⑧ 妊婦または妊娠している可能性のある患者
⑨ その他、担当医師が不適当と判断した患者

（4）臨床試薬
被験薬：合成ヒトアドレノメデュリン

（5）臨床試験の方法：オープン試験
被験者の末梢静脈ラインを確保し、アドレノメデュリン 1.5 pmol/kg/min を 8 時間/日（午前 9 時～午後 5 時）の投与スケジュールにより最大 14 日間投与する。血圧、脈拍数を持続モニターし、患者の状況（血圧が規定以上に低下した場合など）によっては患者の安全を優先し、1 日当たりの投与量ないしは投与時間を短縮したり、投与期間を短縮する場合がある。

（6）観察・検査項目
- 自覚症状および QOL の改善度 (Visual analog scale:VAS, IBDQ)
- 身体所見の変化（結膜充血等）
- 血圧、脈拍
- 心電図
- 血液検査（血算、生化学、各種サイトカイン、アドレノメデュリン血中濃度）
- 臨床重症度評価（DAI スコアないし IOIBD スコア）
- 大腸内視鏡所見による重症度評価（アドレノメデュリン投与前、投与開始後 1 週間）
- 大腸内視鏡、生検による病理学的評価（アドレノメデュリン投与前、投与開始後 1 週間目）
- 大腸癌スクリーニング（アドレノメデュリン投与前）

（7）評価項目
＜有効性＞
主要評価項目
有効性
排便回数
血便の状態
腹痛
下部消化管内視鏡所見
臨床検査値

（8）目標例数　10 例

（9）臨床試験予定期間
2010 年 1 月 1 日から 2012 年 12 月 31 日

D．考察
AM は抗炎症作用を有する事が判明しており、大腸潰瘍モデルラットおよび大腸炎モデルマウスに対しても粘膜障害の改善作用を有する。その機序として IL-6 や TNF-α などの炎症性サイトカイン産生抑制が関与している

と考えられている。今回の探索的臨床研究では、ヒトや動物実験により安全性が検証されている持続静注法によりAMを投与する。AMは炎症性腸疾患モデル動物に対しては、注腸投与ばかりでなく、腹腔投与や経静脈投与でも有効性が確認されており、今回の静注法での有効性は十分期待できる。血圧への影響や治療効果を勘案して、アドレノメデュリン 1.5 pmol/kg/min x 8時間（午前9時～午後5時）を1日の投与量とし、最大14日間行うこととした。アドレノメデュリン投与中は、血圧、脈拍数を持続モニターし、患者の状況（血圧が規定以上に低下した場合や治療効果など）によっては患者の安全を優先し、1日当たりの投与量ないしは投与時間を短縮したり、投与期間を短縮する方針とした。

E．結論

臨床研究のプロトコールが完成し、倫理委員会にて承認されたことで、臨床研究への準備が順調に進んでいる。

F．健康危険情報

なし

G．研究発表

1．論文発表

1. Ashizuka S, Inagaki-Ohara K, Kuwasako K, Kato J, Inatsu H, Kitamura K: Adrenomedullin treatment reduces intestinal inflammation and maintains epithelial barrier function in mice administered dextran sulphate sodium. *Microbiol Immunol. 53:* 573-581 (2009)

2．学会発表

1. Kitamura K, Ashiduka S, Inagaki-Ohara K, Kuwasako K, Kato J, Inatsu H: Adrenomedullin treatment reduces intestinal inflammation in mice administered dextran sulphate sodium. 14th International Congress of Endocrinology (ICE2010). (2010年3月 京都)

2．中島孝治、彦坂ともみ、星子新理、松本英丈、早稲田文子、芦塚伸也、押川勝太郎、稲津東彦、北村和雄、堺　雅彦：MALTリンパ腫疑診症例から直腸炎型潰瘍性大腸炎へと変化した一例．第95回日本消化器病学会総会、2009年5月

H．知的財産権の出願・登録状況

なし

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kita T, Suzuki Y, Kitamura K.	Hemodynamic and hormonal effects of exogenous adrenomedull in administration in humans, and relationship to insulin resistance.	Hypertens Res.		in press	
Ashizuka S, Inagaki-Ohara K, Kuwasako K, Kato J, Inatsu H, Kitamura K.	Adrenomedullin treatment reduces intestinal inflammation and maintains epithelial barrier function in mice administered dextran sulphate sodium.	Microbiol Immunol.	53	573-581	2009
Baba A, Fujimoto S, Kikuchi M, Kita T, Kitamura K.	Effects of uroguanylin on natriuresis in experimental nephrotic rats.	Nephrology (Carlton).	14	80-85	2009
Kuwasako K, Kitamura K, Nagata S, Kato J.	Flow cytometric analysis of the calcitonin receptor-like receptor domains responsible for cell-surface translocation of receptor activity-modifying proteins.	Biochem Biophys Res Commun.	384	249-254	2009
Masuyama H, Tsuruda T, Sekita Y, Hatakeyama K, Imamura T, Kato J, Asada Y, Stasch JP, Kitamura K.	Pressure-independent effects of pharmacological stimulation of soluble guanylate cyclase on fibrosis in pressure-overloaded rat heart.	Hypertens Res.	32	597-603	2009
Nomura I, Kato J, Tokashiki M, Kitamura K.	Increased plasma levels of the mature and intermediate forms of adrenomedullin in obesity.	Regul Pept.	158	127-131	2009
Petersen KA, Birk S, Kitamura K, Olesen J.	Effect of adrenomedullin on the cerebral circulation: relevance to primary headache disorders.	Cephalalgia.	29	23-30	2009
Tsuruda T, Hatakeyama K, Masuyama H, Sekita Y, Imamura T, Asada Y, Kitamura K.	Pharmacological stimulation of soluble guanylate cyclase modulates hypoxia-inducible factor-1alpha in rat heart.	Am J Physiol Heart Circ Physiol.	297	H1274-1280	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
北村和雄	アドレノメデュリンの発見	日本臨牀	67	67-72	2009
北村和雄	アドレノメデュリン（AM），PAMPとその受容体	Heart View	13	69-75	2009
桑迫健二、加藤丈司、北村和雄	アドレノメデュリン遺伝子	日本臨牀	67	400-404	2009

Hypertension Research (2010) 33, 314–319

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ORIGINAL ARTICLE

Hemodynamic and hormonal effects of exogenous adrenomedullin administration in humans and relationship to insulin resistance

Toshihiro Kita, Yoshihiko Suzuki[1] and Kazuo Kitamura

Although adrenomedullin (AM) is a potent hypotensive peptide that acts mainly as a vasodilative and proliferation inhibitory factor, there have been few hemodynamic studies on AM in humans, especially concerning arterial stiffness and hormonal effects. In addition, AM is a suppressive factor in insulin resistance, suggesting that the effects of AM in a state of insulin resistance are important. To evaluate the effects of AM in humans, 28 participants were intravenously administered AM (5 pmol min^{-1} kg^{-1}) for 90 min. They also received a representative vasodilator drug, nicardipine, as a reference drug. Blood pressure, heart rate, pulse wave velocity (PWV) and blood flow were monitored throughout the experiment. Hormonal changes were also monitored by blood tests. The effects of AM were compared with those of nicardipine. In addition, the effects of AM were re-evaluated against insulin resistance state. AM and nicardipine produced the same level of hypotension, but AM showed a more potent ability to increase heart rate, blood flow and cardiac output and reduce PWV. AM and nicardipine similarly stimulated plasma noradrenaline and renin activity. However, in the state of insulin resistance, favorable effects of AM on aortic stiffness were blunted and differences between AM and nicardipine disappeared. Furthermore, there was a significant correlation between maximum changes in the PWV induced by AM and the homeostasis model assessment of insulin resistance index (r=0.58, P=0.001). Our results suggest that AM may improve arterial stiffness and act as a compensatory factor against arterial sclerosis. Moreover, decreased reactivity of AM may participate in the progression of arterial sclerosis in insulin resistance.
Hypertension Research (2010) **33**, 314–319; doi:10.1038/hr.2009.236; published online 22 January 2010

Keywords: adrenomedullin; insulin resistance; nicardipine; pulse wave velocity

INTRODUCTION

Adrenomedullin (AM) is a potent hypotensive peptide found ubiquitously in tissues and organs, especially in cardiovascular tissues, the kidneys, lungs and endocrine glands. AM has multiple functions in a wide range of tissues and acts mainly as a vasodilative and proliferation inhibitory factor.[1] AM also has a role in the development of arterial sclerosis as an inflammatory modulator.[2,3] Recently, it was shown that endogenous AM has a protective effect against cardiovascular injury, possibly through the inhibition of oxidative stress.[4] Morphologically, dense manifestation of AM has been detected in macrophages within plaques of atherosclerotic lesions.[5] Shinomiya *et al.*[6] reported an association between plasma AM concentration and carotid atherosclerosis in patients with stroke. Furthermore, we previously reported a relationship between plasma AM levels and pulse wave velocity (PWV), an indicator of arterial stiffness, in patients.[7] In addition, AM may counteract insulin resistance development through an antioxidative stress factor.[8,9] Insulin resistance is well recognized as a major pathogenetic factor of arterial disorders, including hypertension and arterial sclerosis. Accumulating data suggest that AM acts as an important modulator against arterial sclerosis and organ damage.

Exogenous AM administration has been shown to have beneficial effects in various stages of cardiovascular disease. In normotensive and hypertensive subjects, short-term AM infusion produced hypotension through vasodilation and increased cardiac output.[10,11] AM also improved hemodynamics in heart failure patients. Specifically, AM administration reduced arterial pressure and cardiac filling pressure and also increased cardiac output and renal sodium excretion.[12] However, the effect of AM on the arteries, especially large arteries, has not been determined in humans. A sustained increase in arterial stiffness, as demonstrated by increased PWV, is closely correlated with morbidity and mortality in cardiovascular events.[13–15] In this study, we investigated the effect of AM on arterial stiffness in human subjects. In addition, because the effect of AM may be affected by insulin resistance, we evaluated the relationship between the effects of AM and insulin resistance state in these subjects.

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E-mail: t-kita@po.sphere.ne.jp
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METHODS

Study subjects

Twenty-eight subjects that were normotensive (n=11), hypertensive (n=9) or diabetic (n=8) received AM. In addition, all subjects received nicardipine as a reference drug at least 1 week after AM. Subjects with one or more of the following conditions were excluded: (1) heart failure (ejection fraction <50%), (2) severe valvular diseases, (3) renal insufficiency (serum creatinine >1.0 mg per 100 ml), (4) peripheral artery diseases and (5) history of cardiovascular events. All subjects were completely free from any kind of drugs. The study was approved by the ethics committee of the institute, and all participants gave written informed consent.

Preparation of human AM

Chemically synthesized human AM was purchased from the Peptide Institute, Osaka, Japan. The homogeneity of human AM was confirmed by reverse-phase high-performance liquid chromatography and amino acid analysis. AM was dissolved in distilled water with 3.75% D-mannitol and 0.05% aminoacetic acid, then sterilized by passage through a 0.22-μm filter (Millipore, Bedford, MA, USA). The chemical nature and content of the human AM in vials were verified by reverse-phase high-performance liquid chromatography.

Study protocol

All experiments began at 0900 hours with subjects in a fasted state. Experiments were conducted in our outpatient office, which provided a quiet environment with a constant temperature. A 20-gauge cannula was inserted into the forearm vein for infusion of 0.9% saline. Saline was infused at a rate of 100 ml h^{-1} throughout the experiments (Figure 1). Baseline measurements were obtained after a 30-min equilibration period. Then AM (5 pmol min^{-1} kg^{-1}) was intravenously administered at a rate of 5 ml h^{-1} for 1.5 h followed by saline infusion for 1.5 h. One week after AM infusion, the same subjects were infused with nicardipine (1–1.5 μg min^{-1} kg^{-1}) as a reference drug. Blood pressure and pulse rate were monitored every 10 min by an automated hemodynamometer on a brachial cuff. Every 15 min, blood pressure, heart rate and PWV were measured using an automatic waveform analyzer (form PWV/ABI, BP-203RPE; Omron Colin, Komaki, Japan), as reported in our previous study.[7] Carotid artery pulsation was measured using echo equipment at three time points, as indicated in Figure 1, and the elastic property[16] was calculated. Using the Doppler echo method, blood flow in the common carotid artery and segmental renal artery were measured, as was cardiac output. In addition, blood samples were taken at three time points, namely, before, during and after AM infusion (Figure 1). Plasma total and mature AM were measured by specific immunoradiometric assay kits (Shionogi, Osaka, Japan). Plasma concentrations of other hormones were measured using a commercially available laboratory testing service (SRL, Hachioji, Japan).

Statistical analyses

All data were expressed as the mean ± s.e.m. Comparisons of parameters between the two groups (AM *vs.* nicardipine) were carried out using paired Student's *t*-tests. Comparisons of the time course of parameters between the two groups were carried out by two-way repeated measures analysis of variances followed by Bonferroni/Dunn's multiple comparison tests. A value of $P<0.05$ was the criterion for statistical significance.

RESULTS

Table 1 presents the baseline characteristics of the participants. AM and nicardipine achieved the same levels of systolic blood pressure reduction in all subjects, as shown in Figure 2. However, AM produced a stronger diastolic blood pressure reduction and heart rate increase when compared with nicardipine (Figures 2a and b). Most interestingly, AM caused a significantly larger reduction of PWV and elastic property of the carotid artery when compared with nicardipine (Figures 2c and d). These changes rapidly recovered after termination of AM or nicardipine administration, except for the prolonged decrease in systolic blood pressure induced by nicardipine. Table 2

Table 1 Baseline characteristics of participants

	Normotensive	*Hypertensive*	*Diabetic*
N	11	9	8
Age (years)	40.8±2.5	50.0±2.7*	48.5±3.3
BMI (kg m^{-2})	24.3±0.5	24.1±0.8	25.2±0.9
SBP (mm Hg)	118.9±1.8	155.4±4.8**	131.3±3.2**
DBP (mm Hg)	74.9±2.6	95.0±3.6**	81.0±2.2
Heart rate (b.p.m.)	59.5±2.4	65.8±3.6	63.1±2.5
PWV (cm s^{-1})	1263±52	1598±73**	1435±35*
Elastic property (kPa)	97.7±8.5	154.2±9.7**	107.1±7.6
Peak CAF (cm s^{-1})	87.1±2.7	76.8±3.0*	87.9±6.9
Mean CAF (cm s^{-1})	37.5±1.7	36.1±2.7	38.1±2.0
Peak RAF (cm s^{-1})	49.1±4.1	40.0±2.6	48.1±5.2
Mean RAF (cm s^{-1})	28.9±1.8	25.1±1.7	28.5±3.3
Cardiac output (l min^{-1})	4.52±0.28	4.55±0.28	4.73±0.25
IRI (μU ml^{-1})	7.7±1.7	8.2±2.0	6.3±1.5
Blood sugar (mg per 100 ml)	99.4±2.2	104.9±3.3	165.0±11.5**
Total AM (fmol ml^{-1})	12.9±0.7	13.5±1.0	15.4±2.3
Mature AM (fmol ml^{-1})	2.0±0.2	1.9±0.2	1.7±0.2
Adrenaline (pg ml^{-1})	20.4±2.9	32.8±7.6	25.3±2.6
Noradrenaline (pg ml^{-1})	255±39	282±32	188±24
Renin activity (ng ml^{-1} h^{-1})	0.76±0.28	0.73±0.15	1.24±0.26
Aldosterone (pg ml^{-1})	70.2±11.0	76.0±7.5	77.6±8.9
ANP (pg ml^{-1})	17.2±2.8	29.1±8.2	19.1±5.5
BNP (pg ml^{-1})	12.1±2.9	36.7±22.0	10.2±3.9
cAMP (pmol ml^{-1})	11.7±0.6	11.9±0.5	11.6±0.7
cGMP (pmol ml^{-1})	2.9±0.3	4.4±1.0	2.9±0.5

Abbreviations: AM, adrenomedullin; ANP, atrial natriuretic peptide; BMI, body mass index; BNP, brain natriuretic peptide; CAF, common carotid artery flow; DBP, diastolic blood pressure; IRI, immunoreactive insulin; PWV, pulse wave velocity; RAF, renal segmental artery flow; SBP, systolic blood pressure.
*P<0.05, **P<0.01 *vs.* normotensive.

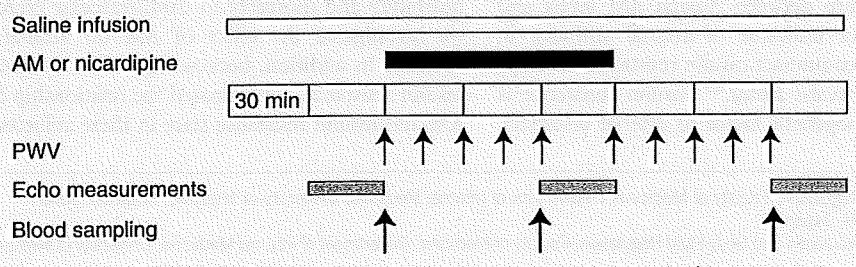

Figure 1 Experimental protocol. After a 60-min baseline period, AM (5 pmol min^{-1} kg^{-1}) or nicardipine (1–1.5 μg min^{-1} kg^{-1}) was intravenously administered for 90 min followed by a 90 min post-infusion period.

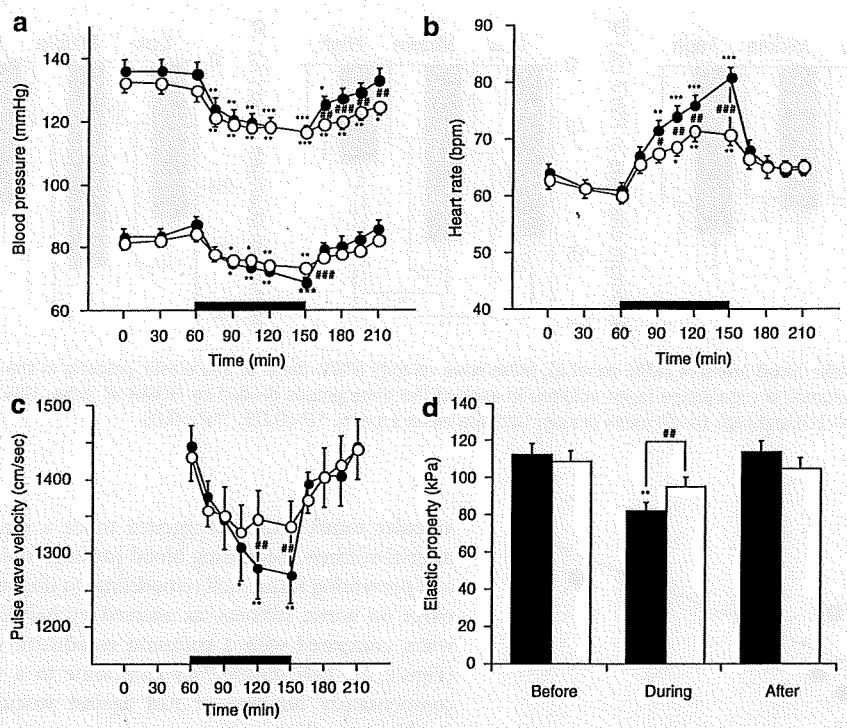

Figure 2 Changes in blood pressure (**a**), heart rate (**b**), pulse wave velocity (**c**) and elastic property of the carotid artery (**d**) during infusion of AM (closed symbols) or nicardipine (open symbols). Data are mean±s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$ *vs.* each baseline; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.0001$ *vs.* nicardipine.

Table 2 Changes of blood flow velocity and cardiac output

	Before	*During*	*After*	P *in trend*
Peak CAF ($cm\,s^{-1}$)				
AM	84.0±2.5	130.2±3.0$^{**,\,\#\#}$	80.3±2.5	<0.0001
Nicardipine	83.9±2.6	107.2±2.5**	84.2±2.0	<0.0001
Mean CAF ($cm\,s^{-1}$)				
AM	37.2±1.2	53.8±1.4$^{**,\,\#\#}$	34.3±1.1	<0.0001
Nicardipine	36.4±1.1	42.7±1.2**	34.7±1.0	<0.0001
Peak RAF ($cm\,s^{-1}$)				
AM	45.9±2.4	61.2±3.3**	40.9±2.1$^{\#}$	<0.0001
Nicardipine	46.1±2.5	56.9±2.8**	45.0±2.2	0.0017
Mean RAF ($cm\,s^{-1}$)				
AM	27.6±1.3	34.0±1.6$^{**,\,\#}$	24.8±1.1$^{\#\#}$	<0.0001
Nicardipine	27.7±1.4	31.6±1.6	27.3±1.3	0.066
Cardiac output ($l\,min^{-1}$)				
AM	4.59±0.15	7.63±0.25$^{**,\,\#\#}$	4.77±0.14	<0.0001
Nicardipine	4.58±0.14	6.18±0.30**	4.90±0.27	<0.0001

Abbreviations: AM, adrenomedullin; CAF, common carotid artery flow; RAF, renal segmental artery flow.
Date are mean±s.e.m.
$^{**}P<0.01$ *vs.* before.
$^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* nicardipine.

summarizes the increase in blood flow and cardiac output after AM or nicardipine administration. Both reagents clearly increased blood flow, but AM was more potent than nicardipine. AM induced significantly larger increases in cardiac output when compared with nicardipine.

The responses of these parameters were similar among normotensive, hypertensive and diabetic groups of subjects (data not shown). Next, subjects were divided into three groups according to the homeostasis model assessment of insulin resistance (HOMA-IR) index, according to which the highest tertile (HOMA-IR≥2.0) corresponds to an insulin-resistant state in Japan. HOMA-IR values for each group were as follows: low=0.31–1.39 ($n=9$), middle=1.43–1.78 ($n=10$) and high=2.00–6.96 ($n=9$). As shown in Figure 3, only the reduction in PWV induced by AM was blunted in subjects with the highest HOMA-IR, despite the nearly identical reduction in systolic blood pressure. In this group, the reductions in PWV were similar for AM and nicardipine treatments. This phenomenon was also confirmed by the significant correlation between maximum changes in PWV induced by AM and HOMA-IR (Figure 4). The difference in elastic property reduction between AM and nicardipine treatment was not significant in the high HOMA-IR group (Figure 3c).

Table 3 summarizes the changes in humoral factors. AM administration produced significant increases in total AM (approximately 2.5-fold) and mature AM (approximately 7-fold) as well as an approximately 40% increase in the second messenger cAMP. AM and nicardipine produced the same degree of increase in noradrenaline and renin activity. There was no significant difference among the HOMA-IR groups for all humoral factor alterations (data not shown). Finally, AM and nicardipine had no effect on insulin and glucose levels.

DISCUSSION

In this study, we confirmed the hemodynamic effects of AM as a vasodilative agent in humans. AM increased heart rate and cardiac output and decreased blood pressure. In addition, AM increased blood flow in carotid and renal arteries. These effects were similar

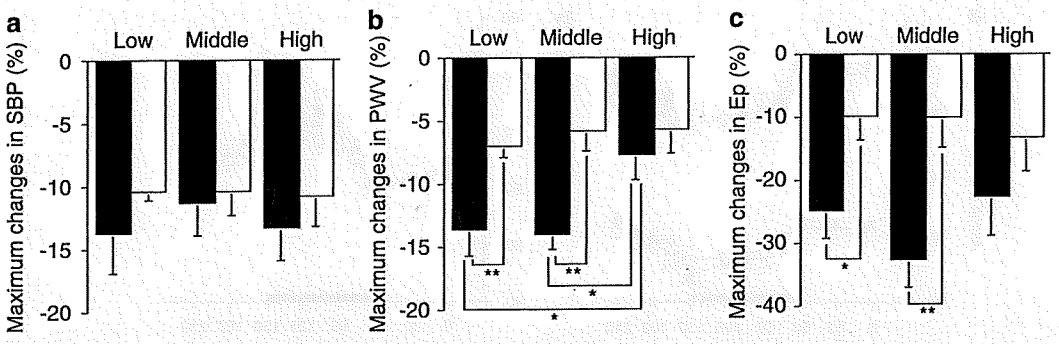

Figure 3 Maximum changes in systolic blood pressure (SBP, panel **a**), pulse wave velocity (PWV, panel **b**) and elastic property of the carotid artery (Ep, panel **c**) during infusion of AM (closed column) or nicardipine (open column) in each of the three groups divided by HOMA-IR index. Groups divided by HOMA-IR index include low (n=9), middle (n=10) and high (n=9) index groups. Data are mean ± s.e.m. $*P<0.05$, $**P<0.01$.

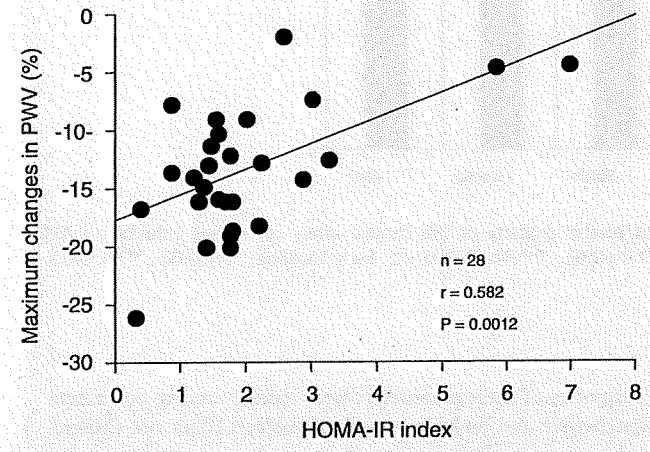

Figure 4 Relationship between maximum changes in pulse wave velocity (PWV) induced by AM administration and the HOMA-IR index.

to those of the common vasodilator nicardipine. However, AM produced greater increases in cardiac output and heart rate when compared with nicardipine (Table 2). The ventricular myocardium has abundant AM-binding sites; therefore, AM increases cardiac cAMP.[17,18] This cAMP-dependent mechanism mediates the positive inotropic action of β-adrenergic stimulants. AM also enhances angiotensin-II-induced improvement of systolic function, resulting in a further increase in left ventricular ejection fraction.[19] In addition, a cAMP-independent mechanism for the positive inotropic action of AM has been reported.[20] These data suggest that increased cardiac output, and probably heart rate, may be attributable not only to the decrease in cardiac afterload but also to the direct positive inotropic action of AM.

PWV is a convenient indicator of arterial stiffness and is applicable in the casual and prognostic estimation of risk for cardiovascular events. We used brachial ankle PWV (baPWV) in this study. As shown for authentic PWV, baPWV correlates well with blood pressure and aging,[21] and increased baPWV is associated with cardiovascular diseases and risk factors.[22] More importantly, high baPWV values predict poor prognosis in subjects.[23] Conversely, improvement of PWV by antihypertensive therapy may reduce the incidence of cardiovascular events.[15] Strong expression of AM is found ubiquitously in blood vessels where AM functions as a vasodilator, coordinating with other vasodilators, such as nitric oxide, to regulate vascular tonus.[1] AM is suggested to be a significant modulator of arterial stiffness, decreasing blood pressure and vascular wall tension and preventing future wall remodeling. In this study, AM had a greater effect on aortic stiffness, as assessed by baPWV or elastic property, when compared with a common vasodilator Ca^{2+} channel blocker (Figure 2). This feature may contribute to a vascular protective or compensatory function of AM against vascular deterioration and resulting vascular events.

baPWV is mainly altered by blood pressure, but other factors, such as increased heart rate, cardiac output and sympathetic nerve activity, may also increase baPWV. Indeed, the effect of nifedipine on baPWV was reduced by increased sympathetic activity.[24] Although decreases in systolic blood pressure were well matched in nicardipine and AM treatments, AM showed greater baPWV reduction despite larger increments in heart rate and cardiac output when compared with nicardipine (Figure 2 and Table 2). The increases in catecholamines and renin activity were equivalent in both treatments (Table 3). These alterations probably reduced or inhibited the decrease in baPWV in nicardipine treatment. Alternatively, it is conceivable that AM has greater potency against negative alterations to increase baPWV when compared with nicardipine.

AM and nicardipine produced a larger blood pressure reduction in hypertensive participants when compared with normotensive or diabetic participants, which is a common feature of hypotensive reagents (data not shown). However, other hemodynamic effects of AM and nicardipine evaluated within each treatment were essentially the same for each subgroup of participants. We evaluated potentially influential factors in the effects of AM, and we found that only the insulin resistance interfered with the effects of AM. Insulin resistance is an aggravating factor in vascular function and is an underlying cause of cardiovascular diseases. Insulin resistance also influences the sensitivity or efficacy of many drugs and bioactive substances. More importantly, increased arterial stiffness is commonly found in representative insulin resistance states, namely, metabolic syndrome and diabetes.[25–27] As shown in Figures 3 and 4, favorable effects of AM on arterial stiffness were blunted in a state of insulin resistance. AM is thought to function as a suppressive factor against insulin resistance.[8,9] As such, plasma concentration of AM was progressively increased in patients with impaired glucose tolerance, diabetes and diabetes with nephropathy.[28] In addition, an increase in AM was related to multiple metabolic factors.[28] AM and insulin resistance may conflict with each other. Specifically, decreased reactivity of AM may contribute to increased arterial stiffness during insulin resistance, and this alteration may accelerate the progression of arterial sclerosis in insulin resistance.

Table 3 Hormonal responses to adrenomedullin or nicardipine

	Before	*During*	*After*	P *in trend*
Total AM, fmol ml^{-1}				
AM	13.9±0.8	32.8±1.8**	15.9±0.6	<0.0001
Nicardipine	13.1±0.4	12.4±0.4##	12.8±0.5##	NS
Mature AM, fmol ml^{-1}				
AM	1.9±0.1	12.2±0.8**	2.6±0.1	<0.0001
Nicardipine	1.6±0.2	1.8±0.2##	1.7±0.2##	NS
cAMP, pmol ml^{-1}				
AM	11.8±0.3	16.5±0.5**	12.9±0.4	<0.0001
Nicardipine	11.1±0.4	11.0±0.4##	11.1±0.4##	NS
cGMP, pmol ml^{-1}				
AM	3.5±0.4	3.7±0.3	3.4±0.4	NS
Nicardipine	3.6±0.3	3.7±0.3	3.0±0.2	NS
Renin activity, ng ml^{-1} h^{-1}				
AM	0.9±0.1	1.7±0.3**	1.0±0.1	0.006
Nicardipine	0.9±0.1	1.7±0.3**	1.1±0.1	0.016
Aldosterone, pg ml^{-1}				
AM	74.4±5.2	69.7±5.3	65.3±3.7	NS
Nicardipine	78.2±4.7	77.2±5.3#	71.0±4.5	NS
Noradrenaline, pg ml^{-1}				
AM	245±20	429±33**	267±21	<0.0001
Nicardipine	247±19	423±33**	346±25**, ##	<0.0001
Adrenaline, pg ml^{-1}				
AM	27.6±3.3	32.1±3.8	25.8±2.2	NS
Nicardipine	21.8±2.1#	24.4±3.2##	31.8±3.2*, #	0.04
ANP, pg ml^{-1}				
AM	21.8±3.2	25.4±4.1	22.7±3.7	NS
Nicardipine	17.0±1.8	19.0±2.0#	14.0±1.2##	NS
BNP, pg ml^{-1}				
AM	12.4±2.0	11.4±1.8	14.0±2.2	NS
Nicardipine	9.4±1.6	10.0±1.7	10.1±1.6	NS
IRI, μIU ml^{-1}				
AM	7.2±1.0	7.1±0.9	6.0±0.7	NS
Nicardipine	8.0±0.8	7.1±0.7	6.4±0.6	NS
Glucose. mg per 100 ml				
AM	119±6	116±6	110±5	NS
Nicardipine	124±8	121±8	116±7	NS

Abbreviations: AM, adrenomedullin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; IRI, immunoreactive insulin; NS, not significant.
Date are mean±s.e.m.
*P<0.05, **P<0.01 *vs.* before.
#P<0.05, ##P<0.01 *vs.* AM.

However, this intervention is only a temporary treatment, so further studies are required to clarify the relationship between AM and insulin resistance.

Plasma concentration of total AM was increased approximately 2.4-fold above the control value after AM administration (Table 3). This level of AM concentration has been found in renal failure or heart failure patients,[29] so the level of AM used was pathophysiological, not pharmacological. AM administration also increased cAMP, which is a second messenger of AM, approximately 1.4-fold above the control value. Similar changes in AM and cAMP have been reported in previous studies.[10–12] AM and nicardipine also produced similar hormonal alterations, namely, stimulated sympathetic activity and renin release, which was also observed in another study.[10,11] The only difference between the effects of AM and nicardipine in our study was on aldosterone release. AM tended to inhibit aldosterone release despite increased renin activity, although this difference was not significant (P=0.051, Table 3). AM did not change aldosterone levels in healthy volunteers or patients with essential hypertension,[10,11] but AM suppressed increased aldosterone levels in patients with heart failure.[12] Furthermore, AM may have renin-independent suppressive potency for aldosterone release, and this feature should be elucidated in future studies.

In conclusion, exogenous AM and Ca^{2+} channel blocker nicardipine caused similar vasodilations in humans, accompanied with resemble interactions with the renin–angiotensin and sympathetic nervous systems. However, AM had a greater potency in its cardiac inotropic action when compared with nicardipine. AM also more effectively decreased arterial stiffness, but the effect was weakened to a similar level as for nicardipine in a state of insulin resistance. Our results support the hypothesis that AM may modulate vasoactive substances and vascular tonus and also have a role in pathophysiological conditions, such as an insulin resistance state.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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1 Eto T, Kato J, Kitamura K. Regulation of production and secretion of adrenomedullin in the cardiovascular system. *Regl Pept* 2002; **112**: 61–69.
2 Elsasser TH, Kahl S. Adrenomedullin has multiple roles in disease stress: development and remission of the inflammatory response. *Microsc Res Tech* 2002; **57**: 120–129.
3 Wong LY, Cheung BM, Li Y-Y, Tang F. Adrenomedullin is both proinflammatory and anti-inflammatory: its effects on gene expression and secretion of cytokines and macrophage migration inhibitory factor in NR8383 macrophage cell line. *Endocrinology* 2005; **146**: 1321–1327.
4 Shimosawa T, Shibagaki Y, Ishibashi K, Kitamura K, Kangawa K, Kato S, Ando K, Fujita T. Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. *Circulation* 2002; **105**: 106–111.
5 Marutsuka K, Hatakeyama K, Sato Y, Yamashita A, Sumiyoshi A, Asada Y. Immunohistological localization and possible functions of adrenomedullin. *Hypertens Res* 2003; **26** (Suppl): S33–S40.
6 Shinomiya K, Ohmori K, Ohyama H, Hosomi N, Takahashi T, Osaka K, Kohno M. Association of plasma adrenomedullin with carotid atherosclerosis in chronic ischemic stroke. *Peptides* 2001; **22**: 1873–1880.
7 Kita T, Kitamura K, Hashida S, Morishita K, Eto T. Plasma adrenomedullin is closely correlated with pulse wave velocity in middle-aged and elderly patients. *Hypertens Res* 2003; **26**: 887–893.
8 Shimosawa T, Ogihara T, Matsui H, Asano T, Ando K, Fujita T. Deficiency of adrenomedullin induces insulin resistance by increasing oxidative stress. *Hypertension* 2003; **41**: 1080–1085.
9 Xing G, Shimosawa T, Ogihara T, Matsui H, Itakura K, Qingyou X, Asano T, Ando K, Fujita T. Angiotensin II-induced insulin resistance is enhanced in adrenomedullin-deficient mice. *Endocrinology* 2004; **145**: 3647–3651.
10 Lainchbury JG, Troughton RW, Lewis LK, Yandle TG, Richards AM, Nicholls MG. Hemodynamic, hormonal, and renal effects of short-term adrenomedullin infusion in healthy volunteers. *J Clin Endocrinol Metab* 2000; **85**: 1016–1020.
11 Troughton RW, Levis LK, Yandle TG, Richards AM, Nicholls MG. Hemodynamic, hormone, and urinary effects of adrenomedullin infusion in essential hypertension. *Hypertension* 2000; **36**: 588–593.

12 Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, Oya H, Kyotani S, Nakanishi N, Goto Y, Masuda Y, Miyatake K, Kangawa K. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation* 2000; **101**: 498–503.
13 Blacher J, Asmar R, Djane S, London GM, Safar ME. Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension* 1999; **32**: 570–574.
14 Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, Ducimetiere P, Benetos A. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 2001; **37**: 1236–1241.
15 Guerin AP, Blacher J, Pannier B, Marchais SJ, Safar ME, London GM. Impact of aortic stiffness attenuation on survival of patients in end-stage renal failure. *Circulation* 2001; **103**: 987–992.
16 Blankenhorn DH, Chin HP, Conover DJ, Nessim SA. Ultrasound observation on pulsation in human carotid artery lesions. *Ultrasound Med Biol* 1988; **14**: 583–587.
17 Owji AA, Smith DM, Coppock HA, Morgan DG, Bhogal R, Ghatei MA, Bloom SR. An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. *Endocrinology* 1995; **136**: 2127–2134.
18 Ihara T, Ikeda U, Tate Y, Ishibashi S, Shimada K. Positive inotropic effects of adrenomedullin on rat papillary muscle. *Eur J Pharmacol* 2000; **390**: 167–172.
19 Luodonpää M, Leskinen H, Ilves M, Vuolteenaho O, Ruskoaho H. Adrenomedullin modulates hemodynamic and cardiac effects of angiotensin II in conscious rats. *Am J Physiol* 2004; **286**: R1085–R1092.
20 Szokodi I, Kinnunen P, Tavi P, Weckstrom M, Toth M, Ruskoaho H. Evidence for cAMP-independent mechanisms mediating the effects of adrenomedullin, a new inotropic peptide. *Circulation* 1998; **97**: 1062–1070.
21 Tomiyama H, Yamashina A, Arai T, Hirose K, Koji Y, Chikamori T, Hori S, Yamamoto Y, Doba N, Hinohara S. Influences of age and gender on results of noninvasive brachial-ankle pulse wave velocity measurement—a survey of 12517 subjects. *Atherosclerosis* 2003; **166**: 303–309.
22 Yamashina A, Tomiyama H, Arai T, Hirose H, Koji Y, Hirayama Y, Yamamoto Y, Hori S. Brachial-ankle pulse wave velocity as a marker of atherosclerotic vascular damage and cardiovascular risk. *Hypertens Res* 2003; **26**: 615–622.
23 Kitahara T, Ono K, Tsuchida A, Kawai H, Shinohara M, Ishii Y, Koyanagi H, Noguchi T, Matsumoto T, Sekihara T, Watanabe Y, Kanai H, Ishida H, Nojima Y. Impact of brachial-ankle pulse wave velocity and ankle-brachial blood pressure index on mortality in hemodialysis patients. *Am J Kidney Dis* 2005; **46**: 688–696.
24 Munakata M, Nagasaki A, Nunokawa T, Sakuma T, Kato H, Yoshinaga K, Toyota T. Effects of valsartan and nifedipine coat-core on systemic arterial stiffness in hypertensive patients. *Am J Hypertens* 2004; **17**: 1050–1055.
25 Ghiadoni L, Penno G, Giannarelli C, Plantinga Y, Bernardini M, Pucci L, Miccoli R, Taddei S, Salvetti A, Del Prato S. Metabolic syndrome and vascular alterations in normotensive subjects at risk of diabetes mellitus. *Hypertension* 2008; **51**: 440–445.
26 Kovaite M, Petrulioniene Z, Ryliskyte L, Badariene J, Dzenkeviciute V, Cypiene A, Laucevicius A, Polena S, Gintautas J. Systemic assessment of arterial wall structure and function in metabolic syndrome. *Proc West Pharmacol Soc* 2007; **50**: 123–130.
27 El Feghali R, Topouchian J, Pannier B, Asmar R. Ageing and blood pressure modulate the relationship between metabolic syndrome and aortic stiffness in never-treated essential hypertensive patients. A comparative study. *Diabetes Metab* 2007; **33**: 183–188.
28 Lim SC, Morgenthaler NG, Subramanian T, Wu YS, Goh SK, Sum CF. The relationship between adrenomedullin, metabolic factors, and vascular function in individuals with type 2 diabetes. *Diabetes Care* 2007; **30**: 1513–1519.
29 Nishikimi T. Adrenomedullin in cardiovascular disease. *Adv Pharmacol* 1998; **42**: 599–603.

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ORIGINAL ARTICLE

Adrenomedullin treatment reduces intestinal inflammation and maintains epithelial barrier function in mice administered dextran sulphate sodium

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ABSTRACT

Hyperactivation and hyperpermeability of the intestinal epithelium is a hallmark of IBD. AM has been shown to reduce the severity of colitis in the acetic acid and TNBS-induced colitis model, however the mechanism of the therapeutic effect of AM against the colitis has not been clarified. Here, we show that the protective capability of AM is associated with suppression of inflammation and maintenance of the intestinal epithelial barrier function. In the DSS-induced colitis model, intra-rectal AM-treated mice showed a reduction in loss of body weight and severity of colitis. AM-treatment suppressed phosphorylation of STAT1 and STAT3 in the colonic epithelium, and altered the cytokine balance in the intestinal T cells, with lower levels of IFN-γ and TNF-α but higher levels of TGF-β. Expression of the epithelial intercellular junctions such as tight and adherence junctions were sustained in the AM-treated mice. In contrast, the epithelial junctions were down-regulated in the control mice, leading to loss of epithelial barrier integrity and enhanced permeability. Collectively, these data indicate a broad spectrum of AM-induced effects with respect to protection against DSS-induced colitis, and suggest a potential therapeutic value of this treatment for IBD.

Key words adrenomedullin, colitis, epithelial barrier.

AM was originally isolated from human pheochromocytoma and identified as a vasodilatory peptide (1), and has since been shown to be constitutively produced by a variety of tissues such as gut and bronchial epithelium of humans (2, 3) and gut of mice and rats (4, 5). In addition, AM also exerts anti-inflammatory (6) and antimicrobial activity (7). Since the expression of AM and its receptor are up-regulated during inflammation (6, 8), AM has potential therapeutic value in inflammatory diseases as well as several other categories of disease such as hypertension, cardiovascular and renal disorders, cancer, and diabetes.

IBD, including CD and UC, is a chronic, relapsing, and remitting condition with unknown etiology which exhibits various features of dysregulated immunological

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List of Abbreviations: AM, adrenomedullin; AP, allophycocyanin; CD, Crohn's disease; DSS, dextran sulfate sodium; DTT, dithiothreitol; EC, epithelial cells; HE, hematoxylin and eosin; IBD, inflammatory bowel diseases; IEL, intestinal intraepithelial T lymphocytes; IFN-γ, interferon-γ; IL, Il-4, interleukin-4; JAK, Janus kinase; JAM, junctional adhesion molecule; mAb, monoclonal Ab; MHC, major histocompatibility complex; r, recombinant; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TER, transepithelial electrical resistance; TGF-β, transforming growth factor-β; Th, T helper; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF-γ, tumor necrosis factor-γ; UC, ulcerative colitis, ZO, zonula occludens.

systems (9, 10). The pathogenesis of IBD involves interplay of the genetic, microbial, and immune systems, resulting in chronic intestinal inflammation with disruption of epithelial integrity and barrier function and increased sensitivity to intestinal flora (11–14). Interaction between IEL and EC is necessary for maintenance of epithelial integrity and a physical barrier to prevent potential inflammatory responses and infectious challenges in the intestinal epithelium (14–19). Recently, we and others have reported that AM is a potent anti-inflammatory agent in rat acetic acid-induced colitis (20) and murine TNBS-induced colitis (21). However, the mechanisms of AM-mediated amelioration of colitis have not been clarified. In this study, we have investigated the effect of AM treatment on suppression of DSS-induced colitis in the context of maintenance of epithelial barrier function.

MATERIALS AND METHODS

Animals and peptide

Male C57BL/6 mice (7–9 weeks old) were purchased from Japan SLC (Hamamatsu, Japan), housed under specific pathogen-free conditions and maintained on standard pellet chow and water *ad libitum*. The recombinant (r) human AM used in this study was provided by Shionogi, (Osaka, Japan). All experiments were carried out in accordance with the regulations of the Animal Research Committee of the University of Miyazaki and University of the Ryukyus.

Treatment with AM and induction of colitis

AM (0.05 μg of AM diluted in 0.2 ml of saline) was delivered into the lumen of the colon via a 5 cm long animal feeding tube inserted into the rectum to a depth of approximately 3 cm from the anal verge. Non AM-treated (control) mice were administered 0.2 ml of saline without AM. AM and saline were administrated once a day for 7 days. To induce colitis, mice were given 1.8% DSS (molecular weight, 36 000–50 000 daltons; ICN Biomedicals, Aurora, Ohio, USA) in their drinking water for 7 days (from day 0 to 6). On day 7, their water was switched to regular drinking water.

Detection of anaerobes

Detection of anaerobes was performed as described previously (16).

Histological analysis

Paraffin-embedded colonic sections of 10% formalin-fixed tissues were stained with HE.

Disease evaluation

Intestinal inflammation in the mice was evaluated according to previously described criteria (22). The disease score was determined by a combination of ulceration (0, no ulceration; 1, presence of erosion; 2, presence of focal ulceration; 3, presence of multiple ulcerations) and histological scores (0–3, inflammatory cell infiltration; 0–3, epithelial cell elongation).

Preparation of EC, IEL and cell culture for cytokine ELISA

IEL and EC were isolated and prepared according to a previously published method (16). Briefly, dissected small segments of the intestines were incubated at 37°C for 40 min in an RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FCS and 1 mM DTT with vigorous shaking. The tissue suspension was passed through a nylon mesh to remove debris and centrifuged through a 25/40/75% discontinuous Percoll (Sigma-Aldrich) gradient at 600 $\times$ g at 20°C for 20 min. The cells collected from the interface of 25/40% were EC, and that of 40/75% were IEL. IEL were cultured for 48 hr with anti-CD3 mAb in RPMI 1640 supplemented with 10% FCS. The supernatants were collected to estimate the cytokine contents by ELISA using mouse IFN-γ, IL-4, and TNF-α (e-Bioscience, San Diego, CA, USA) and TGF-β (Biosource International, Camarillo, CA, USA) according to the manufacturer's instructions.

Western blot analysis

Lysates were prepared from colonic EC and analyzed by Western blotting according to a published method (14). Abs used in the Western blotting were as follows: rabbit anti-PY-STAT3, anti-PY-STAT1, anti-STAT3 and anti-STAT1 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-ZO-1 and anti-occludin (Zymed, South San Francisco, CA, USA), mouse anti-E-cadherin (BD Transduction Laboratories, Lexington, KY, USA), mouse anti-β-catenin (BD Transduction Laboratories), and mouse anti-β-actin (Sigma, St Louis, MO, USA).

FACS analysis

IEL were stained with FITC-conjugated TCR$\gamma\delta$ (GL3), PE-conjugated TCRβ (H57–597), AP-conjugated CD3ε (145-2C11) mAb as described previously (16). Flow cytometry analysis was performed on a FACS calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). All mAbs were purchased from BD Pharmingen (San Jose, CA, USA).

RT-PCR and real-time PCR

Total RNA was extracted from freshly isolated EC using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and primed with 20 pmol of a random primer in mixtures for reverse transcription. The synthesized cDNA was amplified by PCR using primers specific for the murine junctional molecules. The primer sets for detection of these junctional molecules have previously been described (23). For quantitative analysis of mRNA, the synthesized cDNA was amplified by using primers and probes specific for ZO-1, occludin and β-actin cDNA sequences (TaqMan gene Expression Assays; Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed by using an ABI Prism 7000 Sequence Detector System (Applied Biosystems Foster, CA, USA). Results are expressed as the n-fold difference relative to the expression of β-actin.

Measurement of TER

The functional integrity of tight junctions in cell layers established on filter inserts was assessed by measuring TER using a Millicel ERS Volt-ohm meter (Millipore, Bedford, MA, USA) as described previously (19). CMT93 cells, a murine intestinal epithelial cell line, were seeded on the apical chamber of a transwell using BD BioCoat Intestinal Epithelium Differentiation Environment (BD Biosciences Bedford, MA, USA) for 72 hr in a 5% CO_2 incubator according to the manufacturer's instructions, and developed a TER around 450 Ohm $\times$ cm^2. The culture medium was then removed from the apical and basolateral chambers and replaced with either fresh medium or medium containing 100 ng/ml rIFN-γ (Chemicon, Temecula, CA, USA) and AM (10^{-7}, 10^{-8} and 10^{-9} M). Values of TER are expressed as percentage of the initial resistance as follows:

% of initial resistance = ([resistance from each point]− [resistance from a blank]/[resistance from non-treated cells] − [resistance from a blank]) × 100. [1].

Statistical analysis

Data are presented as means ± SD. Statistical parameters were ascertained with Statview 4.51 software (Abacus Concepts, Berkeley, CA, USA). A P value of less than 0.05 was taken as significant.

RESULTS

AM administration abrogates colitis in DSS-treated mice

We investigated the therapeutic effect of intrarectally administered AM on the severity of DSS-induced colitis. Mice given DSS showed profound and sustained weight loss, a major symptom of colitis (Fig. 1a). In contrast, AM-treated mice exhibited maintenance of body weight and suppression of other clinical symptoms such as overt bleeding and diarrhea. The AM-treated mice remained healthy and experienced none of the long-term effects of colitis seen in the control mice by day 28, while all of the control mice succumbed (data not shown). These results indicate that the administration of AM significantly abrogates severe symptoms of DSS colitis.

Histological analysis revealed neutrophil and lymphocyte infiltration on day 3; furthermore, remarkable thickening of the colonic wall, excessive crypt abscesses, EC erosions, and destruction of epithelial integrity were observed in the control mice on day 10 after DSS administration (Fig. 1b). By contrast, the AM-treated mice exhibited lesions that were much less severe, although there was mild thickening of the colon wall. On day 14 after AM administration, no evidence of inflammation was observed in the AM-treated mice, whereas crypt hyperplasia and infiltrated inflammatory cells were still evident in the control mice. This finding was further supported by assessment of changes in disease score (Fig. 1c). These results indicate that treatment with AM reduces development of DSS-induced colitis and strikingly improves inflammatory symptoms.

In addition to anti-inflammatory activity, AM displays potent antimicrobial activity against a variety of Gram-negative and Gram-positive bacteria (7, 24). After induction of DSS colitis the number of anaerobes was significantly decreased in the AM-treated compared with the control mice (Fig. 2). Since increased numbers of facultative anaerobes can be associated with IBD in humans (13), these results suggest that AM protects against disruption of the mucosal epithelium through suppression and translocation of anaerobes into the intestinal mucosa, leading to recruitment and activation of immune cells.

AM administration decreases inflammatory cytokines and increases regulatory cytokines

Hyperactivation due to deregulation of cytokine signaling has been suggested as a cause of colitis in the murine model and in IBD patients (12). Th1-type cytokine and cytokines which belong to the IL-6 cytokine family have been reported to induce acute bowel inflammation in IBD patients (25, 26) and in DSS-treated mice (27). Suppression of colitis can also be attributed to cell-to-cell interaction between T cells and enterocytes causing changes in the cytokine profile (15, 16). The effect of AM-treatment was also seen at the level of cytokine production from

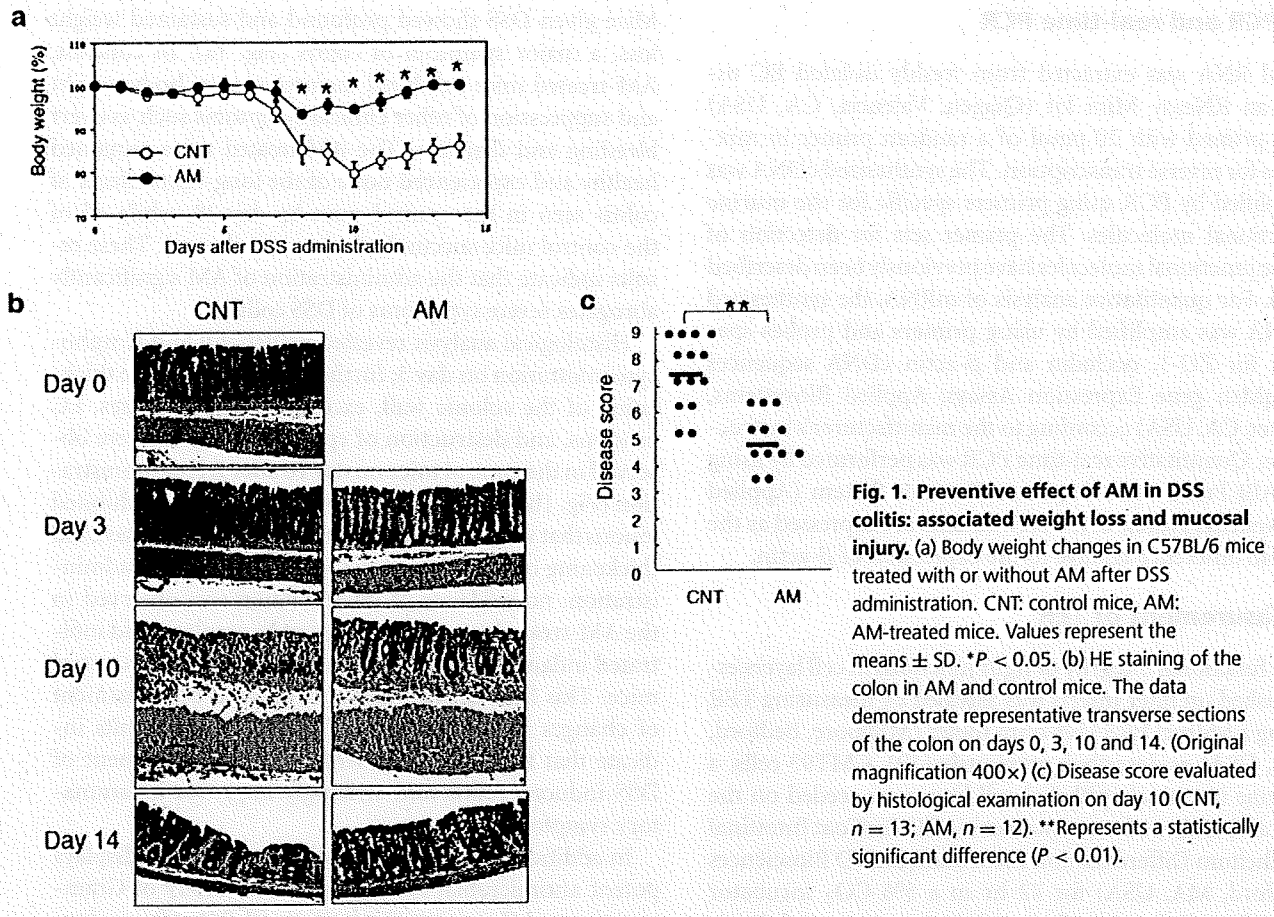

Fig. 1. Preventive effect of AM in DSS colitis: associated weight loss and mucosal injury. (a) Body weight changes in C57BL/6 mice treated with or without AM after DSS administration. CNT: control mice, AM: AM-treated mice. Values represent the means ± SD. $^*P < 0.05$. (b) HE staining of the colon in AM and control mice. The data demonstrate representative transverse sections of the colon on days 0, 3, 10 and 14. (Original magnification 400×) (c) Disease score evaluated by histological examination on day 10 (CNT, $n = 13$; AM, $n = 12$). **Represents a statistically significant difference ($P < 0.01$).

IEL of the large intestine. IEL isolated from the control mice consistently produced high concentrations of IFN-γ, TNF-α, and IL-6, with peak concentrations detected on day 10 after DSS administration (Fig. 3a). In contrast, AM treatment induced substantial TGF-β production, which did not occur in the IEL taken from the control mice. IL-4 production after DSS administration was not affected by AM treatment.

Cytokines bind to specific cell-surface receptors and activate cytoplasmic signal transduction pathways such as the JAK/STAT pathway (28). Continuous activation of STAT3 and STAT1 is often observed in patients with IBD as well as other inflammatory diseases (29, 30), and experimental and clinical data point toward aberrant activation of the Th1-dominant cytokine network. Since STAT3 is preferentially activated by IL-6 and its related cytokines and STAT1 is activated by IFN-γ (31), we examined both STAT3 and STAT1 activation. As shown in Figure 3b, in control mice STAT3 and STAT1 activation in the intestinal mucosa was observed on day 10; in contrast, their suppression was clearly shown in the AM-treated mice.

Since interaction between IEL and EC is important for the maintenance of intestinal homeostasis and both TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells have been implicated in the development of colitis (16, 32, 33), we examined changes in the number of IEL and the proportion of TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells in the IEL compartment of the large intestine. Although colonic CD3-positive IEL increased for 7 days following DSS administration in both control and AM-treated mice (Fig. 4a), TCR$\gamma\delta$ IEL decreased in the control mice, but not in the AM-treated mice (Fig. 4b). The number of IEL recovered to the initial amount in the AM-treated mice but not in the control mice. The induction of DSS colitis led to changes in the IEL profile, with a relative decrease in the TCR$\gamma\delta^+$ population on days 7 to 10 after DSS administration. Treatment of mice with AM prevented these alterations in the TCR$\alpha\beta$ and TCR$\gamma\delta$ IEL populations. Collectively, these results suggest that AM suppresses the production of proinflammatory cytokines by maintaining the TCR $\gamma\delta$ IEL and increasing the production of TGF-β expressed by the TCR $\gamma\delta$ IEL, thus leading to suppression of continuous STAT3 and STAT1 activation in EC.

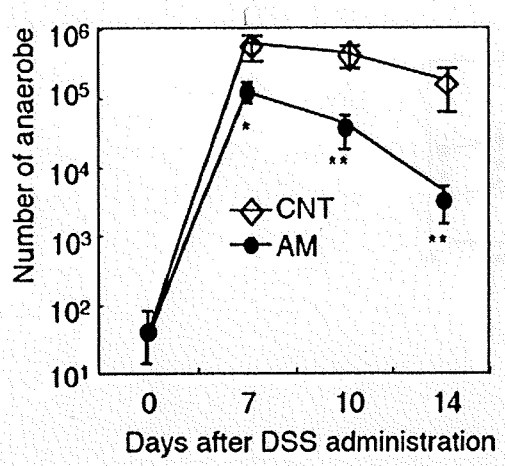

Fig. 2. The number of intestinal anaerobes in mice with DSS induced-colitis is decreased by AM treatment. Number of anaerobes was determined on day 7, 10 and 14 after AM administration. Three mice were used for each point. Values represent the means ± SD of three individual experiments. * and ** represent a statistically significant difference (*$P < 0.05$, **$P < 0.01$).

Treatment of mice with AM leads to recovery of junctional molecules in EC

To maintain the integrity of the intestinal epithelial barrier, EC express various junctional molecules including those associated with a tight junction; (ZO-1, occludin, and JAM); an adherens junction (β-catenin and E-cadherin); desmosomes (desmoglein-2) and a gap junction (connexin 26). The combined function of these molecules is to maintain the epithelial layer as a continuous impermeable belt to prevent paracellular crossing of a variety of luminal contents (34, 35). Expression of the junctional molecules is decreased in the course of colitis (23).

To evaluate the protective effect of AM against cytokine-induced impairment of the epithelial barrier *in vitro*, we examined TER by using CMT93 cells, a murine intestinal epithelial cell line. The TER of CMT93 cells was decreased by the addition of IFN-γ. The addition of AM increased the TER of the cells treated with IFN-γ in a dose-dependent manner (Fig. 5a). We next examined the degree of expression of specific mRNA for a variety of junctional molecules in large intestinal EC *in vivo*. Following induction of DSS-colitis, mRNA expression levels of junctional molecules in the tight junction, adherens junction, and desmosomes were drastically decreased on day 10 and had incompletely recovered on day 14 in the control mice (Fig. 5b). In contrast, these changes were much smaller in the AM-treated mice. There was a slight transient reduction in the expression of junctional molecule mRNA on day 10 of colitis induction, but full recovery was noted by day 14 in the AM-treated mice. Since ZO-1 and occludin are crucial for the formation of TER, we further examined their expression by real-time RT-PCR. ZO-1 expression in the control and AM-treated mice decreased and recovered in parallel. Occludin expression in the control mice continued to decrease, whereas that in the AM-treated mice was sustained (Fig. 5c). This result is coincident with the RT-PCR data shown in Figure 5a. Western blot data also showed decreased expression of junctional molecules in the control mice but maintenance of the initial degree of expression in the AM-treated mice, except in the case of ZO-1 (Fig. 5d). These results indicate that AM plays a role in maintaining the expression of junctional molecules and epithelial barrier function in the large intestine after induction of colitis.

DISCUSSION

Since IBD are refractory diseases of unknown etiology in humans, the development of new therapies is important. Recently, AM has been suggested as a candidate for IBD treatment because it acts as an anti-inflammatory agent in the rodent colitis model (20, 21). However, the mechanism of AM's protective effect against destruction of the epithelial barrier, which is a remarkable feature of IBD, has yet to be elucidated. This study is the first showing that the therapeutic effect of AM against colitis is correlated with maintenance of epithelial barrier integrity. The effects of AM on DSS-induced colitis include changes in cytokine production from IEL, and in expression of junctional molecules of EC and microbial flora, suggesting that AM exerts wide beneficial effects which protect against IBD.

The oral administration of DSS stimulates nonlymphoid cells such as EC and phagocytes, causing loss of body weight, mucosal ulcers, and neutrophil infiltration, and resulting in release of proinflammatory cytokines and destruction of the epithelial layer. Because DSS colitis occurs in mice lacking T cells, B cells, and NK cells, T cell-mediated immunity is not essential for induction of DSS colitis (36, 37). However, Th1-biased T cell-mediated immunity is activated in this model (38). We have clearly shown that AM suppresses Th1-type cytokines and subsequent proinflammatory responses associated with production of regulatory cytokines by IEL. These responses might be a mechanism for the therapeutic effect of AM treatment in colitis. Another therapeutic effect of AM based on a murine model of Crohn's disease has been reported: AM treatment was associated with down-regulation of both inflammatory and Th1-driven autoimmune responses, including regulation of a wide spectrum of inflammatory mediators mediated by $CD4^+CD25^+$ regulatory T cells (21). In the IBD models, colitis is ameliorated by TGF-β, an immunosuppressive

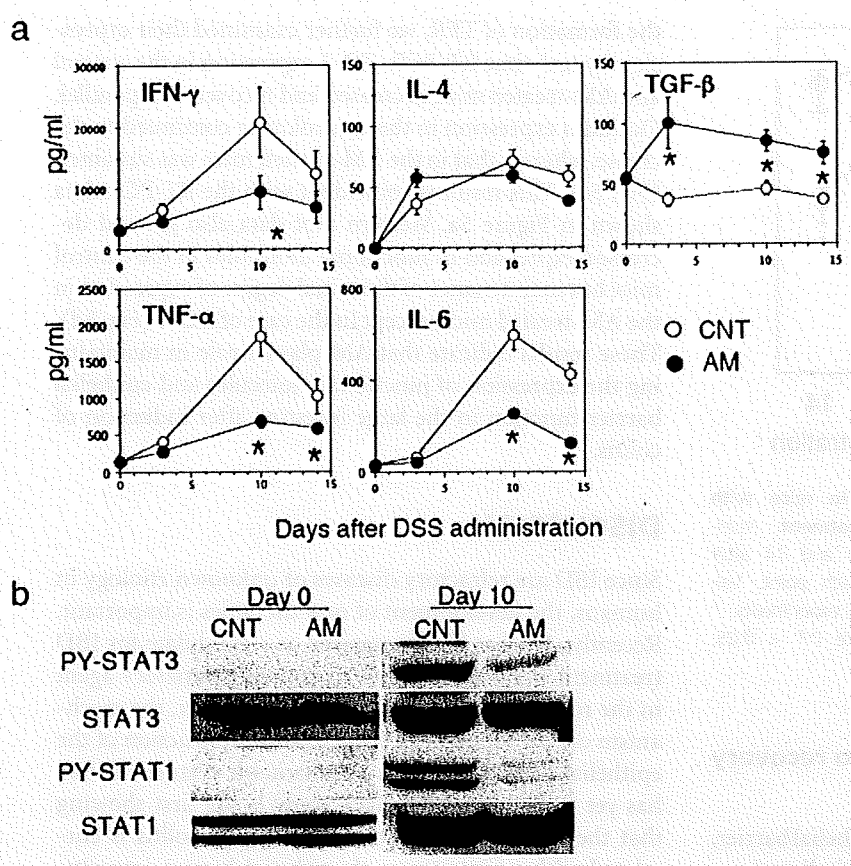

Fig. 3. AM administration suppresses the production of Th1- and inflammatory cytokines in the large intestine. (a) Changes in cytokine production of IEL. Three mice were used for each point. Values represent the means ± SD of three individual experiments. *Represents a statistically significant difference ($P < 0.05$). (b) Phosphorylation of STAT1 and STAT3 in colonic EC on days 0 and 10 after DSS administration.

cytokine produced by regulatory T cells (39, 40). TGF-β exerts a protective role against Th1-type colitis by suppressing the activated T cells (39, 41). Therefore, TGF-β produced by IEL of AM-treated mice also suppressed colitis in our experimental system.

TCR$\gamma\delta$ IEL can produce TGF-β and may suppress inflammation as regulatory T cells (42). In fact, C$\delta^{-/-}$ mice, which lack $\gamma\delta$ T cells, show a reduction in TGF-β production (16). In the present study, a decrease in TGF-β production by IEL may have been caused in part by a decrease in TCR$\gamma\delta$ IEL after DSS treatment of mice (Figs. 3a and 4). Because TCR$\gamma\delta$ IEL were maintained in AM-treated mice (Fig. 4b), the TCR$\gamma\delta$ IEL were assumed to be regulatory cells which suppress the Th1-type inflammatory response through cytokines or cell-to-cell interactions between EC and TCR$\alpha\beta$ IEL (15, 16). The IEL and EC reciprocally regulate their development and growth mediated by a variety of cytokines and their receptor signaling (15, 43, 44). C$\delta^{-/-}$ mice show a reduction in the turn-over of EC and down-regulation of expression of the MHC class II molecule (45). TCR$\gamma\delta$ IEL-mediated exertion of the suppressive function of EC may be an important mechanism of TCR$\gamma\delta$ T cell-mediated suppression of TCR$\alpha\beta$ IEL in the murine colitis model (16). Taken together, these results indicate that it is of great worth to investigate when and how AM regulates the function of TCR$\gamma\delta$ IEL during the development of colitis.

A possible alternative mechanism of AM-mediated suppression of colitis is modification of intestinal microbial flora through its antibacterial activity. Although the etiology of IBD is unknown, an abnormal inflammatory response directed against enteric microbial flora in susceptible hosts has been reported (9). AM has potent antibacterial activity against a variety of Gram-positive and Gram-negative bacteria that are frequently detected in the skin, digestive tract and airways (7, 24), and it has been suggested that the antibacterial activity is enhanced by post-secretory processing of the AM protein (24). Our results show that, after induction of DSS colitis, the number of intestinal anaerobes decreases in the AM-treated compared to the control mice, suggesting that AM suppresses colitis induction through modification of intestinal microbial flora by its antibacterial activity. Since AM is present in epithelium and mucosa in the gastrointestinal tract, it could also prevent the development of colitis in healthy individuals through its antibacterial activity.

Colitis may influence EC immune functions such as the production of cytokines, chemokines, and defensins

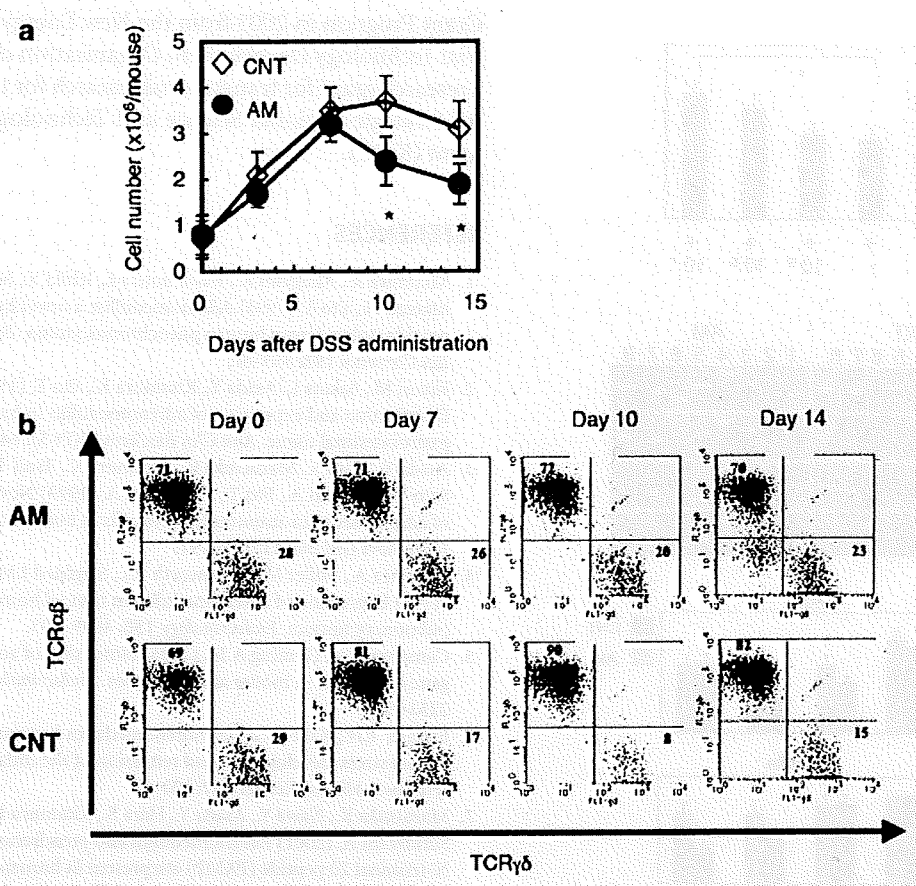

Fig. 4. Alteration of the number and proportion of IEL in the large intestine of mice with DSS-induced colitis treated with AM. (a) Number of CD3-positive IEL. IEL were collected on days 3, 7, 10 and 14 with or without AM administration. IEL were stained with antibodies against CD3, TCRβ and TCR$\gamma\delta$, followed by FACS analysis. Absolute CD3-positive cell number was calculated by multiplying total IEL number by proportion of CD3+-cells. *Represents a statistically significant difference ($P < 0.05$). (b) The proportion of IEL bearing TCR $\alpha\beta$ and TCR $\gamma\delta$. These TCR $\alpha\beta$ and TCR $\gamma\delta$ expressions on IEL are shown after the analysis gate was set on CD3$^+$ cells. Three mice were used for each point. Representative data are shown.

(46, 47), resulting in damage to the physical barrier function of EC. To maintain homeostasis and preserve the integrity of the epithelial barrier, the intercellular junctional complex, consisting of tight junctions, adherens junctions, desmosomes and gap junctions, is thought to facilitate appropriate communication between the external and internal environments. Using the human intestinal cell line, HT-29/B6, inflammatory cytokines such as IFN-γ and TNF-α have been reported to down-regulate the occludin promoter (48). Indeed, abnormal mucosal permeability attributed to inflammation and altered epithelial paracellular permeability has been observed in IBD patients with diarrhea (49, 50). Increased paracellular permeability enhances antigenic exposure to underlying immune cells, further compromising barrier function. In the present study, it was shown that mRNA concentrations of E-cadherin and occludin, which were decreased in the DSS-treated mice, recovered through AM administration, indicating that AM is linked to maintenance of the epithelial barrier against a Th1-type response.

The present results indicate that AM is a potential new therapeutic agent for colitis, and works through anti-inflammatory activity and maintenance of the colonic epithelial barrier concomitantly with its antibacterial effect.

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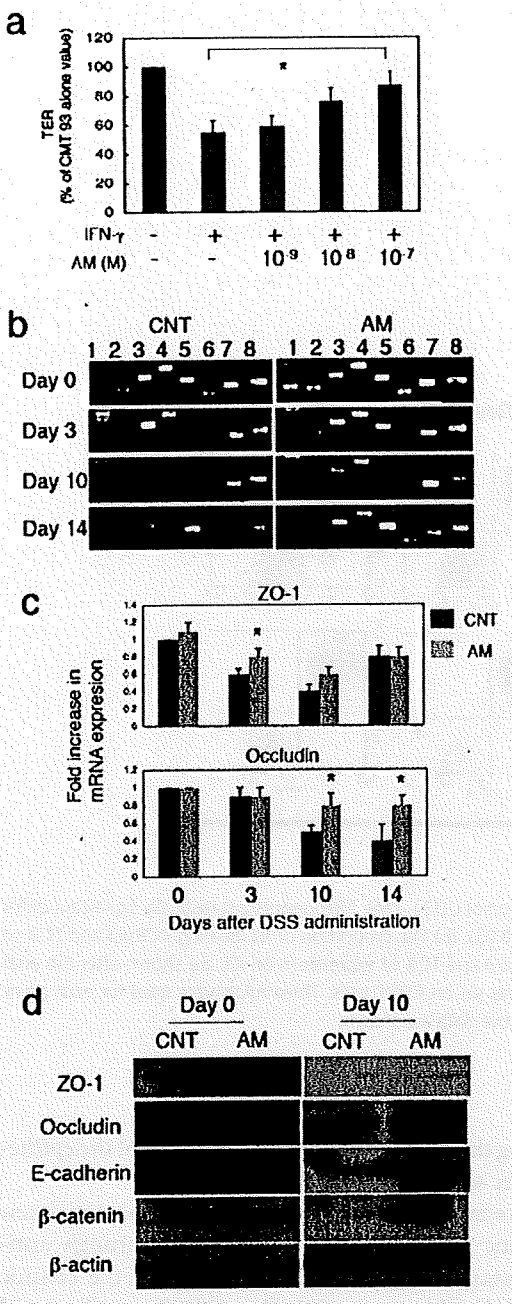

Fig. 5. The colonic epithelial barrier is maintained due to AM administration. (a) Recovery of TER of CMT93 cells by addition of AM. CMT93 cells were exposed to 100 ng/ml of rIFN-γ and a variety of concentration of AM for 24 hr. (b) Recovery of expression of junctional molecules on EC by AM administration to mice with DDS-induced colitis. The amount of mRNA of junction molecules in EC was analyzed by RT-PCR. Lane 1, ZO-1; lane 2, occludin; lane 3, JAM; lane 4, β-catenin; lane 5, E-cadherin; lane 6, desmoglein-2; lane 7, connexin 26; lane 8, β-actin. (c) mRNA expression of ZO-1 and occludin was analyzed quantitatively by real-time PCR. Data is presented as the ratio of degree of expression at each time-point to the data on day 0 in the control mice. *Represents a statistically significant difference between control and AM-treated mice ($P < 0.05$). (d) Western blot analysis of junctional molecules of colonic EC on days 0 and 10 after DSS administration.

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REFERENCES

1. Kitamura K., Kangawa K., Kawamoto M., Ichiki Y., Nakamura S., Matsuo H., Eto T. (1993) Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* **192:** 553–60.
2. Kitani M., Sakata J., Asada Y., Kitamura K., Eto T. (1998) Distribution and expression of adrenomedullin in human gastrointestinal tissue. *Ann Clin Biochem* **35**(Pt 5): 643–8.
3. Asada Y., Hara S., Marutsuka K., Kitamura K., Tsuji T., Sakata J., Sato Y., Kisanuki A., Eto T., Sumiyoshi A. (1999) Novel distribution of adrenomedullin-immunoreactive cells in human tissues. *Histochem Cell Biol* **112:** 185–91.
4. Martinez A., Miller M.J., Unsworth E.J., Siegfried J.M., Cuttitta F. (1995) Expression of adrenomedullin in normal human lung and in pulmonary tumors. *Endocrinology* **136:** 4099–105.
5. Cameron V.A., Fleming A.M. (1998) Novel sites of adrenomedullin gene expression in mouse and rat tissues. *Endocrinology* **139:** 2253–64.
6. Elsasser T.H., Kahl S. (2002) Adrenomedullin has multiple roles in disease stress: development and remission of the inflammatory response. *Microsc Res Tech* **57:** 120–9.
7. Marutsuka K., Nawa Y., Asada Y., Hara S., Kitamura K., Eto T., Sumiyoshi A. (2001) Adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP) are present in human colonic epithelia and exert an antimicrobial effect. *Exp Physiol* **86:** 543–5.
8. Ueda S., Nishio K., Minamino N., Kubo A., Akai Y., Kangawa K., Matsuo H., Fujimura Y., Yoshioka A., Masui K., Doi N., Murao Y., Miyamoto S. (1999) Increased plasma levels of adrenomedullin in patients with systemic inflammatory response syndrome. *Am J Respir Crit Care Med* **160:** 132–6.
9. Fiocchi C. (1998) Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* **115:** 182–205.
10. Blumberg R.S., Saubermann L.J., Strober W. (1999) Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr Opin Immunol* **11:** 648–56.
11. Gassler N., Rohr C., Schneider A., Kartenbeck J., Bach A., Obermuller N., Otto H.F., Autschbach F. (2001) Inflammatory bowel disease is associated with changes of enterocytic junctions. *Am J Physiol Gastrointest Liver Physiol* **281:** G216–28.
12. Bouma G., Strober W. (2003) The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* **3:** 521–33.
13. Cummings J.H., Macfarlane G.T., Macfarlane S. (2003) Intestinal bacteria and ulcerative colitis. *Curr Issues Intest Microbiol* **4:** 9–20.
14. Inagaki-Ohara K., Sasaki A., Matsuzaki G., Ikeda T., Hotokezaka M., Chijiiwa K., Kubo M., Yoshida H., Nawa Y., Yoshimura A. (2006) Suppressor of cytokine signalling 1 in lymphocytes regulates the development of intestinal inflammation in mice. *Gut* **55:** 212–9.
15. Yamamoto M., Fujihashi K., Kawabata K., McGhee J.R., Kiyono H. (1998) A mucosal intranet: intestinal epithelial cells down-regulate intraepithelial, but not peripheral, T lymphocytes. *J Immunol* **160:** 2188–96.
16. Inagaki-Ohara K., Chinen T., Matsuzaki G., Sasaki A., Sakamoto Y., Hiromatsu K., Nakamura-Uchiyama F., Nawa Y., Yoshimura A. (2004) Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *J Immunol* **173:** 1390–8.

17. Cheroutre H. (2005) IELs: enforcing law and order in the court of the intestinal epithelium. *Immunol Rev* **206:** 114–31.
18. Komori H.K., Meehan T.F., Havran W.L. (2006) Epithelial and mucosal gamma delta T cells. *Curr Opin Immunol* **18:** 534–8.
19. Inagaki-Ohara K., Dewi F.N., Hisaeda H., Smith A.L., Jimi F., Miyahira M., Abdel-Aleem A.S., Horii Y., Nawa Y. (2006) Intestinal intraepithelial lymphocytes sustain the epithelial barrier function against *Eimeria vermiformis* infection. *Infect Immun* **74:** 5292–301.
20. Ashizuka S., Ishikawa N., Kato J., Yamaga J., Inatsu H., Eto T., Kitamura K. (2005) Effect of adrenomedullin administration on acetic acid-induced colitis in rats. *Peptides* **26:** 2610–5.
21. Gonzalez-Rey E., Fernandez-Martin A., Chorny A., Delgado M. (2006) Therapeutic effect of urocortin and adrenomedullin in a murine model of Crohn's disease. *Gut* **55:** 824–32.
22. Sugimoto K., Ogawa A., Mizoguchi E., Shimomura Y., Andoh A., Bhan A.K., Blumberg R.S., Xavier R.J., Mizoguchi A. (2008) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* **118:** 534–44.
23. Inagaki-Ohara K., Sawaguchi A., Suganuma T., Matsuzaki G., Nawa Y. (2005) Intraepithelial lymphocytes express junctional molecules in murine small intestine. *Biochem Biophys Res Commun* **331:** 977–83.
24. Allaker R.P., Grosvenor P.W., McAnerney D.C., Sheehan B.E., Srikanta B.H., Pell K., Kapas S. (2006) Mechanisms of adrenomedullin antimicrobial action. *Peptides* **27:** 661–6.
25. Fuss I.J., Neurath M., Boirivant M., Klein J.S., de la Motte C., Strong S.A., Fiocchi C., Strober W. (1996) Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* **157:** 1261–70.
26. Podolsky D.K. (1991) Inflammatory bowel disease (1). *N Engl J Med* **325:** 928–37.
27. Rogler G., Andus T. (1998) Cytokines in inflammatory bowel disease. *World J Surg* **22:** 382–9.
28. Leonard W.J., O'Shea J.J. (1998) Jaks and STATs: biological implications. *Annu Rev Immunol* **16:** 293–322.
29. Atreya R., Neurath M.F. (2008) Signaling molecules: the pathogenic role of the IL-6/STAT-3 trans signaling pathway in intestinal inflammation and in colonic cancer. *Curr Drug Targets* **9:** 369–74.
30. Schreiber S., Rosenstiel P., Hampe J., Nikolaus S., Groessner B., Schottelius A., Kuhbacher T., Hamling J., Folsch U.R., Seegert D. (2002) Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* **51:** 379–85.
31. Inagaki-Ohara K., Hanada T., Yoshimura A. (2003) Negative regulation of cytokine signaling and inflammatory diseases. *Curr Opin Pharmacol* **3:** 435–42.
32. Mombaerts P., Mizoguchi E., Grusby M.J., Glimcher L.H., Bhan A.K., Tonegawa S. (1993) Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell* **75:** 274–82.
33. Chen Y., Chou K., Fuchs E., Havran W.L., Boismenu R. (2002) Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* **99:** 14338–43.
34. Berkes J., Viswanathan V.K., Savkovic S.D., Hecht G. (2003) Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. *Gut* **52:** 439–51.
35. Schneeberger E.E., Lynch R.D. (2004) The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol* **286:** C1213–28.
36. Axelsson L.G., Landstrom E., Goldschmidt T.J., Gronberg A., Bylund-Fellenius A.C. (1996) Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice: effects in CD4(+) -cell depleted, athymic and NK-cell depleted SCID mice. *Inflamm Res* **45:** 181–91.
37. Dieleman L.A., Ridwan B.U., Tennyson G.S., Beagley K.W., Bucy R.P., Elson C.O. (1994) Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* **107:** 1643–52.
38. Egger B., Bajaj-Elliott M., MacDonald T.T., Inglin R., Eysselein V.E., Buchler M.W. (2000) Characterisation of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency. *Digestion* **62:** 240–8.
39. Powrie F., Carlino J., Leach M.W., Mauze S., Coffman R.L. (1996) A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* **183:** 2669–74.
40. Neurath M.F., Fuss I., Kelsall B.L., Presky D.H., Waegell W., Strober W. (1996) Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. *J Exp Med* **183:** 2605–16.
41. Kitani A., Fuss I.J., Nakamura K., Schwartz O.M., Usui T., Strober W. (2000) Treatment of experimental (Trinitrobenzene sulfonic acid) colitis by intranasal administration of transforming growth factor (TGF)-beta1 plasmid: TGF-beta1-mediated suppression of T helper cell type 1 response occurs by interleukin (IL)-10 induction and IL-12 receptor beta2 chain downregulation. *J Exp Med* **192:** 41–52.
42. Ehrhardt R.O., Strober W., Harriman G.R. (1992) Effect of transforming growth factor (TGF)-beta 1 on IgA isotype expression. TGF-beta 1 induces a small increase in sIgA+ B cells regardless of the method of B cell activation. *J Immunol* **148:** 3830–6.
43. Fujihashi K., Kawabata S., Hiroi T., Yamamoto M., McGhee J.R., Nishikawa S., Kiyono H. (1996) Interleukin 2 (IL-2) and interleukin 7 (IL-7) reciprocally induce IL-7 and IL-2 receptors on gamma delta T-cell receptor-positive intraepithelial lymphocytes. *Proc Natl Acad Sci U S A* **93:** 3613–8.
44. Inagaki-Ohara K., Nishimura H., Mitani A., Yoshikai Y. (1997) Interleukin-15 preferentially promotes the growth of intestinal intraepithelial lymphocytes bearing gamma delta T cell receptor in mice. *Eur J Immunol* **27:** 2885–91.
45. Komano H., Fujiura Y., Kawaguchi M., Matsumoto S., Hashimoto Y., Obana S., Mombaerts P., Tonegawa S., Yamamoto H., Itohara S., *et al.* (1995) Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc Natl Acad Sci USA* **92:** 6147–51.
46. Campbell N., Yio X.Y., So L.P., Li Y., Mayer L. (1999) The intestinal epithelial cell: processing and presentation of antigen to the mucosal immune system. *Immunol Rev* **172:** 315–24.
47. O'Neil D.A., Porter E.M., Elewaut D., Anderson G.M., Eckmann L., Ganz T., Kagnoff M.F. (1999) Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol* **163:** 6718–24.
48. Mankertz J., Tavalali S., Schmitz H., Mankertz A., Riecken E.O., Fromm M., Schulzke J.D. (2000) Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma. *J Cell Sci* **113**(Pt 11): 2085–90.
49. Teahon K., Smethurst P., Levi A.J., Menzies I.S., Bjarnason I. (1992) Intestinal permeability in patients with Crohn's disease and their first degree relatives. *Gut* **33:** 320–3.
50. Irvine E.J., Marshall J.K. (2000) Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. *Gastroenterology* **119:** 1740–4.

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Original Article

Effects of uroguanylin on natriuresis in experimental nephrotic rats

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SUMMARY:

Aim: Uroguanylin, isolated from human and opossum urine, is a candidate intestinal natriuretic hormone that controls the sodium and water balance between the intestine and the kidneys. Levels of immunoreactive (ir)-uroguanylin in the plasma and urine are increased in rats and humans with nephrotic syndrome, which is physiologically characterized by sodium retention with massive proteinuria. The present study evaluates the effect of natriuresis induced by uroguanylin on nephrotic rats.

Methods: Normal rats and rats rendered nephrotic by injections of puromycin aminonucleoside (PAN) were treated with uroguanylin (0.5 nmol/h, delivered by an osmotic pump) or with vehicle during the sodium retention phase. All rats consumed the same quantity of sodium.

Results: Uroguanylin did not increase urinary excretion of sodium and water in normal rats, but significantly increased urinary sodium excretion during the sodium retention phase in nephrotic rats (untreated vs uroguanylin-treated nephrotic rats in mmol/mmol creatinine; 2.92 ± 0.65 vs 8.93 ± 2.53 on day 6, $P < 0.05$; 3.55 ± 0.47 vs 10.37 ± 1.73 on day 7, $P < 0.01$; 14.88 ± 2.32 vs 24.47 ± 2.86 on day 8, $P < 0.05$). Plasma levels of ir-uroguanylin in uroguanylin-treated nephrotic rats on day 6 were significantly increased compared with those in uroguanylin-treated control and untreated nephrotic rats.

Conclusion: Uroguanylin increased urinary sodium excretion in rats with PAN-induced nephrosis, and might be useful for treating sodium retention in patients with nephrotic syndrome.

KEY WORDS: **natriuresis, nephrotic syndrome, PAN-induced nephrosis, sodium retention, uroguanylin.**

The endogenous peptide uroguanylin, belonging to the growing family of natriuretic peptides that activate transmembranous guanylate cyclase (GC)-C, influences cellular function via the intracellular second messenger, cyclic guanosine 3′-5′ monophosphate (cGMP) and affects water and electrolyte transport.[1–3] This mechanism of uroguanylin in the intestine is established, but the situation is quite different in the kidney. Indeed, uroguanylin stimulates the urinary excretion of sodium, potassium and water in the isolated perfused rat kidney[4] and in mice *in vivo*,[5,6] but the effects of uroguanylin are unaltered in GC-C-deficient mice.[7] This finding suggests that uroguanylin functions through GC-C independent signalling pathways in the kidney.

Although the detailed mechanism is unknown, accumulating data indicates that uroguanylin could be an important natriuretic factor in various states including nephrosis. We previously found that uroguanylin excretion in urine is increased in humans and rats on a high-salt diet compared with a low-salt one, and that uroguanylin mRNA expression is increased in the intestine and kidneys of rats given a high-salt diet.[8,9] In addition, natriuresis induced by oral salt loading is decreased in uroguanylin-deficient mice.[10] Uroguanylin levels are also increased in the circulation of patients with renal disease and congestive heart failure.[11–14] We recently examined relationships between urinary immunoreactive (ir)-uroguanylin and sodium excretion in rats with puromycin aminonucleoside (PAN)-induced nephrosis.[15] That study showed that urinary excretion of ir-uroguanylin increased by approximately eightfold or more in rats with PAN-induced nephrosis compared with controls during the natriuretic phase, in accordance with an increase

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