS, not significant.

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Table 4 Basal profiles of the male and female residents with or without BW gain of 10 kg or more. Means±s.D.

	Men		Women	
BW gain ≧10 kg	(−)	(+)	(−)	(+)
n	112	60	122	52
Age (years)	62.7±9.4	61.1±8.4	61.3±8.8	63.2±8.1
BW (kg)	60.7±7.3	69.7±8.6*	49.1±5.9	56.8±7.0*
BMI (kg/m^2)	22.4±1.9	25.5±2.5*	21.0±2.5	24.4±2.6*
Waist circumference (cm)	82.3±5.6	90.4±6.6*	79.3±8.0	88.4±8.0*
Mean blood pressure (mmHg)	95±11	98±13	89±12	91±11
Fasting blood glucose (mg/dl)	96±15	97±11	91±8	94±10
HbA1c (%)	5.4±0.3	5.5±0.3	5.4±0.2	5.5±0.3
eGFR (ml/min per 1.73 m^2)	73±14	75±14	76±13	76±15

eGFR, estimated glomerular filtration rate. *P<0.01 vs without body weight (BW) gain ≧10 kg.

unlikely that sex steroids are involved in the gender difference in BW gain-induced AM production: neither testosterone nor estradiol has much effect on AM production (26). Clearly, further studies are necessary to clarify the mechanism behind the gender difference in BW gain-induced alteration in plasma AM levels.

BNP has natriuretic and vasodilatory effects, exerting cardiovascular protective actions, and plasma levels of BNP are elevated in patients with hypertension and heart failure, as are those of AM (14). The increased BNP levels are thought to be a mechanism counteracting blood pressure elevation and excess body fluid retention in patients with hypertension or heart failure (12, 14). In contrast to these phenomena, plasma BNP level has been shown to be decreased in obesity, where reduced BNP action is assumed to be involved in BW gain-induced elevation of blood pressure (27). Indeed, higher BNP or NT-proBNP levels were found to be associated with favorable adipose tissue distribution by a population-based study (28). Chainani-Wu *et al.* (29) reported increased plasma BNP levels in obese patients with coronary heart disease (CHD) or high risk of CHD following comprehensive life style modification, suggesting that the BNP elevation associated with BW reduction does not necessarily indicate deterioration of heart disease.

In this study, consistent with the notions discussed above, plasma levels of BNP and NT-proBNP in the subjects with BW gain were slightly lower than in those without BW gain in both genders. In addition, plasma levels of these peptides were inversely correlated with BMI or WC in the female subjects; however, the differences between the residents with or without BW gain were not statistically significant in both genders. This study also showed the higher plasma BNP and NT-proBNP levels in women than in men, a finding accordant with the previous notions (12), while those gender difference were less clear as compared with plasma AM. Thus, the present results suggest that both gender- and BW gain-related alterations in plasma levels of the peptides are clearer in AM than in BNP.

Next, we need to discuss the biological or clinical significance of the present findings. AM has been shown to exert a wide range of biological actions including blood pressure lowering, cardiovascular protection, and alleviation of insulin resistance (3, 5). As mentioned above, in the case of BNP, BW gain-related reduction in plasma BNP levels is assumed to be involved in obesity-induced elevation of blood pressure (27). In contrast to this, we speculate, based on the AM actions, that BW gain-induced increase in AM level in the female subjects is a counter-regulatory mechanism against obesity-related disorders such as insulin resistance and hypertension.

Lastly, there are limitations we need to mention in this study. First, a lack of statistical power may need to be taken into account, because we examined a relatively small number of subjects with BW gain data based on the simple questionnaire. For example, differences in the plasma BNP or NT-proBNP levels between two genders or between those with and without BW gain were statistical insignificant. Meanwhile, a significant finding of this study is that the gender-related alterations were clearly seen in the plasma levels of AM despite insignificant differences in those of the BNP peptides. Second, we have been unable to completely exclude residents with inflammatory, respiratory, or liver diseases, which had possibly affected the AM measurement from the study subjects (3, 6, 8). In our health check-up, when physicians notice

Table 5 Plasma levels of AM, BNP, and NT-proBNP of the male and female residents with or without BW gain of 10 kg or more. Means±s.D.

	Men		Women	
BW gain ≧10 kg	(−)	(+)	(−)	(+)
AM (pmol/l)	7.11±1.22	7.20±1.42	6.53±1.02†	7.34±1.33*
BNP (pg/ml)	19.6±26.8	18.2±15.5	22.8±15.9	19.2±14.4
NT-proBNP (pg/ml)	64.7±120	52.6±51.8	70.4±47.8	62.6±49.4

AM, adrenomedullin; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP. *P<0.01 vs without body weight (BW) gain in the identical gender and $^\dagger P$<0.01 vs men without BW gain.

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possibilities of these diseases in history taking or physical examination, they are supposed to describe it on the medical files; but there were no reports about such a disease. Thirdly, this study lacks parameters or clinical tests, with which we could seek further the relationships between AM levels and low-grade inflammation associated with obesity or alterations of body fat distribution (6, 11), such as C-reactive protein and magnetic resonance imaging, and these points need to be clarified in future studies.

In summary, there appear to be gender-related differences in the plasma AM levels and in the BW gain–plasma AM relationship in the general population. The AM levels in the female residents without BW gain during the adolescent period were partly attributed to the lower plasma AM of women.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EC-14-0131.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Biological properties of adrenomedullin conjugated with polyethylene glycol

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ABSTRACT

Adrenomedullin (AM) is a vasodilator peptide with pleiotropic effects, including cardiovascular protection and anti-inflammation. Because of these beneficial effects, AM appears to be a promising therapeutic tool for human diseases, while intravenous injection of AM stimulates sympathetic nerve activity due to short-acting potent vasodilation, resulting in increased heart rate and renin secretion. To lessen these acute reactions, we conjugated the N-terminal of human AM peptide with polyethylene glycol (PEG), and examined the biological properties of PEGylated AM in the present study. PEGylated AM stimulated cAMP production, an intracellular second messenger of AM, in cultured human embryonic kidney cells expressing a specific AM receptor in a dose-dependent manner, as did native human AM. The pEC50 value of PEGylated AM was lower than human AM, but no difference was noted in maximum response (Emax) between the PEGylated and native peptides. Intravenous bolus injection of 10 nmol/kg PEGylated AM lowered blood pressure in anesthetized rats, but the acute reduction became significantly smaller by PEGylation as compared with native AM. Plasma half-life of PEGylated AM was significantly longer than native AM both in the first and second phases in rats. In summary, N-terminal PEGylated AM stimulated cAMP production in vitro, showing lessened acute hypotensive action and a prolonged plasma half-life in comparison with native AM peptide in vivo.

1. Introduction

Adrenomedullin (AM) is a pluripotent bioactive peptide initially isolated from human pheochromocytoma, while AM circulates in the blood and is expressed in a number of tissues and organs, including the heart and blood vessels in humans [9–11,13,14]. The characteristic feature initially found was blood pressure-lowering action due to potent vasodilation [14], but AM has been shown to possess various biological properties: inhibition of cardiac hypertrophy and fibrosis, inhibition of vascular remodeling, neovascularization, inhibition of apoptosis, and anti-inflammation [9–11,13]. A number of experimental studies have been carried out to test where this endogenous peptide exerts beneficial effects by using animal models of various human diseases [1,2,9,10,19]. For example, AM was shown to inhibit cardiac remodeling following acute myocardial infarction and to alleviate hind limb ischemia in rats or mice, and also suppressed inflammatory cytokines, facilitating the healing of colon ulcer, in rat models of inflammatory bowel diseases [1,2,9,10,19]. These findings suggest the potential of AM as a therapeutic tool in the treatment of ischemic heart disease, peripheral vascular disease, and inflammatory bowel diseases, and indeed, AM was shown to be effective in treating patients with those diseases [3,8]. Meanwhile, AM should be infused continuously with careful dose settings in human patients because the short-acting potent vasodilator property of AM could result in acute hypotension, activated sympathetic nerve activity, and increased renin secretion [12]. In an attempt to reduce these acute unfavorable actions, we synthesized molecularly modified AM by conjugating the N-terminal of the peptide with polyethylene glycol (PEG), and characterized the biological effects of the PEGylated AM peptide using cultured cells in vitro and anesthetized rats in vivo.

Abbreviations: AM, adrenomedullin; PEG, polyethylene glycol; Boc, t-butyloxycarbonyl; HEK, human embryonic kidney; CLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein.

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2. Materials and methods

2.1. Preparation of peptide

N^{ε}-t-butyloxycarbonyl-lysyl^{25,36,38,46} human AM (H_2N-[Lys(Boc)]$_4$-human AM) was chemically synthesized by the solution method with the Boc strategy according to a previous report [6]. H_2N-[Lys(Boc)]$_4$-human AM solved in dimethyl sulfoxide (DMSO) was reacted overnight with PEG5000-NHS (SUNBRIGHT ME-050-HS; NOF Corporation, Tokyo, Japan) at room temperature in the presence of diisopropylethylamine. PEG5000-[Lys(Boc)]$_4$-human AM was purified by high-performance liquid chromatography, and then N-terminal PEGylated human AM peptide was obtained by treatment with 95% trifluoroacetic acid for 40 min and further purification with high-performance liquid chromatography.

2.2. Cell culture experiments

To test the pharmacological effects of PEGylated AM in vitro, we used human embryonic kidney (HEK)-293 cells stably expressing the AM type I receptor (AM1 receptor), which had been prepared as previously described [16]. This receptor subtype is highly specific to AM and formed by co-expression of calcitonin receptor-like receptor (CLR) and activity-modifying protein-2 (RAMP2) [15,18]. The HEK-293 cell were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G, 100 units/ml streptomycin, 500 μg/ml amphotericin B, 0.4 mg/ml hygromicin B, and 0.25 μg/ml geneticin in a 24-well plate coated with human fibronectin (Invitrogen) in 5% CO_2 at 37 °C. After culturing for 3 days, 90% confluent cells were subjected to experiments stimulating intracellular cAMP accumulation by PEG-conjugated or native AM peptides. The culture media were replaced with Hanks' buffer containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 0.1% bovine serum albumin. The cells were then incubated with the indicated concentrations of the peptides in the presence of 0.5 mM isobutylmethylxanthine for 15 min at 37 °C. The reactions were terminated by the addition of cell-lysis buffer, and cAMP levels of the supernatants were measured with an enzyme immunoassay kit (GE Healthcare UK Limited, UK). The pEC50 value and maximum response (Emax) were calculated based on intracellular cAMP concentrations stimulated by the AM peptides at 10^{-11} to 10^{-6} mol/L.

2.3. Animal experiments

Animal experiments were performed in accordance with the Animal Welfare Act and with approval from the University of Miyazaki Institutional Animal Care and Use Committee (No. 2012-501-3). Male Wistar rats of 7–8 weeks of age were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and maintained under a 12-h light/dark cycle in specific pathogen-free conditions with standard chow. After anesthetizing rats by inhalation of 1.5–2.5% isoflurane at a flow volume of 0.6–0.8 L/min, tracheotomy was performed and the trachea was intubated with a PE-240 catheter. A PE-10 catheter was inserted into the left jugular vein for intravenous injection of AM peptides. Similarly, the carotid artery was cannulated with a PE-50 catheter for either blood pressure monitoring or blood sampling before and after peptide injections.

In the blood pressure-monitoring experiment, the catheter inserted into the carotid artery was connected to a pressure transducer (MLT0699; ADInstruments, Australia), the outputs of which were analyzed with a blood pressure-monitoring system (PowerLab and LabChart; ADInstruments) before and after intravenous bolus injections of 3 or 10 nmol/kg PEGylated AM or native human AM peptides. Mean blood pressure prior to the injections was 88.9 ± 2.1 mmHg (mean ± S.D.) and, throughout the experiment, 0.9% saline was infused at a constant rate of 4.8 ml/kg/h. To determine plasma half-lives, either PEGylated or native AM was injected through the jugular vein catheter. Three hundred microliters of blood were drawn via the carotid artery with 21 μg aprotinin and 0.3 mg EDTA-2Na at the indicated time points, and plasma samples were obtained by centrifugation at 3000 rpm. Human AM levels in plasma were measured by a fluorescence enzyme immunoassay using two anti-human AM antibodies: one binds to the ringed structure and the other to the amidated C-terminal of the AM peptide [20]. Cross-reactivity of this method for PEGylated AM was found to be 78% on a molar basis by assaying PEGylated peptide added to rat plasma.

2.4. Statistical analysis

All data were analyzed with IBM SPSS software version 21.0 (IBM, Armonk, NY, USA). Plasma half-lives of the AM peptides were calculated by the two-compartment model. Unpaired *t*-test was used to compare two parameters, and multiple comparisons were made by analysis of variance (ANOVA) followed by Tukey's HSD test. All data are expressed as the means ± S.E.M., unless otherwise indicated, and $P < 0.05$ was considered to be significant.

3. Results

First, we tested the biological activity of PEGylated AM to stimulate intracellular accumulation of cAMP in HEK-293 cells stably expressing AM1 receptor. As shown in Fig. 1, native human AM peptide elevated intracellular cAMP levels in a dose-dependent manner with pEC50 and Emax values of 8.59 ± 0.90 and 9.30 ± 0.26 nmol/well, respectively. PEGylated AM exerted similar effects, augmenting cAMP production with pEC50 of 8.19 ± 0.10 and Emax of 9.44 ± 0.30 nmol/well. pEC50 of PEGylated AM was significantly ($P < 0.05$) smaller than that of native AM, while Emax values of the two forms of peptide were similar.

Fig. 2 shows the time course of mean blood pressure following bolus intravenous injection of PEGylated or native human AM into anesthetized rats. At the dose of 3 nmol/kg, slight blood pressure reductions were observed, while no significant differences were noted in the hypotensive effects between PEGylated and native peptides. Meanwhile, injection of 10 nmol/kg native AM resulted in a substantial reduction of mean blood pressure of −24.5 ± 3.2 mmHg at 2 min, followed by a gradual rise. An identical dose of PEGylated AM exerted hypotensive effects, which were significantly less than native AM 2 and 4 min after injection. This difference in the hypotensive effects became insignificant at 6 min

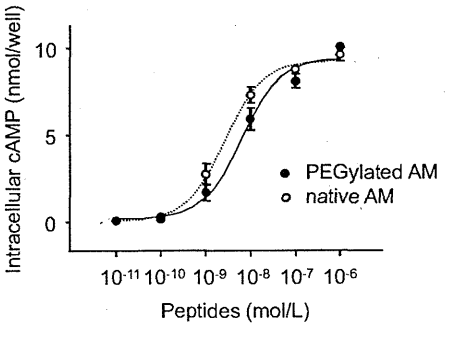

Fig. 1. Intracellular cAMP accumulation by PEGylated or native AM in cultured cells. HEK-293 cells stably expressing AM1 receptor were incubated with the indicated concentrations of PEGylated or native human AM peptides for 15 min, and intracellular cAMP levels were measured with enzyme immunoassay. Data are presented as the means ± S.E.M. of 6 samples examined.

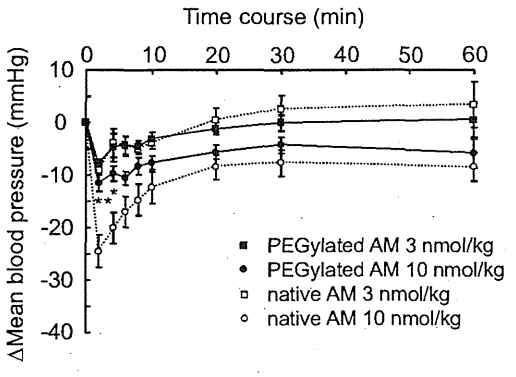

Fig. 2. Reduction in blood pressure following intravenous bolus injection of PEGylated or native AM. Indicated doses of either PEGylated or native human AM peptides were injected intravenously at time 0 into anesthetized rats. Data are presented as the means ± S.E.M. of 5 rats examined. *$P < 0.05$, **$P < 0.01$, compared with an identical dose of native human AM.

or later; meanwhile, in contrast to the groups of 3 nmol/kg, reductions of mean blood pressure by the native AM injection remained larger throughout the experiment in comparison with the PEGylated AM injection. No differences in heart rate were noted among the four groups throughout the experiment.

Fig. 3A and B is plasma disappearance curves of PEGylated and native human AM following bolus intravenous injection of 3 nmol/kg into anesthetized rats. The first and second plasma half-lives of PEGylated AM were 4.87 ± 0.68 and 108 ± 12 min (Fig. 3A), while those of native AM were 0.62 ± 0.02 and 15.2 ± 1.9 min (Fig. 3B), respectively. The half-lives of the PEGylated peptide were significantly prolonged when compared with the native peptides ($P < 0.05$).

4. Discussion

Consisting of 52 aminoacids, AM is an endogenous bioactive peptide with a wide range of biological actions [13,14]. Both amidated C-terminal Tyr^{52} and a ringed structure formed by Cys^{16}-Cys^{21} were shown to be essential for the actions of AM [13,14]. We therefore conjugated Tyr^{1} of the N-terminus of human AM peptide with polyethylene glycol (PEG) and looked at the biological properties of the PEGylated human AM peptide in the present study. PEGylated AM stimulated intracellular cAMP production in cultured cells stably expressing AM type I receptor (AM1 receptor) in vitro, exerting lower acute hypotensive action at a high dose of 10 nmol/kg and a longer plasma half-life in comparison with the native human AM peptide. This is the first report on the pharmacological features of AM molecularly modified by PEG.

Biological actions of AM are mediated by a unique receptor system: calcitonin receptor-like receptor (CLR) can function as AM type 1 and 2 (AM1 or AM2) receptors when co-expressed with receptor activity-modifying protein-2 and 3 (RAMP2 and RAMP3), respectively [15,18]. AM1 receptor (CLR/RAMP2) has been shown to be highly specific to AM in humans and rats [15]. In the present study, we found that PEGylated AM stimulated the production of cAMP, an intracellular second messenger of AM, as did native AM in cultured cells stably expressing the AM1 receptor. Consistent with the previous findings [7], we observed slight reductions of blood pressure following bolus injection of 3 nmol/kg PEGylated or native AM into anesthetized rats. There was no significant difference in blood pressure reduction at a low dose of 3 nmol/kg, but the acute hypotensive action of PEGylated AM was lower than that of native AM at a high dose of 10 nmol/kg. This reduced action of PEGylated AM in vivo is comparable with the result in vitro showing that intracellular cAMP accumulation by PEGylated AM is lower than native AM at 10^{-9} mol/L. According to the results of the plasma half-life study, this concentration of peptide is expected to be reached in the blood of rats at an acute phase following bolus injection in vivo.

A notable finding of this study is that the first and second phases of the plasma half-life of PEGylated AM were prolonged 7.9- and 7.1-fold, respectively. PEGylation has been used as a method to enhance or prolong the pharmacological efficacy of peptides or proteins used to treat patients with various diseases. Examples are interferon-α for viral hepatitis, growth hormone (GH) receptor antagonist for acromegaly, and erythropoietin for renal anemia, while there has been no report on AM peptides [4,17,21,22]. In the present study, the mechanisms of the prolonged plasma half-life of PEGylated AM remain to be specified, while those proposed so far are reduced renal clearance due to a larger molecular size and protection from enzymatic degradation [5]. As shown in Fig. 2, the acute hypotension by native AM was lessened by PEGylation, while it remains unproven in the present study whether the prolonged plasma half-life results in the prolonged duration of the action: no significant differences were noted in the hypotensive effects between native and PEGylated AM at 6 min or later. AM has been shown to exert pharmacological actions, including cardiovascular protection, angiogenesis or anti-inflammation, that seem beneficial in the treatment of particular diseases [1,10,11]. Indeed, there are a number of reports implying the potential of AM as a therapeutic tool for patients with acute myocardial infarction, peripheral vascular disease and inflammatory bowel disease [3,8–10]. Meanwhile, when used as an intravenous agent, AM doses should be carefully monitored, because of the acute hypotensive action which activates sympathetic nerve activity, resulting in increased heart rate and renin secretion [12]. The present findings suggest that PEGylation can lessen these acute unfavorable effects and possibly prolong the duration of the action when compared with native

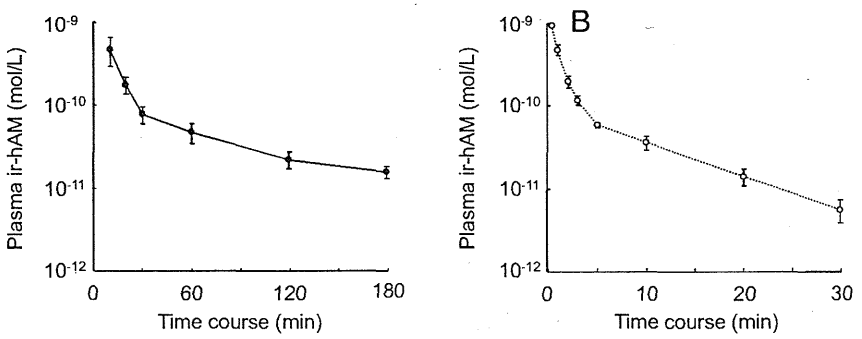

Fig. 3. Plasma disappearance curves of PEGylated (A) and native (B) AM peptides. Either PEGylated or native human AM peptide at a dose of 3 nmol/kg was injected intravenously into anesthetized rats, and blood samples were collected at the indicated time points. Data are presented as the means ± S.E.M. of 3 rats examined.

AM peptide. Further studies will be aimed to test whether the longer plasma half-life can be translated into better outcomes in the above-mentioned, diseased settings.

In summary, PEGylated AM peptide stimulated cAMP production in cultured cells expressing AM1 receptor, as did native human AM peptide, while it showed lower acute hypotensive action and longer plasma half-lives than native peptide. These results suggest the potential of PEGylated AM as a therapeutic tool devoid of the unfavorable effect of acute hypotension of native AM.

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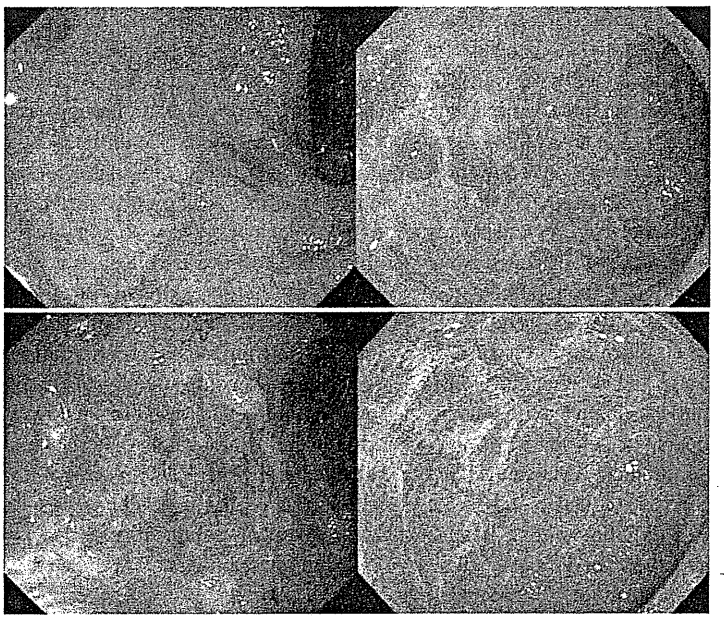

FIGURE 1. Colonoscopic findings. Wide and deep ulcers were observed in the transverse colon (a) and the sigmoid colon (b) before AM therapy. Two weeks after AM therapy (c,d), significant neovascularization and mucosal regeneration were observed at the margin and base of deep ulcers in the transverse colon (c). In the sigmoid colon, fibrosis (scarring) and vasodilation were seen in relatively shallow ulcers. Scarred regions had a reticulated, spider web-like appearance (d).

Adrenomedullin: A Novel Therapy for Intractable Ulcerative Colitis

To the Editor:

In May 2010, a 68-year-old woman undergoing treatment for diabetes presented with a 3-year history of ulcerative colitis (UC). The patient was steroid-dependent, so a regimen of mesa-lazine (5-aminosalicylic acid [5-ASA]), prednisolone (PSL), and azathioprine (AZA) was prescribed. Despite this therapy the patient experienced a rapid deterioration, with severe abdominal pain and bloody stool (>10 times/day). Further evaluation revealed deep ulceration and erosion throughout the large intestine, which

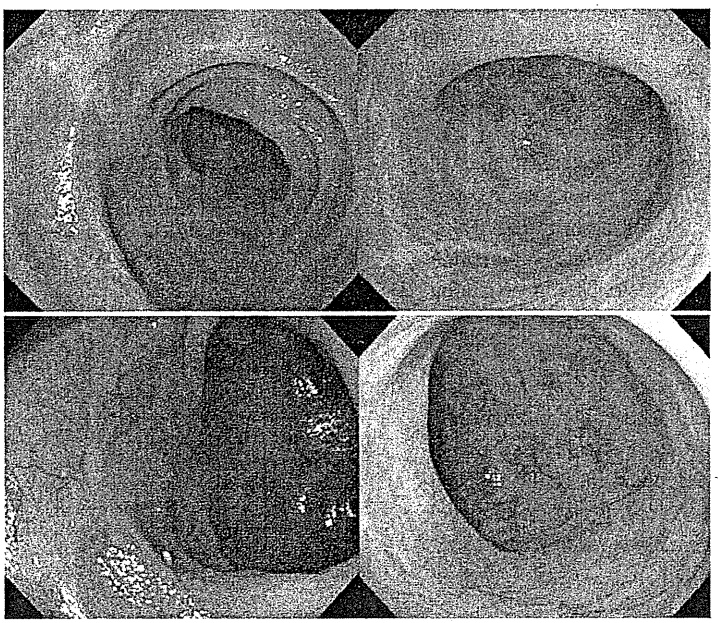

FIGURE 2. Colonoscopic findings. Three months after treatment with AM the ulcers had disappeared and ulcer scars were observed in the transverse colon (a) and the sigmoid colon (b). One year after the treatment with AM the mucosa of the transverse colon (c) and sigmoid colon (d) remained in remission without steroid therapy.

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gave an Ulcerative Colitis Disease Activity Index (UCDAI) score of 12. Higher doses of PSL and AZA in combination with leukocytapheresis failed to induce remission (UCDAI score: 7). Addition of immunosuppressants or biologics was deemed unfeasible due to the patient's age, impaired glucose tolerance, and old tuberculosis, as well as the significant risk of concomitant infectious disease. After ruling out the presence of ischemic heart disease, cerebrovascular disorder, or other cardiovascular or malignant disease, adrenomedullin (AM; 1.5 pmol/kg/min) was intravenously administered for 8 hours per day for 12 days. A few days after starting the AM treatment the patient's abdominal pain and bloody stool appeared to go into remission. No adverse events were observed apart from a slight decline in blood pressure. Endoscopy at 2 weeks revealed significant mucosal regeneration (Fig. 1) and spider web-like scarring in some ulcers (Fig. 2), and the patient's UCDAI score had declined to 2. After 3 months, all of the patient's colonic lesions had healed with scarring and her UCDAI score had reached 0, so the PSL was discontinued.

AM was first identified as a biologically active peptide with potent vaso-dilating action,[1] but is now known to exert a wide range of physiological effects, including cardiovascular protection,[2] neovascularization, and suppression of inflammation and apoptosis. We previously reported that AM therapy was effective in an animal colitis model,[3] and that AM's mechanism of action is likely attributable to its suppression of inflammatory cytokines and activation of regulatory cytokines in intestinal intraepithelial lymphocytes, as well as to its protection of intercellular junctions and its antibacterial activity.[4] In addition, AM reportedly suppresses cytokine production in trinitrobenzene sulfonic acid (TNBS)-induced colitis,[5] and exerts beneficial effects on microvascular function[6] and the reepithelialization[7] of ulcers in an experimental model of colitis.

Although AM has potent hypotensive activity, we observed only minor hemodynamic effects after administering a dose of 1.5 pmol/kg/min, which we considered safe based on human dose-response data in our possession.

Conventional treatment of active UC focuses on steroids, immunosuppressants, and biologics, but the use of these drugs is restricted in geriatric and immunocompromised patients.[8] AM, on the other hand, is a physiological peptide and is therefore anticipated to have excellent safety. Here we present the first reported case in which AM was used to treat a patient with intractable UC. AM treatment produced mucosal regeneration accompanied by marked neovascularization and vasodilation visible on endoscopic examination. These findings are suggestive of AM's potential to be a ground-breaking modality with a novel mechanism of action that differs from existing immunomodulation therapy.

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Potential Oncogenic Properties of Mobilized Stem Cells in a Subpopulation of Inflammatory Bowel Disease Patients Infected with *Helicobacter pylori*

To the Editor:

Marlicz et al[1] concluded that Crohn's disease (CD) triggers the mobilization of various types of stem cells, such as hematopoietic stem progenitor cells, into peripheral blood in patients suffering from this disease, while the significance and precise role of these mobilized cells in repair of damaged intestine requires further study. However, the authors did not discuss the possibility of potential oncogenic properties of the mobilized stem cells, at least in the subgroup of patients possibly infected with *Helicobacter pylori (H. pylori)*.

In this regard, although relative data indicate an absence or inverse association between *H. pylori* and inflammatory bowel disease (IBD), the prevalence of *H. pylori* infection in the IBD patients appears to be 38.2%–47% in Europe.[2] Moreover, enterohepatic and gastric *Helicobacter* species have been documented in fecal specimens from children with CD using polymerase chain reaction (PCR), and *H. pylori,* for example, was recently found in the intestinal mucosa of a patient affected by CD.[3]

Experimental data indicate that *H. pylori* infection leads to development of chronic inflammation, hyperplasia, metaplasia, dysplasia, and recruitment

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Big angiotensin-25: A novel glycosylated angiotensin-related peptide isolated from human urine

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ABSTRACT

The renin–angiotensin system (RAS), including angiotensin II (Ang II), plays an important role in the regulation of blood pressure and body fluid balance. Consequently, the RAS has emerged as a key target for treatment of kidney and cardiovascular disease. In a search for bioactive peptides using an antibody against the N-terminal portion of Ang II, we identified and characterized a novel angiotensin-related peptide from human urine as a major molecular form. We named the peptide Big angiotensin-25 (Bang-25) because it consists of 25 amino acids with a glycosyl chain and added cysteine. Bang-25 is rapidly cleaved by chymase to Ang II, but is resistant to cleavage by renin. The peptide is abundant in human urine and is present in a wide range of organs and tissues. In particular, immunostaining of Bang-25 in the kidney is specifically localized to podocytes. Although the physiological function of Bang-25 remains uncertain, our findings suggest it is processed from angiotensinogen and may represent an alternative, renin-independent path for Ang II synthesis in tissue.

1. Introduction

The renin–angiotensin system (RAS) plays key roles in the regulation of blood pressure and electrolyte and body fluid dynamics. According to the classical concept, the systemic RAS consists of renin, angiotensinogen (Aogen), angiotensin-converting enzyme (ACE), angiotensin (Ang) I, Ang II, and the Ang II type 1 and 2 receptors. Upon activation of the systemic RAS, renin is secreted from the juxtaglomerular apparatus in the kidneys into the circulation, where it acts on Aogen secreted from the liver to produce Ang I, which is in turn converted to Ang II by ACE [1,2]. Ang II then binds to Ang II type 1 and 2 receptors to exert its biological effects. In addition, it is now recognized that a wide variety of tissues and organs, including the heart, vasculature, kidney and nervous system, among others, produce Ang II, which then acts in an autocrine/paracrine fashion independently of the systemic RAS [3,4]. Within the heart, for example, local Ang II appears to contribute to both the maintenance of myocardial homeostasis and to adaptive responses induced by cardiac stress, such as those caused by prolonged hypertension. Elevated levels of cardiac Ang II activity are also associated with diseases of the heart and vasculature, including cardiac hypertrophy, coronary artery disease and myocardial infarction. Similarly, excess local Ang II in the kidney is associated with glomerular sclerosis, diabetic nephropathy and renal arterial stenosis.

Over the past 20 years, novel components of the RAS, including the (pro)renin receptor, ACE2, Ang (1–7), Ang III and Ang IV, as well as their receptors, have been identified and studied [5–8]. To examine the biosynthesis of Ang II within tissue, we developed a radioimmunoassay (RIA) that recognizes the N-terminal sequence of Ang II. Then using that assay, we isolated and characterized proangiotensin-12 (proang-12), as a major molecular form in rat small intestine [9]. Proang-12 is an angiotensin-related peptide with the same amino acid sequence as Ang I plus two additional amino acids. When measuring tissue levels of proang-12 and Ang II in rats treated with RAS inhibitors or fed a low-salt diet, we found that the tissue levels of proang-12 and Ang II did not correspond to the circulating RAS activity. Instead, Proang-12 appears to be an important intermediate involved in the regulation of rat tissue Ang II [10,11].

Up to now similar angiotensin-related peptides had never been reported in humans. In the present study, however, we describe an angiotensin-related peptide isolated from human urine and assess the potential function of the novel peptide.

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2. Materials and methods

2.1. Ethics statement

Urine samples were collected from healthy volunteers. Tissue samples were obtained from the Miyazaki University Hospital. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional review board (No. 817).

2.2. Peptides and enzymes

Ang I and II were purchased from Peptide Institute Inc. (Osaka, Japan) and Ang II + Cys was synthesized by Bex Co., Ltd. (Tokyo, Japan). Renin and chymase substrate were synthesized by Peptide Institute Inc. (Osaka, Japan). Aogen was purchased from Calbiochem. Renin and mast cell chymase were purchased from AnaSpec, Inc. (Fremont, CA, USA) and Elastin Products Company, Inc. (Owensville, MO, USA), respectively.

2.3. Radioimmunoassay for the N-terminal portion of Ang II

The radioimmunoassay (RIA) for the N-terminal portion of Ang II was performed using the method described previously [9]. The standard buffer was 50 mM sodium phosphate (pH 7.4) containing 0.5% bovine serum albumin (BSA), 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA–2Na, 0.05% NaN_3 and 500 KIU/ml of aprotinin. The RIA incubation mixture was composed of 100 μl of standard Ang II or the sample to be determined, 50 μl of antiserum against the N-terminal of Ang II at a dilution of 1:5000, and 50 μl of ^{125}I-labeled ligand (18,000 cpm). After incubation for 24 h, the reaction was stopped by adding 50 μl of 1% γ-globulin and 200 μl of 23% polyethyleneglycol (#6000) in the standard buffer. After vigorous shaking, the mixture was incubated at 4 °C for 15 min and centrifuged at 2000×*g* for 30 min. The radioactivity in the resultant pellet was measured in a gamma counter (Aloka ARC-600, Tokyo). All assay procedures were performed in duplicate at 4 °C, as with the RIA for the C-terminal region of adrenomedullin [12]. This RIA cross-reacted with Ang I and Ang III at levels of 50% and 12.5%, respectively, but did cross-react with Ang IV or Ang (1–7).

2.4. Purification procedure

After collecting 5.5 L of urine from three healthy subjects, the urine was applied to a Sep-Pak C18 cartridge (35 ml, Waters) and eluted with 60% acetonitrile in 0.1% trifluoroacetic acid. The eluted sample was lyophilized, dissolved in 10 mM NH_4COOH (pH 4.0), and then applied to a CM 52 column (2.5 × 75 cm). After washing the column with 10 mM NH_4COOH (pH 4.0), the fraction containing immunoreactivity was eluted with 100 mM NH_4COOH (pH 4.0). The eluate was concentrated and subjected to gel filtration on a Sephadex G-50 column (3.0 × 150 cm), after which the fraction showing immunoreactivity was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a diphenyl column (0.46 × 25 cm, Grace Vydac Inc., Deerfield, IL, USA). The fraction with immunoreactivity was then further purified on an affinity column (Affi-Gel 10 Active Ester Agarose; Bio Rad, Tokyo, Japan), which had been prepared with anti-Ang II N-terminal antiserum. Finally, the purified peptide was subjected to RP-HPLC using an ODS-120A column (0.46 × 15 cm, Tosho, Tokyo, Japan). During these purification steps, levels of immunoreactive Ang II N-terminal were monitored using RIA described previously [9].

2.5. Structural determination

To determine the amino acid sequence and molecular weight of the purified peptide, a tandem mass spectrometric analysis was performed using a positive electrospray ionization with a QSTAR Elite Hybrid LC/MS/MS System (AB SCIEX, USA), and the amino acid sequence was determined using a Procise 494 HT Protein Sequencing System (Applied Biosystems, USA). For mass spectroscopy, the sample was dissolved in a solution of 50% acetonitrile in 0.1% formic acid. To characterize the glycosyl chain, the conditions used for the enzymatic release, fluorescent labeling, separation and structural identification of N-glycan were the same as reported previously [13,14]. Briefly, the delipidated extract was subjected to proteolysis catalyzed by pepsin plus glycoamidase A, and the resultant peptides were further digested to amino acids using pronase. After purification on a Bio-Gel P-2 column, the reducing ends of the released N-glycans were labeled with fluorescent 2-aminopyridine, and any excess reagents were then removed by Sephadex G-15 chromatography. The pyrimidylamino (PA)-glycans were then separated based on charge on a TSK-gel diethylaminoethyl (DEAE)-5PW column (Tosoh, Tokyo, Japan), after which each separated fraction was applied to a Shim-pack HRC octadecyl silica (ODS) column (Shimadzu, Kyoto, Japan) for separation based on hydrophobicity, and the elution time of each peak was expressed as a glucose unit (GU) value. The molecular mass of each PA-glycan fraction was then analyzed using Maldi time-of-flight mass spectrometry (MALDI-TOF-MS). Fractions containing two types of glycan were further subfractionated based on molecular size using a TSK-gel Amide-80 column (Tosoh), and the elution positions were calibrated in GU values. Sample PA-glycans were mapped on the basis of their GU and molecular mass values, and their coordinates were compared with those of reference PA-glycans in the GALAXY database.

2.6. Renin and chymase kinetics

Human Bang-25 and NonG-Bang-25 were synthesized by Peptide Institute, Inc. (Osaka, Japan). Renin substrates (Bang-25, NonG-Bang-25, Aogen) at a concentration of 1–200 μM were incubated with 25 ng of recombinant human renin for 0, 30 or 60 min at 37 °C in 0.5 M PBS buffer (pH 7.4) containing 0.02% BSA. The reaction was stopped by addition of 4 volumes of acetic acid and boiling for 5 min. Ang I was then quantified using the previously described RIA for Ang I [9].

Chymase substrates (Bang-25, NonG-Bang-25, Aogen) at a concentration of 1–200 μM were incubated with 10.85 mU of mast cell chymase for 0, 30 or 60 min at 25 °C in 0.6 M Tris–HCl + 3 M NaCl (pH 8.0). The reaction was stopped by addition of 4 volumes of HPLC Buffer A (10% CH_3CN, 0.1% trifluoroacetic acid) and boiling for 5 min. Ang II was quantified using the aforementioned RIA for Ang II [9].

2.7. Immunohistochemical staining of human Bang-25

Human Bang-25 was detected using antiserum raised in rabbit against the C-terminal portion of the peptide. Thereafter, the polyclonal antibody was affinity purified by Scrum Inc. (Tokyo, Japan) using Bang-25-(18–25) as the antigen. For subsequent immunohistochemical staining, formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm-thick sections and labeled using the anti-Bang-25 antibody (100× dilution in PBS). As a negative control, the antibody was replaced with non-immune rabbit serum (Dako Japan, Inc.). Then after blocking endogenous peroxidase activity using hydrogen peroxide, the sections were incubated with an EnVision+/HRP System (Dako Japan, Inc.), and the staining was developed using 3,3′-diaminobenzidine. Finally the sections were counterstained with Meyer's hematoxylin.

2.8. Statistical analysis

Values are presented as means ± S.E. Comparisons of enzyme kinetics data were made using analysis of variance (ANOVA) followed by Bonferroni's test. Values of $P < 0.05$ were considered significant.

3. Results and discussion

When we screened human urine gel filtration fractions for peptides using an antibody raised against the N-terminal portion of Ang II, we found a highly immunoreactive peptide with an apparent molecular weight of about 5000 (Fig. 1A). Notably, this peptide was present at concentrations estimated to be more than 100 times higher than those of Ang I and Ang II.

We next purified this peptide from 5.5 L of human urine using ion-exchange, gel-filtration and affinity chromatography steps, and then finally RP-HPLC, which yielded a single peak (Fig. 1B). With this protocol we obtained 200 pmol of purified peptide from the 5.5 L of urine. When the sample of purified peptide was subjected to mass spectrometry, we found a hexahydric charged ion with $m/z = 780.18$, a pentavalent ion with $m/z = 936.02$, and a quadrivalent ion with $m/z = 1169.76$ (Fig. 1C), which collectively indicate the molecular weight of the purified peptide to be 4675.1. The amino-acid sequence of the peptide was then determined to be DRVYIHPFHLVIHX^{1}ESTX^{2}EQLAKAN (X, not identified), and from the known amino acid sequence of Aogen [15], X^1 and X^2 were identified as Asn and Cys, respectively (Fig. 1D). In addition, because we found a MS/MS peak for a glycosyl chain, the purified peptide was digested with N-glycosidase and again subjected to mass spectrometry. Under these conditions, the pentavalent ion had a $m/z = 611.71$ and the quadrivalent ion had a $m/z = 764.39$ (data not shown), which indicates the molecular weight of the deglycosylated peptide to be 3053.6 and confirms that X^2 is Cys–Cys.

Structural analysis of the glycosyl chain was delegated to GLYENCE Co., Ltd. After using diethylaminoethyl (DEAE) and ODS column chromatography to purify the glycosyl to a single peak (Fig. 1E), the resultant ODS fraction was subjected to MALDI-TOF-MS glycoanalysis using the three MS axes and the chromatography database (GALAXY) [14,16]. The results showed the peptide in this fraction to have a molecular mass of 1720 Da and to give rise to an oligosaccharide of 9.8 (ODS) and 6.9 (DEAE) GU, which corresponds to reference PA-glycan code No. 200.4 in

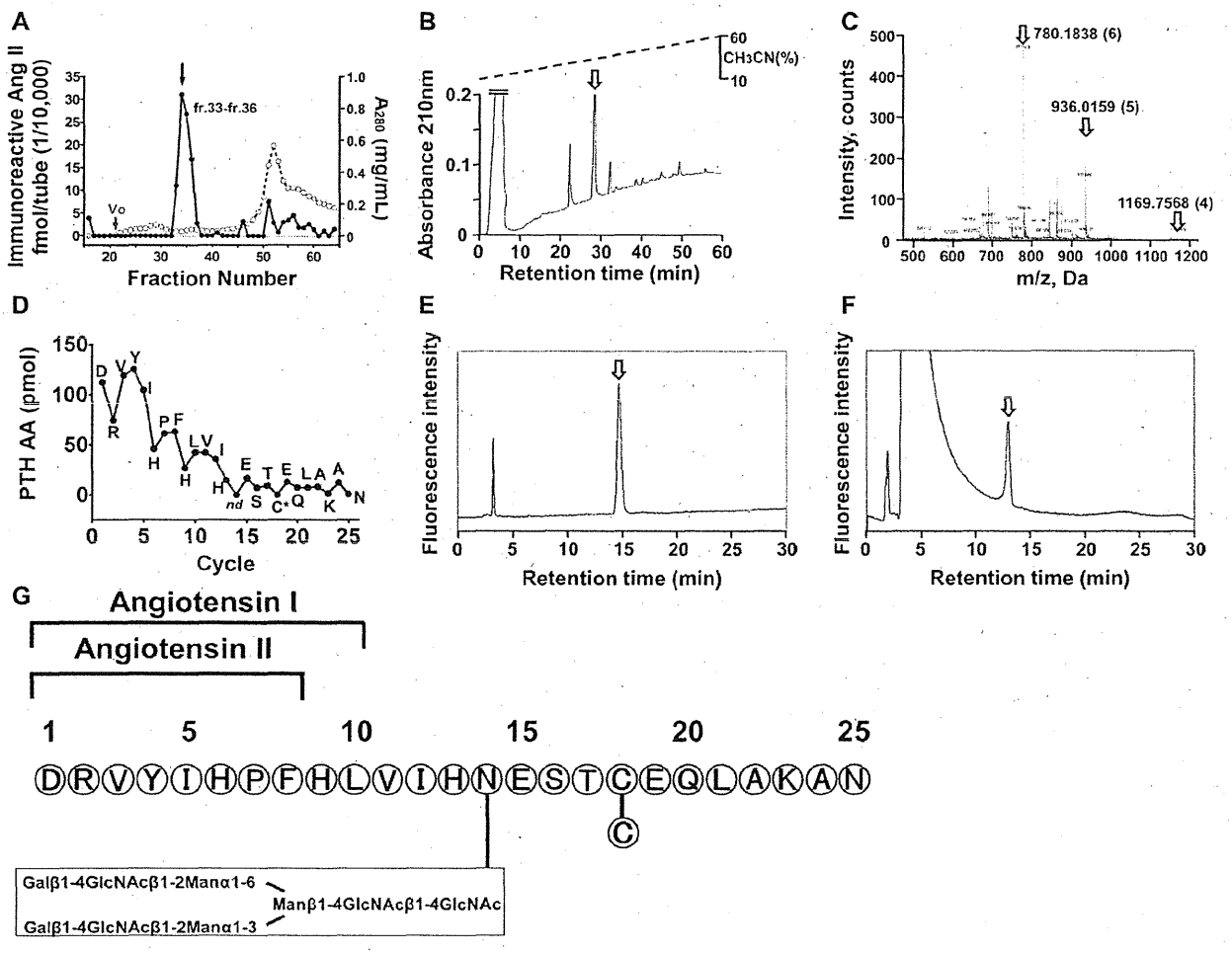

Fig. 1. (A) Immunoreactive Ang II N-terminal in human urine. Extract of human urine was subjected gel filtration (Sephadex G50), and the immunoreactive Ang II N-terminal in each fraction was assayed using a specific RIA: closed circles, immunoreactive Ang II N-terminal (fmol/tube); open circles, absorbance at 280 nm (mg/ml). The arrow indicates the highly immunoreactive Ang II N-terminal fraction. (B) Final purification by RP-HPLC. An elution gradient of 10–60% CH_3CN in 0.1% trifluoroacetic acid was run for over 60 min at a flow rate of 1 ml/min. (C) Determination of the molecular weight of the purified peptide based on quadrupole time-of-flight (Q-TOF) mass spectrometry. (D) Amino acid sequence analysis using a protein sequencer; 25 amino acid residues were detected. (E) Primary structural analysis of the glycosyl chain. A single peak was detected. (F) Secondary structural analysis of the glycosyl chain. Both the candidate (arrow) and GALAXY glycosyl chains were run on an ODS column. (G) Structure of human Bang-25 isolated from urine. GlcNAc: *N*-acetylglucosamine, Gal: galactose, Man: mannose.

the GALAXY database. The identity of this sample was then confirmed using co-chromatography (Fig. 1F) and MALDI-TOF-MS analyses, which showed the glycosyl chain to be a complex sugar chain consisting of nine sugars, which is often found with N-linked glycosylation. The complete structure of the molecule is shown in Fig. 1G. We are calling this novel peptide Big angiotensin-25 (Bang-25), because it consists of a 25-amino acid peptide that is N-glycosylated on Asn14 and has a cysteine linked to Cys18. Human Aogen contains four putative asparagine-linked glycosylation sites (Asn14, 137, 271, 295) and contains four cysteines (Cys18, 138, 232, 308) [15,17–19], with Cys18 and Cys138 linked by a disulfide bridge. From its structure it appears clear that Bang-25 is a derivative of Aogen.

Knowing the structure, we synthesized Bang-25 using a combination of solid phase and liquid phase techniques, which will be described elsewhere. The synthetic peptide was confirmed by amino acid analysis, MALDI-TOF-MS analysis and RP-HPLC. With the RP-HPLC, for example, native and synthetic Bang-25 emerged at the exact same elution position.

Using the synthetic peptide, we found that renin processed Bang-25 into Ang I, but the rate was slow (Fig. 2A and C). By contrast, chymase rapidly digested Bang-25 to produce Ang II (Fig. 2B and D). When we compared Aogen, Bang-25 and nonglycosylated Bang-25 (NonG-Bang-25), each at a concentration of 1 μM, as substrates for renin and chymase ($n = 6$ for each reaction), we found that renin rapidly produced Ang I from Aogen, but production of Ang I from Bang-25 was much slower (Fig. 2A). Consistent with that result, the K_m for Aogen was estimated to be 1.2 μM, which is comparable to the value reported previously [17,19], while the K_m and V_{max} for Bang-25 were 95 μM and 340 pmol/min/ml (Fig. 2C). In addition, the K_m and V_{max} for NonG-Bang-25 were 24 μM and 307 pmol/min/ml (Fig. 2C). It therefore seems doubtful that Bang-25 functions as a substrate for renin in vivo. Moreover, renin catalyzed significantly greater production of Ang I from NonG-Bang-25 than from Bang-25, indicating the glycosyl chain protects the peptide from renin (Fig. 2A and C). This is consistent with the earlier observation that N-linked glycosylation on Asn14 is an important inhibitor of binding to renin. Still, renin does not cleave NonG-Bang-25 as efficiently as it cleaves Aogen. Given that the Aogen N-terminus is linked to the rest of the molecule by a disulfide bridge between Cys-18 and Cys-138, which plays a key role in the peptide's binding to renin [19] and is absent from Bang-25, it is not surprising that Bang-25 is cleaved less efficiently by renin than is Aogen, with or without glycosylation.

Chymase, by contrast, does not act on human Aogen (Fig. 2B) [20], but it efficiently digests both Bang-25 and NonG-Bang-25 (Fig. 2B and D). The K_m and V_{max} were 9.6 μM and 129 pmol/min/ml for Bang-25 and 7.5 μM and 155 pmol/min/ml for NonG-Bang-25 (Fig. 2D). And although production of Ang II from NonG-Bang-25 tended to be greater than from Bang-25, the difference was not statistically significant in the present experiment (Fig. 2D). This suggests Bang-25 functions as a substrate for chymase, which is consistent with our finding that the tissue Ang II concentration was much higher than the Ang I concentration.

We then assessed the distribution of Bang-25 in tissues by immunostaining with an antiserum raised against the C-terminal portion of the peptide. We found that Bang-25 was abundantly expressed in a number of human tissues, including kidney (Fig. 3G and H), heart (Fig. 3C), adrenal gland (Fig. 3A), pancreas (Fig. 3B) and placenta (Fig. 3D–F), among others (Table 1). For practical reasons, we used the placenta as an exemplar for further study. There we found Bang-25 to be localized to extravillous trophoblasts. The

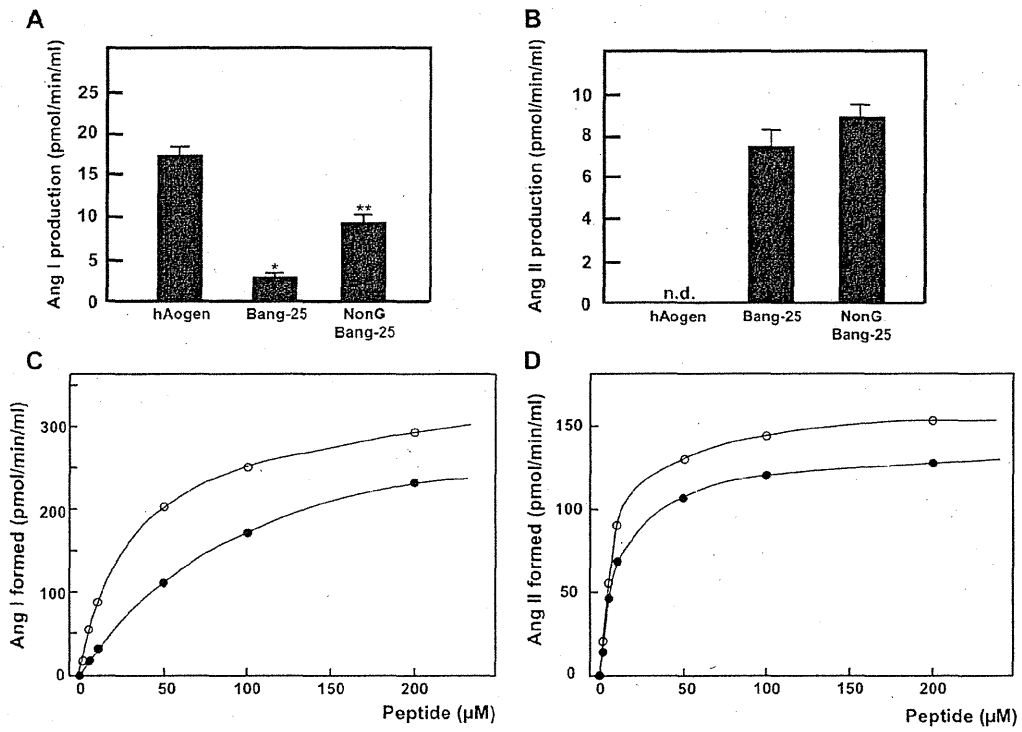

Fig. 2. (A) Velocity of renin-catalyzed Ang I formation at a substrate concentration of 1 μM ($n = 6$). The results are shown as means ± SEM. *$P < 0.05$ vs. NonG-Bang-25 and $P < 0.01$ vs. hAogen. **$P < 0.01$ vs. hAogen. (B) Velocity of chymase-catalyzed Ang II formation at a substrate concentration of 1 μM ($n = 6$). The results are shown as means ± SEM. (C) Michaelis–Menten plots for the renin-catalyzed release of Ang I from Bang-25 (closed circles) and NonG-Bang-25 (open circles) ($n = 2$–6). The K_m and V_{max} were 95 μM and 340 pmol/min/ml for Bang-25 and 24 μM and 307 pmol/min/ml for NonG-Bang-25. (D) Michaelis–Menten plots for the chymase-catalyzed release of Ang II from Bang-25 (closed circles) and NonG-Bang-25 (open circles) ($n = 2$–6). The K_m and V_{max} were 9.6 μM and 129 pmol/min/ml for Bang-25 and 7.5 μM and 155 pmol/min/ml for NonG-Bang-25.

placenta was also highly immunoreactive to our anti-Ang II N-terminal antibody, and gel filtration showed the molecular weight of the antigen to be approximately 5000, just as in human urine (Fig. 3I). Moreover, although gel filtration produced a clear Ang II peak, there was little Ang I peak, indicating Ang II was produced via a renin-independent pathway.

The abundance of Bang-25 over such a wide range of tissues suggests it is important in the production of Ang II in tissue. Particularly interesting to us was the strong Bang-25 staining in endocrine tissues, including pancreatic cells and the adrenal gland medulla. It has been reported that Ang II contributes to oxidative stress, inflammation and apoptosis in pancreatic cells [21], and that local Ang II may stimulate catecholamine release from the adrenal medulla [22]. Within that context, the possible conversion of Bang-25 to Ang II in tissue suggests Bang-25 may indirectly contribute to the regulation of endocrine cell function through Ang II production. Alternatively, the observation that it is highly localized in endocrine cells suggests Bang-25 may itself function as an endocrine hormone without conversion to Ang II. In the kidney, Bang-25 is localized predominantly to podocytes (Fig. 3G and H). This is noteworthy, as it is known that pathological production of Ang II by podocytes causes injury [23] related to such disease states as primary glomerulopathy, hypertension and diabetes mellitus [24], and reduced podocyte loss can entirely account for the renoprotective effect of Ang II blockade [25]. Although it is not presently clear whether podocytes are the origin of Bang-25 in urine, it could be that Bang-25 in urine is marker of podocyte injury.

Chymase, the enzyme that cleaves Bang-25 to produce Ang II, is released into interstitial tissues from mast cell granules following tissue injury and during inflammation, and chymase expression is upregulated after kidney injury and in heart disease [26]. In human vascular extracts, chymase inhibition reduces Ang II synthesis by more than 90% [27], suggesting chymase-dependent Ang II synthesis is stimulated by tissue injury or inflammation. One possibility is that, in tissue, Aogen is subject to cleavage by a different enzyme to form Bang-25, which is in turn cleaved by chymase to form Ang II.

Table 1
Result of the immunohistochemistory.

Kidney	Podocyte	++
Heart	Left ventricle, cardiocytes	++
Lung	Pulmonary artery, SMCs	+
	Bronchiole, epithelium	+
Esophagus	Stratified squamous epithelium (basal layer)	+
Stomach	Glands (endocrine cells)	+
	Nerve (submucosal and Auerbach)	+
Large intestine	Crypts (endocrine cells)	+
Liver	Hepatocytes	+
Spleen	Histiocytes	+
Pancreas	Islets	++
	Exocrine (acinus/duct)	–
Adrenal gland	Medulla	++
	Cortex	–
Lymph nodes	Histiocytes	+
	Dendritic cells	+
Testis	Sertoli cells	++
Salpinx	SMCs	++
	Tubal epithelium	+
Placenta	Extravilous trophoblasts	++
	Villi	–

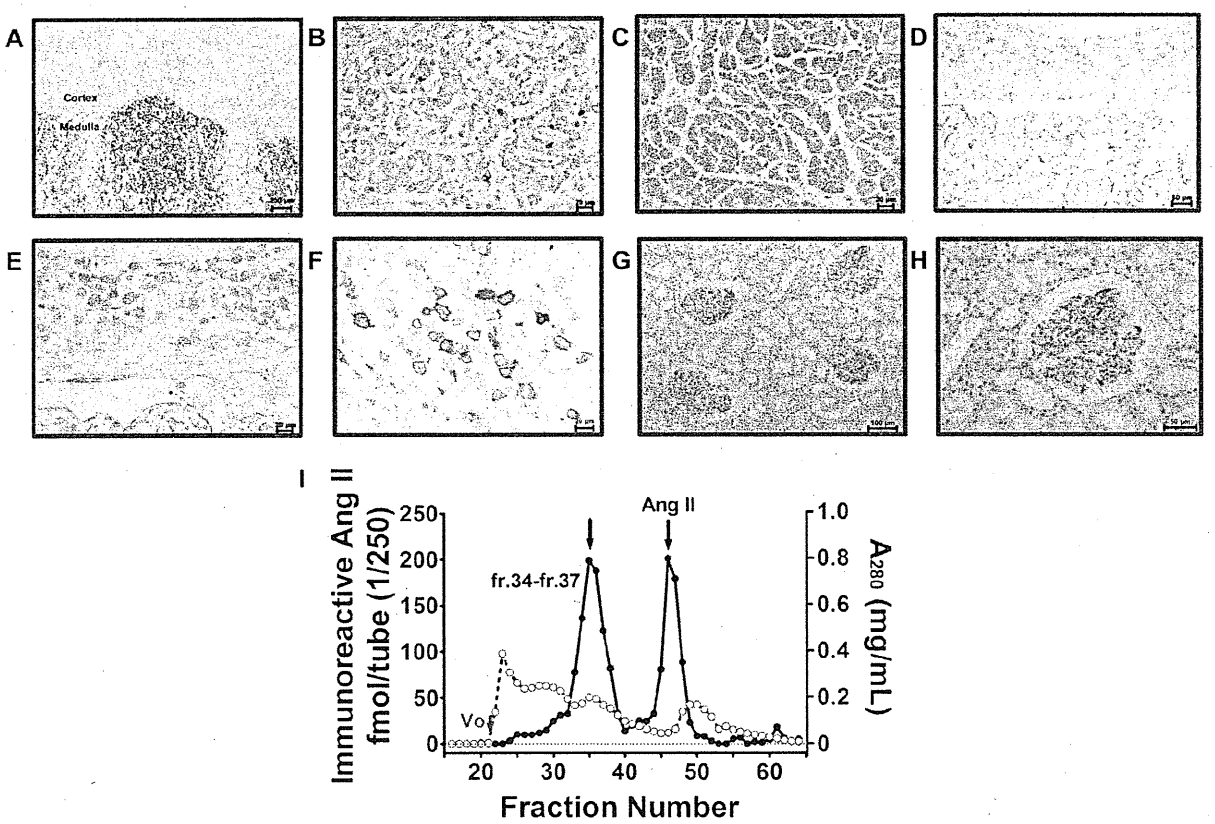

Fig. 3. (A–H) Immunohistochemical staining of Bang-25 in representative sections of human tissue. Bang-25 immunostaining in adrenal gland (A), pancreas (islet cells; B), heart (C), placenta (D–F) and kidney (G and H). The staining in kidney was localized to podocytes (G and H). (I) Immunoreactive Ang II N-terminal in placenta. An extract of human placenta was subjected gel filtration (Sephadex G50), and the immunoreactive Ang II N-terminal in each fraction was assayed using a specific RIA: closed circles, Ang II N-terminal (fmol/tube); open circles, absorbance at 280 nm (mg/ml).

In the present study, we isolated and identified a novel Aogen-derived peptide, Bang-25, which is composed of 25 amino acids, is N-glycosylated on its 14th amino acid (Asn), and has a cysteine linked to its 18th amino acid (Cys). The identification of Bang-25 suggests the existence of a RAS processing cascade different from the renin-catalyzed cleavage of Aogen to Ang I, and provides a potential target for assessing Ang II in tissue and for the development of new therapeutic approaches to related diseases.

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