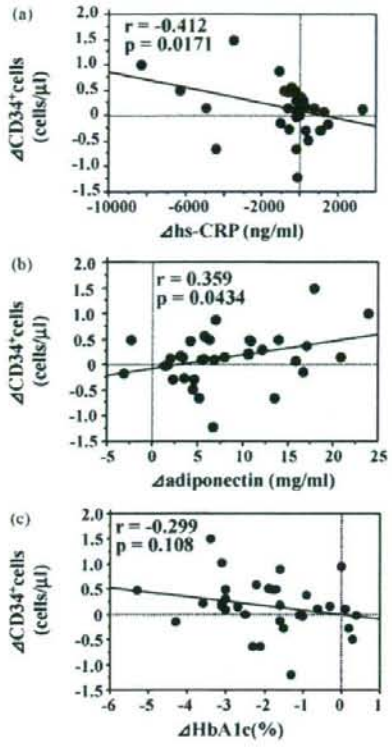

Fig. 2 – Correlation between ΔCD34⁺ cells and ΔhsCRP ($r = -0.412$, $p = 0.017$) (a), correlation between ΔCD34⁺ cells and Δadiponectin ($r = 0.359$, $p = 0.043$) (b), and correlation between ΔCD34⁺ cells and ΔHbA1c ($r = -0.299$, $p = 0.108$) (c).

1.15 ± 0.57 and 1.15 ± 0.65 cells/μl at 0, 12 and 24 weeks, respectively, $n = 18$). There was no significant difference in the change in CD34⁺ cell level (ΔCD34⁺ cells) between 15 mg and 30 mg of pioglitazone (15 mg; 0.07 ± 1.01 vs. 30 mg; 0.14 ± 0.32).

3.4. *Factors involved in the stimulation of CD34⁺ cells*

We next investigated which factors were correlated with the stimulation of CD34⁺ cells. ΔCD34⁺ cells were significantly correlated with Δhs-CRP in univariate analysis ($r = -0.412$, $p = 0.017$) (Fig. 2a). Further, Δadiponectin correlated with Δ CD34⁺ cells ($r = 0.359$, $p = 0.043$) (Fig. 2b). On the other hand, change in HbA1c levels (ΔHbA1c) ($r = -0.299$, $p = 0.108$) (Fig. 2c), change in HDL-C levels (ΔHDL-C) ($r = 0.253$, $p = 0.168$) and change in triglyceride levels (Δtriglycerides) ($r = 0.0072$, $p = 0.969$), were not significantly correlated to Δ CD34⁺ cells.

4. Discussion

Accumulating evidence shows that PPARγ agonists have anti-atherosclerotic actions other than their blood glucose level reduction effects [7,9]. One recent report showed that pioglitazone treatment could stimulate circulating EPCs in patients with coronary artery disease and normal glucose tolerance [12]. In this study, we demonstrated that pioglitazone treatment also increased circulating CD34⁺ cells and this effect continued for 24 weeks in type 2 diabetic patients. We studied the effects of pioglitazone on the stimulation of CD34⁺ cells but not CD34⁺/KDR⁺ cells regarded as EPCs. However, these circulating CD34⁺ cells have the capacity to participate in neovascularization of ischemic tissue. Indeed, their administration enhances the repair of ischemic tissue in ischemic stroke model [13] and improves myocardial circulation in myocardial infarction model [14]. Clinically, circulating CD34⁺ cell levels were reported to be correlated with cerebral blood flow in hypoperfusion area [6] and formation of collateral vessels in stroke patients [15]. These reports suggest that CD34⁺ cells may play a role in the maintenance of microcirculation. One recent clinical trial, PROactive Study, demonstrated that pioglitazone treatment could prevent cardiovascular events including stroke in type 2 diabetic patients [16]. Taken together, it is suggested that the stimulation of CD34⁺ cells may partly contribute to the preventive effects of pioglitazone on cardiovascular diseases. Our study also demonstrated that pioglitazone treatment increased circulating CD34⁺ cells in type 2 diabetic patients irrespective of with or without CVD, suggesting that pioglitazone treatment may be useful for primary prevention as well as secondary prevention of diabetic macroangiopathy.

It has been reported that the number of circulating EPCs is inversely correlated with HbA1c levels [3]. Since pioglitazone treatment significantly decreased HbA1c levels and this study did not have control group, we could not exclude the possibility that the stimulation of CD34⁺ cells was associated with the improvement of glycemic control. However, the results of this study suggest that pioglitazone may be capable of stimulating circulating CD34⁺ cells independently of glycemic control because ΔCD34⁺ cells was not positively correlated with ΔHbA1c at levels that achieved statistical significance.

Adipocyte derived factors and inflammation participate in atherogenesis of type 2 diabetic patients. Accumulating evidence show that adiponectin, one of adipocyte derived factors, has anti-atherogenic properties, and hypoadionectinemia was reported to be associated with endothelial dysfunction [17]. Pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in metabolic syndrome subjects [8], suggesting that these effects contribute to the anti-atherosclerotic action of pioglitazone. In this study, we also demonstrated that pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in type 2 diabetes patients. Interestingly, ΔCD34⁺ cells were significantly correlated with Δhs-CRP and Δadiponectin. An in vitro study showed that CRP impaired EPC migration and function [18]. In clinical study, it has been reported that circulating EPCs were inversely correlated to serum interleukin 6 levels [19]. These reports suggested that chronic inflammation may be involved in the regulation of EPCs. One recent clinical study showed that circulating EPCs were positively correlated to serum adiponectin levels in patients with coronary artery disease [20]. Another report showed that

adiponectin treatment increased EPC number and migration [12]. Taken together, it is suggested that the inhibitory effects on chronic inflammation and the effect on adiponectin regulation of pioglitazone may be directly or indirectly involved in the increase of CD34⁺ cells. However, further study is necessary to delineate this hypothesis.

In conclusion, our study demonstrated that pioglitazone treatment increased circulating CD34⁺ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

Conflict of interest

There are no conflicts of interest.

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Impaired flow-mediated vasodilatation and insulin resistance in type 2 diabetic patients with albuminuria

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ABSTRACT

An elevated urinary albumin excretion is associated with an increased risk of cardiovascular disease due to atherosclerosis, but the pathophysiological mechanism underlying this association is poorly understood. We studied 217 diabetic patients, that is, 121 normoalbuminuric patients, 71 microalbuminuric patients, and 25 macroalbuminuric patients. We evaluated flow-mediated dilatation of brachial artery (%FMD, one endothelial function marker associated with endogenous NO production), von Willebrand factor (vWF, endothelial activation marker), high-sensitive CRP (hsCRP, a low-grade inflammation marker), asymmetric dimethyl arginine (ADMA, an endogenous inhibitor of NO synthesis), and insulin sensitivity by steady-state plasma glucose method. %FMD was apparently decreased in microalbuminuric and macroalbuminuric patients compared with normoalbuminuric patients ($p < 0.001$). Moreover, %FMD was significantly correlated with the degree of albuminuria ($r = -0.38$, $p < 0.05$). On the other hand, vWF and hsCRP did not show significant difference between normoalbuminuric patients and microalbuminuric patients. In diabetic patients with macroalbuminuria, ADMA was significantly elevated compared to those with normoalbuminuria. Insulin sensitivity was significantly associated with urinary albumin excretion rate. These results suggested that endothelial dysfunction which may be due to impaired NO production and insulin resistance underlie the association between diabetic nephropathy and atherosclerosis in diabetic patients.

1. Introduction

Elevated urinary albumin excretion rate (UAER) is strongly associated with an increased risk of cardiovascular diseases, which is independent of conventional risk factors including hypertension, hyperlipidemia, and smoking, among individuals with and without type 2 diabetes [1,2]. This suggests that elevated UAER may be associated with atherosclerosis by the unidentified mechanism.

The endothelium plays a crucial role in the maintenance of vascular tone and structure, and endothelial dysfunction is a key feature of atherosclerosis. Nitric oxide (NO) is one of the important endothelium-derived vasoactive mediators. NO is involved in a wide variety of regulatory mechanisms of cardiovascular system, including vascular tone and vascular structure [3].

Flow-mediated endothelium-dependent vasodilatation (FMD) method is based on the endothelial stimulus of increased shear stress (the tangential force on the vessel wall exerted by flowing blood). Increased shear stress is caused by post-ischemic hyperemia and elicits a slow Ca^{+}-independent two to threefold increase in NO production [4,5]. Indeed,

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Celemajer et al. reported that flow mediate vasodilatation was mainly blocked by *N*-monomethyl-L-arginine (an inhibitor of endothelial NO synthetase) [6].

To clarify the contribution of impaired NO production in vascular endothelium to the association between atherosclerotic disease and diabetic nephropathy, we examined FMD by ultrasonography. In addition, we measured asymmetric dimethyl arginine (ADMA), an endogenous NO synthesis inhibitor [3]. Since low-grade inflammation is another key feature of the pathophysiology of atherosclerosis [7], we further examined high-sensitive CRP, which is an inflammation marker, to investigate whether this feature is involved in the association between atherosclerotic disease and diabetic nephropathy.

It has recently been indicated that microalbuminuria and atherosclerosis are closely associated with insulin resistance [8–10], implying that insulin resistance may underlie these pathophysiological conditions although the causative relationship remains unknown. In the present study, we further examined insulin sensitivity in the type 2 diabetic patients with different stage of albuminuria and analyzed the correlation between insulin sensitivity and FMD, to investigate whether elevated UAER and endothelial dysfunction may be associated with insulin resistance.

2. Methods

2.1. Study subjects

We studied 217 patients with type 2 diabetes who were <75 years of age. Patients with a current acute illness (including clinically significant infectious disease) were excluded from this study. Twenty-four-hour urine collections were performed for two consecutive days to determine the stage of diabetic nephropathy. Creatinine clearance (Ccr) was calculated from the 24-h urine sample and serum creatinine levels. The patients were divided into three groups according to the UAER, as follows: normoalbuminuria (UAER <30 mg/day), microalbuminuria (30 ≤ UAER < 100 mg/day) and macroalbuminuria (UAER ≥ 300 mg/day). To exclude diabetic patients with nondiabetic kidney disease, we excluded patients with hematuria or abnormal urinary sediments. This study was conducted with the approval of National Cardiovascular Center Trust Ethics Committee, and patients gave written informed consent before participation.

2.2. Brachial artery flow-mediated dilatation

Using ultrasonography, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasoundsonographic measurements were carried out according to the method described by Celemajer et al. [6]. Brachial artery diameter was measured from B-mode ultrasound images using 10-MHz liner array transducer (ProSound SSD-5500; Aloka, Japan) while an ECG trace was simultaneously recorded. The right brachial artery was scanned in longitudinal sections 1–10 cm above elbow, after at least 15 min of rest in the supine position, the skin surface was marked and the arm was kept in the same position during the study.

Baseline measurements of the diameter were carried out. Endothelium-dependent vasodilatation (flow-mediated dilatation) was determined by scans during reactive hyperemia. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after dilation. After 10 min rest, the second control scan of the diameter was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to posterior "m" line (interface between the media and adventitia) at end-diastole, coincident with the R wave on a continuously recorded ECG. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the FMD were carried out 45–60 s after the cuff release to measure a maximal diameter. Vasodilatation by reactive hyperemia or glyceryl trinitrate (NTG) was expressed as the percent change in diameter compared with the baseline values.

2.3. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated with a newly modified steady-state plasma glucose (SSPG) method with octreotide acetate (Sandostatin; Novartis) after an overnight fasting period of 12 h [11]. Sandostatin (9.8-pmol bolus followed by a constant infusion of 73.5 pmol/h) and Humulin R insulin (45 pmol/kg bolus followed by a constant infusion at a rate of 4.62 pmol/(kg min); Eli Lilly) were infused intravenously for 120 min. Glucose in a final 12% solution containing KCl (0.5 μmol/(kg min)) was infused at a rate of 0.033 mmol/(kg min) (6 mg/(kg min)) through an antecubital vein via a constant infusion pump. Blood samples were drawn routinely at 0 and 120 min (9:00 and 11:00 a.m.) for the determination of glucose, insulin, and lipids. The value of glucose at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose utilization. High SSPG levels showed peripheral insulin resistance.

Another marker of insulin resistance (IR) was estimated by calculating homeostasis model assessment (HOMA-IR) index ((fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/l))/22.5) [12].

2.4. Measurement of vWF, hsCRP, and ADMA

vWF was determined in citrated plasma using a homemade enzyme-linked immunosorbent assay. Data are given as the percentage of pooled human plasma (set at 100%). Serum hsCRP concentration was determined by latex nephelometry method (SRL, Tokyo, Japan). Serum ADMA concentration was determined by high-performance liquid chromatography method (SRL, Tokyo, Japan).

2.5. Statistical analysis

Values are expressed as means ± S.D. Statistical analysis was performed by use of ANOVA followed by Scheffes' test. The

Table 1 – Characteristics of diabetic patients with normoalbuminuria, microalbuminuria, and overt nephropathy

Parameter	Stage of nephropathy		
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
n	121	71	25
Age (years)	62 ± 9	65 ± 8	66 ± 7
Men/women	76/45	34/37	12/13
Duration of diabetes (years)	12 ± 8	14 ± 8	18 ± 8*
BMI (kg/m^2)	25.0 ± 3.7	25.1 ± 3.7	25.1 ± 3.9
SBP (mmHg)	128 ± 13	133 ± 15	141 ± 19*
DBP (mmHg)	74 ± 10	73 ± 9	76 ± 10
FBS (mmol/l)	7.4 ± 1.4	7.5 ± 1.5	7.5 ± 1.9
HbA1c (%)	8.3 ± 1.5	8.9 ± 1.7*	8.8 ± 1.4
HOMA-IR	1.62 ± 0.98	1.71 ± 2.06	2.29 ± 1.47
Total cholesteroyl (mmol/l)	4.86 ± 0.90	4.86 ± 0.90	4.73 ± 0.75
Serum creatinine (μmol/l)	70 ± 20	60 ± 20	110 ± 40
Urinary albumin (mg/day)	10 ± 7	85 ± 79**	583 ± 576**
Creatinine clearance (ml/s)	1.43 ± 0.52	1.50 ± 0.63	0.73 ± 0.43**
ACEI or ARB (yes/no)	36/85	24/47	11/14*
Statin (yes/no)	45/76	25/46	10/15
Current smoker (yes/no)	11/110	7/64	6/19

*$p < 0.05$, **$p < 0.01$ vs. normoalbuminuria, mean ± S.D.

strength of correlation between variables was tested by linear correlation and multiple regression analysis. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Patients characteristics*

Table 1 shows the clinical characteristics of three groups. There was no significant difference in age, gender, BMI, FBS and total cholesterol among the three groups. HbA1c of diabetic patients with microalbuminuric patients was significantly higher than normoalbuminuric patients. Systolic blood pressure of macroalbuminuric patients was significantly higher than normo- and micro-albuminuric patients. Creatinine clearance was significantly decreased in macroalbuminuric patients compared with normo- and micro-albuminuric patients. There is no significant difference in rate of patients taking ACE/ARB between normo- and micro-albuminuric patients whereas the rate of patients taking ACE/ARB of macroalbuminuric patients were significantly large compared with other two groups. On the other hand, there is no significant difference in rate of patients taking statin among three groups.

3.2. *%FMD of diabetic patients*

We studied the endothelial function by FMD using brachial artery echography. %FMD (Δhyperemia) of diabetic patients with microalbuminuria (4.5 ± 3.7%) and macroalbuminuria (4.2 ± 2.4%) was apparently decreased compared with those of diabetic patients with normoalbuminuria (6.6 ± 3.7%) (Fig. 1A). Moreover, %FMD was significantly correlated with UAER in normo- and micro-albuminuric patients independent of age, HbA1c, and systolic blood pressure by multiple regression analysis ($r = -0.38$, $p < 0.05$) (Fig. 2). Dilatation of brachial artery by NTG (ΔNTG) showed no difference among three groups (Fig. 1B).

3.3. *vWF, hsCRP, and ADMA of diabetic patients*

We studied other atherosclerotic markers, that is, vWF, hsCRP, and ADMA. There was no significant difference of the levels of vWF and hsCRP between normoalbuminuric and microalbu-

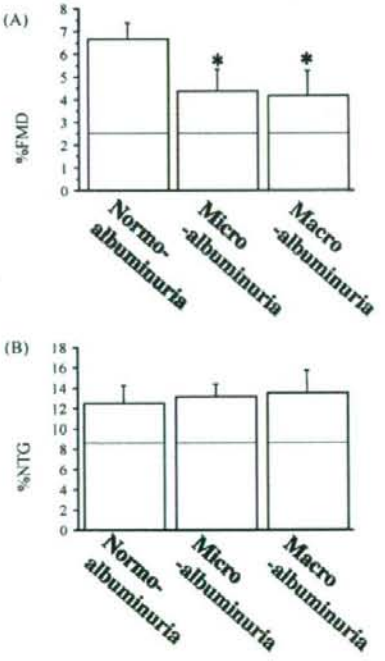

Fig. 1 – %FMD (A) and %NTG (B) in diabetic patients with normoalbuminuria, microalbuminuria and macroalbuminuria. Each value means (means ± S.D.), *$p < 0.001$.

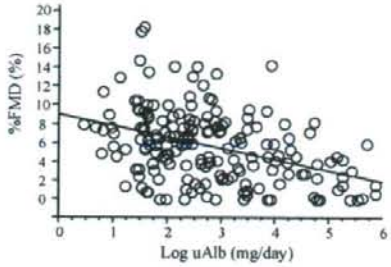

Fig. 2 – Correlation between degree of UAE and %FMD in normo- and micro-albuminuric diabetic patients. There was a significant correlation between both variables ($r = -0.38$, $p < 0.05$, $n = 192$).

minuric patients (Table 2). Although the levels of ADMA in microalbuminuric patients did not show significant difference compared with normoalbuminuric patients (Table 2), the levels of ADMA in macroalbuminuric patients were significantly elevated compared with normoalbuminuric patients (Table 2).

3.4. *Insulin sensitivity of diabetic patients*

We studied the insulin sensitivity by SSPG method. The levels of SSPG had weak but significant correlation with both %FMD ($r = -0.175$, $p < 0.05$) and UAER ($r = 0.181$, $p < 0.05$) independent of age, HbA1c, and systolic blood pressure (Fig. 3A, B).

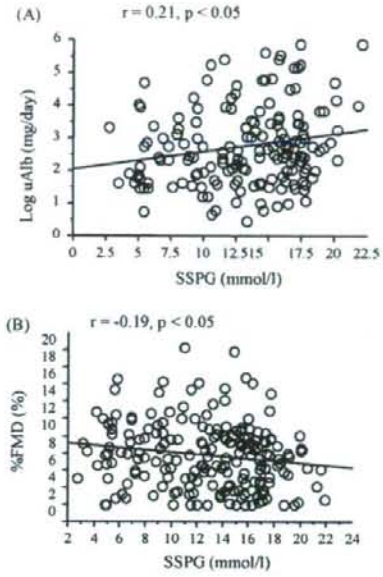

Fig. 3 – Correlation between SSPG and UAE (A), and correlation between SSPG and %FMD (B) in normo- and micro-albuminuric patients.

4. Discussions

There were two main findings from this investigation in type 2 diabetic patients. First, diabetic micro- and macro-albuminuric patients showed significant reduction of %FMD compared with normoalbuminuric patients. This finding suggests that the endothelial dysfunction may account for the association between atherosclerosis and albuminuria in diabetic patients. Second, the level of SSPG was significantly associated with both UAER and %FMD. This finding suggests that insulin resistance may play a role in both atherosclerosis and nephropathy in type 2 diabetic patients.

In diabetic patients, %FMD is decreased compared with healthy control [13,14]. These reports indicated that diabetes mellitus is associated with endothelial dysfunction due to impaired NO production. However the involvement of endothelial dysfunction in diabetic nephropathy has been unclarified. We demonstrated that microalbuminuric and macroalbuminuric patients showed significant decreased %FMD compared with normoalbuminuric patients. In contrast, there was no significant difference of vWF between normoalbuminuric patients and microalbuminuric patients. vWF is a product of vascular endothelial cell, and induces coagulation and platelet aggregation [15]. These findings suggest that endothelial dysfunction due to impaired NO production is specifically induced in micro- and macroalbuminuric patients. One recent report showed that coronary endothelium-dependent dilatation was impaired in a rat model of spontaneous albuminuria [16] supporting this hypothesis. It has been reported that renal NO production was decreased in rodent diabetic model [17]. This report suggests that decrease of NO production may play a role in the

Table 2 – Parameters of atherosclerosis in diabetic patients with normoalbuminuria, microalbuminuria, and overt nephropathy

Parameter	Stage of nephropathy		
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
von Willebrand factor (%)	147 ± 44	146 ± 44	143 ± 41
High-sensitive CRP (ng/ml)	976 ± 1401	951 ± 1110	1113 ± 1187
ADMA (nmol/ml)	0.45 ± 0.06	0.47 ± 0.07	0.55 ± 0.11*

*$p < 0.001$ vs. normoalbuminuria, mean ± S.D.

progression of diabetic nephropathy as well as atherosclerosis. We investigated serum ADMA levels in diabetic patients. There was no significant difference of ADMA levels between normo- and micro-albuminuric patients, suggesting that the reduction of %FMD in microalbuminuric patients might not be resulted from the elevation of ADMA. However, in macroalbuminuric patients, ADMA level was significantly higher than normoalbuminuric patients. Vallance et al. reported that the level of ADMA was elevated in patients with chronic renal failure and suggested the involvement of this in coronary artery disease [18]. They indicate that the elevation of ADMA might be associated with atherosclerosis in patients with chronic renal disease [18]. Thus, this finding suggests that the elevation of ADMA might be associated with atherosclerotic change in diabetic patients with macroalbuminuria.

An association between chronic low-grade inflammation and development of atherosclerotic disease has been observed in basic and clinical studies [7,19–21]. Furthermore, diabetic patients have higher CRP levels than normal subjects, suggesting that chronic inflammation may contribute diabetic atherosclerotic complication [22]. An association between micro- and macro-albuminuria and inflammation has also been reported [23,24]. However, several other studies showed that inflammatory molecules were not associated with micro- and macro-albuminuria [25–27]. Thus the knowledge of this association is still controversial. Also we could not demonstrate the association between CRP and development of microalbuminuria in this study. Our data suggested that chronic low-grade inflammation might not be involved in the association between atherosclerosis and microalbuminuria. However, since this study was performed by cross-sectional analysis and other inflammatory marker was not measured, further study is necessary for demonstrating this hypothesis.

Insulin resistance has been reported to play an important role in the development and progression of atherosclerotic coronary disease [8,9]. Recently the association between insulin resistance and microalbuminuria was also reported [10]. Nakamura et al. demonstrated that administration of pioglitazone to diabetic patients attenuated UAER [28]. In this study, we showed that both the UAER and %FMD were significantly correlated to the level of SSPG. These findings suggest that insulin resistance may be involved in both the elevated urinary albumin excretion and endothelial dysfunction due to impaired NO production. However, HOMA-IR, another insulin sensitivity marker which reflects insulin sensitivity in both the liver and the periphery, did not show significant difference among three groups, suggesting that particularly peripheral insulin resistance may be important for the pathogenesis of atherosclerosis and diabetic nephropathy.

In summary, we showed that %FMD of micro- and macro-albuminuric patients was decreased compared with those of normoalbuminuric patients, without showing significant difference in other various atherosclerotic markers. Furthermore, the level of SSPG was significantly correlated to UAER and %FMD. These findings suggest that endothelial dysfunction which may be due to impaired NO production underlies the mechanism of association between elevated urinary albumin excretion and atherosclerosis in diabetic patients, and that peripheral insulin resistance might be possibly involved in both diabetic nephropathy and atherosclerosis.

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Association between insulin resistance and endothelial dysfunction in type 2 diabetes and the effects of pioglitazone

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Abstract

Endothelial dysfunction is regarded as an early stage of atherosclerosis, and plays a role in the development of atherosclerotic diseases. Insulin resistance is related to the atherosclerotic process. In this study, we examined the association between endothelial function and insulin resistance in 48 subjects with type 2 diabetes. In addition, the effects of pioglitazone treatment on endothelial function and insulin resistance were investigated in a subgroup of subjects. Endothelial function of the brachial artery was non-invasively assessed using ultrasound technique. We measured flow-mediated endothelium-dependent vasodilation (FMD) and glyceryl trinitrate-induced endothelium-independent vasodilation (GTN). Insulin sensitivity was measured by the steady-state plasma glucose (SSPG) method. High SSPG levels indicate insulin resistance. There was a significant inverse correlation ($r = -0.462$, $p < 0.001$) between SSPG and FMD. Systolic blood pressure was inversely correlated with FMD ($r = -0.360$, $p < 0.013$). By multiple regression analysis, insulin resistance was the sole predictor of FMD. The effects of chronic treatment with pioglitazone were assessed in 10 subjects with type 2 diabetes. The increase in FMD significantly correlated with the decrease in SSPG. There is a significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone.

Keywords: Endothelial dysfunction; Insulin resistance; Pioglitazone

1. Introduction

Endothelial dysfunction is thought to be an important early feature in the development of atherosclerosis and occurs in subjects with type 2 diabetes mellitus [1–4]. Insulin resistance is also associated with atherosclerosis and is observed in subjects with type 2 diabetes [5,6].

We previously reported the association between endothelial dysfunction and insulin resistance in patients with essential hypertension [7]. However, the mechanisms responsible for endothelial dysfunction and insulin resistance in hypertension might be different from those of type 2 diabetes. Therefore, we evaluated the relationship between endothelial dysfunction and insulin resistance in patients with type 2 diabetes. Thiazolidinediones, an agonist for the peroxisome proliferator-activated receptor γ (PPARγ), improve insulin resistance. If there is a significant relationship

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between endothelial dysfunction and insulin resistance, thiazolidinediones might influence endothelial function. Therefore, we examined the effects of pioglitazone on endothelial dysfunction and insulin resistance in a subgroup of subjects with type 2 diabetes to verify the relationship between endothelial dysfunction and insulin resistance.

The main purpose of this study was to investigate the relation between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. In addition, the influence of pioglitazone treatment was examined.

2. Subjects and methods

2.1. Subjects

Forty-eight (30 males and 18 females) patients with type 2 diabetes were recruited in the Department of Diabetes and Atherosclerosis of the National Cardiovascular Center. The subjects did not have diabetic retinopathy or nephropathy. Subjects were included on the basis of the following criteria: age between 40 and 79 years, body mass index (BMI) between 17 and 35 kg/m^2, type 2 diabetes confirmed by American Diabetes Association criteria [8]. Subjects were excluded from participation if they had coronary heart, peripheral vascular, renal, hepatic or other endocrine diseases. Subjects were excluded if they had a resting seated blood pressure greater than 150 mmHg systolic or greater than 90 mmHg diastolic, or were taking anti-hypertensive drugs. Diabetes duration was 5.3 ± 1.9 years (3–7 years). Diabetes treatment regimens included diet alone (27 subjects), sulfonylures (18 subjects) and metformin (3 subjects).

The 48 subjects had an average age of 64 ± 1 years, with a mean BMI of 24.6 ± 0.3 kg/m^2, HbA_{1c} of 8.6 ± 0.2%, total cholesterol of 199 ± 5 mg/dl, HDL-cholesterol of 43 ± 2 mg/dl and triglycerides of 137 ± 14 mg/dl. Mean systolic and diastolic blood pressures were 131 ± 3 and 74 ± 2 mmHg, respectively.

Of the 48 diabetic subjects, 10 subjects were started on a single 15 or 30 mg-tablet of pioglitazone (Actos, Takeda Pharmaceuticals, Tokyo, Japan) by mouth each day. Inclusion criteria of the pioglitazone treatment were male, non-smoker, diet alone treatment and mild to severe insulin resistance (SSPG > 160 mg/dl). They received a mean dose of 25.5 ± 2.3 mg/day (30 mg/day: seven subjects and 15 mg/day: three subjects) of pioglitazone for 16.3 ± 1.6 weeks (10–20 weeks). The secondary assessments of endothelial function and insulin sensitivity were performed after the pioglitazone treatments.

The study protocol was approved by the ethics committee of the National Cardiovascular Center. The experiments were conducted with the understanding and the consent of each participant.

2.2. Methods

2.2.1. Assessment of endothelial function

Using the ultrasound method, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasound measurements were carried out based on the method described by Celermajer et al. [9] and our method was reported previously [7]. The assessments were performed after an overnight fast in a quiet air-conditioned room (22–23 °C). The diameter of the brachial artery was measured on B-mode ultrasound images, with the use of a 10-MHz linear array transducer (ProSound SSD-5500, ALOKA, Tokyo, Japan). The right brachial artery was scanned in longitudinal sections 1–10 cm above the elbow, after at least 15 min of rest in the supine position. After the detection of the right transducer position, the skin surface was marked and the arm was kept in the same position during the study. All scans were recorded using a super-VHS videocassette recorder (SONY, SVO-9500MD), and analyzed later.

At first, baseline measurements of the diameter were carried out. Endothelium-dependent vasodilation (flow-mediated dilation) was determined by the scans during reactive hyperemia. Because flow-mediated vasodilation was mainly blocked by *N*-monomethyl-L-arginine (an inhibitor of endothelial nitric oxide synthase) this dilation was regarded as endothelium dependent [10]. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after deflation. After 10–15 min rest, the second control scan of the diameter and the flow velocity was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5–4 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to the posterior 'm' line (interface between the media and adventitia) at endo-diastole, coincident with the R wave on a continuously recorded electrocardiogram. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the flow-mediated dilation were carried out 45–60 s after the cuff release to measure a maximum diameter. Vasodilation by reactive hyperemia (flow-mediated dilation, FMD) or glyceryl trinitrate (GTN) was expressed as the percent change in diameter compared to the baseline values.

2.2.2. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated by a modified steady state plasma glucose (SSPG) method [6,7,11] using Sandostatin (octreotide acetate; Novartis, Basel, Switzerland) after an overnight fasting for at least 12 h. Sandostatin (9.8 pmol in bolus followed by a constant infusion of 73.5 pmol/h) and Novolin R insulin (Novo Nordisk S/A, Tokyo, Japan, 45 pmol/kg [7.5 mU/kg] in a bolus followed by a constant infusion at a rate of 4.62 pmol/kg/min [0.77 mU/kg/min]) were infused intravenously for 120 min.

Glucose in a final 12% solution containing KCl (0.5 μmol/kg/min) were infused at a rate of 0.033 mmol/kg/min [6 mg/kg/min] through an antecubital vein via a constant infusion pump. Blood samples were drawn routinely at 0 and 120 min (9:00 and 11:00 a.m.) for determination of glucose and insulin. Value of glucose at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose utilization. High SSPG levels indicate peripheral insulin resistance. At 120 min SSPG was rapidly measured using a Glucometer (Bayer Corporation, Osaka, Japan) separate from the usual measurement of glucose and insulin. When rapidly measured, if SSPG was found to be lower than 250 mg/dl, oral glucose intake was necessary to prevent hypoglycemia after the insulin sensitivity test. The subjects should have lunch within 30 min after the insulin sensitivity test to prevent hypoglycemia. Homeostasis model assessment (HOMA-IR) was calculated from fasting glucose and insulin concentrations during insulin sensitivity test as follows: HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405.

2.3. Statistical analysis

Values are expressed as mean ± S.E. A probability value of <0.05 was considered to indicate statistical significance. The strength of the correlation between FMD and GTN with respect to risk factors was assessed by Pearson's linear correlation and multiple regression analysis. The effects of pioglitazone on each clinical parameter were assessed by paired t-test and Pearson's linear correlation.

3. Results

3.1. Association between endothelial dysfunction and each parameter in 48 subjects

A significant inverse correlation was observed between FMD and SSPG ($r = -0.462$, $p < 0.001$; Fig. 1). There was no relation between FMD and HbA_{1c} ($p = 0.856$). We also observed a significant inverse correlation between FMD and systolic blood pressure ($r = -0.360$, $p < 0.013$). No significant correlation was found between FMD and diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. There was no relationship between FMD and HOMA-IR ($p = 0.097$).

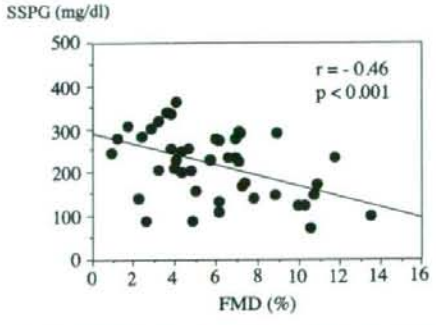

Fig. 1. Relationship between FMD and SSPG in subjects with type 2 diabetes. FMD, flow-mediated vasodilation; SSPG, steady state plasma glucose.

We performed multiple regression analysis to evaluate the independent influence of risk factors including SSPG, systolic blood pressure, HbA_{1c}, total cholesterol, BMI and age on FMD. FMD was independently related to SSPG (regression coefficient: $\beta = -0.419$, $p = 0.0086$) but not to systolic blood pressure ($\beta = -0.254$, $p = 0.0782$), HbA_{1c} ($\beta = -0.090$, $p = 0.5616$), total cholesterol ($\beta = -0.067$, $p = 0.6336$), BMI ($\beta = -0.258$, $p = 0.0863$) or age ($\beta = -0.085$, $p = 0.5650$).

With respect to GTN, no significant correlation was observed between GTN and SSPG or other parameters, including HbA_{1c}, diabetic duration, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI.

3.2. Effects of pioglitazone treatment on endothelial function and insulin resistance

The effects of treatment with pioglitazone were assessed in 10 male subjects with type 2 diabetes (a subgroup of 48 subjects). Table 1 shows the clinical parameters of the 10 subjects before and after pioglitazone treatment. SSPG, HbA_{1c} and fasting plasma glucose decreased and FMD increased significantly due to pioglitazone treatment. However, BMI, total cholesterol, HDL-cholesterol, triglyceride, systolic blood pressure and diastolic blood pressure did not

Table 1
Clinical characteristics of the subjects with type 2 diabetes treated with pioglitazone

	Before Tx	After Tx
Number	10	
Age (years)	65 ± 2	
SSPG (mg/dl)	230 ± 13	185 ± 17*
FMD (%)	4.5 ± 1.1	8.1 ± 1.5***
Body mass index (kg/m^2)	24.4 ± 0.4	24.7 ± 0.4
Fasting plasma glucose (mg/dl)	162 ± 11	133 ± 8*
HbA_{1c} (%)	8.4 ± 0.4	7.0 ± 0.3**
Total cholesterol (mg/dl)	199 ± 8	206 ± 7
HDL cholesterol (mg/dl)	47 ± 4	50 ± 4
Triglyceride (mg/dl)	120 ± 15	129 ± 13
Systolic blood pressure (mmHg)	137 ± 5	137 ± 2
Diastolic blood pressure (mmHg)	78 ± 5	79 ± 1

Values are mean ± S.E. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. before Tx. Tx, Treatments with pioglytazone.

significantly change. GTN was also not significantly altered.

The change in FMD before and after administration of pioglitazone was not significantly correlated with the change in HbA_{1c} ($p = 0.314$) or fasting plasma glucose ($p = 0.717$). The increase in FMD, that is, the improvement in endothelial function, was significantly correlated with the decrease in SSPG ($r = -0.649$, $p < 0.05$).

4. Discussion

In this study we found that vascular endothelial dysfunction was associated with insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone, which improved both endothelial dysfunction and insulin resistance in patients with type 2 diabetes.

The close association between insulin resistance and endothelial dysfunction is our main interest. In a study by Hogikyan et al. [3], insulin resistance as measured by the insulin sensitivity index (minimal model: S_I), was not found to be correlated with endothelial dysfunction in subjects with type 2 diabetes. They measured the forearm blood flow (FABF) using venous occlusion plethysmography and used the FABF response to acetylcholine as an index of endothelial function. The narrow range of S_I values among the subjects might have led to the lack of a relationship between S_I and endothelial dysfunction. In addition, the sensitivity of the techniques using plethysmography might have been low.

Balletshofer et al. [12] reported a significant association between endothelial dysfunction and insulin resistance, as measured by the glucose clamp method, in young normotensive and normoglycemic first-degree relatives of patients with type 2 diabetes. Therefore, this association was observed in a non-diabetic population at future risk of type 2 diabetes.

Insulin causes endothelium-derived nitric oxide (NO)-dependent vasodilation [13]. It is suggested that this insulin action occurs via the phosphatidylinositol 3-kinase and Akt pathway [14,15]. As for insulin action, phosphatidylinositol 3-kinase activation is critical for insulin-mediated glucose uptake into skeletal muscle [16]. Therefore, insulin resistance due to a systemic defect in the phosphatidylinositol 3-kinase pathway might cause a combined defect in insulin-mediated glucose uptake and insulin-mediated endothelial vasodilation.

Among the risk factors for atherosclerosis, insulin resistance was found to be the sole predictor of endothelium dependent vasodilation by multiple regression analysis in the present study. We observed no relationship between FMD and HbA_{1c}. Bagg et al. found that a short-term reduction of HbA_{1c} levels did not appear to affect endothelial function in patients with type 2 diabetes [17]. Furthermore, Mather et al. reported that insulin resistance was the sole predictor of endothelial dysfunction following metformin treatment in type 2 diabetes in stepwise multivariate analysis, and HbA_{1c} and glucose levels were not significant predictors of endothelial dysfunction [18].

Treatment with HMG-CoA inhibitors (statins) has been shown to improve endothelial dysfunction [19–21]. Therefore, statin treatment may have affected the relationship between FMD and risk factors in the present study. In 48 diabetic subjects, 5 were treated with pravastatin and one with simvastatin. We performed statistical analysis in 42 subjects without statin treatment. There was a significant inverse correlation between SSPG and FMD ($r = -0.538$, $p < 0.001$). A significant inverse correlation was observed between FMD and systolic blood pressure ($r = -0.330$, $p < 0.05$). No significant correlation was found between FMD and HbA_{1c}, diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. On multiple regression analysis, FMD was independently related to SSPG (regression coefficient: $\beta = -0.500$, $p = 0.0032$) but not to systolic blood pressure, HbA_{1c}, total cholesterol, BMI or age.

Smoking is associated with endothelial dysfunction [22,23]. Smoking might interfere in the relationship between FMD and risk factors. In 48 diabetic subjects, 13 were smokers in the present study. Statistical analysis was performed in 35 non-smokers. A significant correlation was found between SSPG and FMD ($r = -0.582$, $p < 0.001$). There was a significant inverse correlation between FMD and systolic blood pressure ($r = -0.357$, $p < 0.05$). No significant correlation was observed between FMD and HbA_{1c}, diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. On multiple regression analysis, FMD was independently related to SSPG (regression coefficient: $\beta = -0.591$, $p = 0.0019$) but not to systolic blood pressure, HbA_{1c}, total cholesterol, BMI or age. In the present study, FMD did not correlate with HOMA-IR. SSPG is a more sensitive marker to measure insulin sensitivity than HOMA-IR.

Endothelial dysfunction and insulin resistance were improved by pioglitazone treatment in the present study. SSPG, HbA_{1c} and fasting plasma glucose were decreased and other risk factors were not changed by the treatment. It was reported that hyperglycemia itself inhibits endothelial NO synthase activity [24] and causes endothelial dysfunction [25]. On the other hand,

insulin resistance was also associated with endothelial dysfunction in 48 subjects with type 2 diabetes in this study. The change in FMD before and after treatment with pioglitazone was not significantly correlated with the change in HbA_{1c} or fasting plasma glucose, and the increase in FMD was significantly correlated with the decrease in SSPG in this study. Because of the small number of subjects ($n = 10$), we cannot exclude the possibility that the decreased plasma glucose level improved endothelial dysfunction. The decrease in plasma glucose level might be associated with improved endothelial function if the pioglitazone study was performed with more cases. It can at least be said that insulin resistance is an important factor affecting endothelial function. As previously described, a similar study [18] found that treatment with metformin improved both endothelial function and insulin resistance, and the glucose level and HbA_{1c} were not significant predictors of endothelial dysfunction. Considering generally than the above-mentioned points, it is suggested that increased insulin sensitivity plays an important role in the improvement of endothelial function by pioglitazone treatment.

Pistrosch et al. [26] demonstrated that treatment with rosiglitazone, another PPARγ activator, ameliorated insulin resistance measured by glucose clamp method, and improved endothelial function determined by venous occlusion plethysmography in patients with recently diagnosed type 2 diabetes. They performed a double-blind cross-over trial and treated with rosiglitazone and nateglinide in random order. Glycemic control was comparable under rosiglitazone and nateglinide. Only rosiglitazone improved insulin resistance and endothelial function in the study. Thus, they also showed the relation between insulin sensitivity and endothelial function independent of glucose level in type 2 diabetes.

In conclusion, in the present study we demonstrated significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes, and pioglitazone treatment improved both endothelial dysfunction and insulin resistance with a statistical link. These data support the concept of the important role of insulin resistance in the pathogenesis of endothelial dysfunction in type 2 diabetes mellitus.

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Adrenomedullin induces lymphangiogenesis and ameliorates secondary lymphoedema

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Aims Adrenomedullin (AM) is a multifunctional peptide hormone that plays a significant role in vasodilation and angiogenesis. Lymphoedema is a common but refractory disorder that is difficult to be treated with conventional therapy. We therefore investigated whether AM promotes lymphangiogenesis and improves lymphoedema.

Methods and results The effects of AM on lymphatic endothelial cells (LEC) were investigated. AM promoted proliferation, migration, and network formation of cultured human lymphatic microvascular endothelial cells (HLMVEC). AM increased intracellular cyclic adenosine monophosphate (cAMP) level in HLMVEC. The cell proliferation induced by AM was inhibited by a cAMP antagonist and mitogen-activated protein kinase kinase (MEK) inhibitors. Phosphorylated extracellular signal-regulated kinase (ERK) in HLMVEC was increased by AM. Continuous administration of AM (0.05 μg/kg/min) to BALB/c mice with tail lymphoedema resulted in a decrease in lymphoedema thickness. AM treatment increased the number of lymphatic vessels and blood vessels in the injury site.

Conclusion AM promoted LEC proliferation at least in part through the cAMP/MEK/ERK pathway, and infusion of AM induced lymphangiogenesis and improved lymphoedema in mice.

1. Introduction

Lymphatic system plays an important role in the maintenance of tissue fluid homeostasis,[1] and damage of lymphatic vessels or surgical removal of lymph nodes often triggers the development of lymphoedema.[2,3] Despite substantial advances in surgical and conservative techniques, the therapeutic options for this disease are limited.[4,5] Recently, studies have suggested that vascular endothelial growth factor (VEGF)-C, VEGF-D or hepatocyte growth factor stimulates lymphangiogenesis and promotes oedema recovery in animal models of lymphoedema. The proliferation and migration of lymphatic endothelial cells (LEC) induced by these factors are dependent on activation of extracellular signal-regulated kinase (ERK) and Akt.[6–9]

Adrenomedullin (AM) is a vasodilator and diuretic peptide that was originally isolated from pheochromocytoma cells.[10,11] Earlier studies have shown that AM has protective effects on the cardiovascular system.[12–14] In particular, AM has angiogenic properties through activation of ERK and Akt in vascular endothelial cells.[15–17] Interestingly, a recent study has shown that the AM peptide is widely expressed in breast cancer and the degree of expression is associated with axillary lymph node metastasis.[18] Endogenous AM is necessary for murine lymphatic vascular development during embryogenesis.[19] These findings raise the possibility that AM may play an important role in lymphangiogenesis. However, whether AM promotes lymphangiogenesis and improves lymphoedema remains unknown.

Therefore, the present study was performed to (i) investigate whether AM promotes proliferation, migration, and network formation of cultured LEC *in vitro*, and elucidate the underlying molecular mechanisms and (ii) investigate whether AM promotes lymphangiogenesis and improves lymphoedema in a mouse model of tail lymphoedema *in vivo*.

2. Methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) and human lymphatic microvascular endothelial cells (HLMVEC) were purchased from Lonza (Basel, Switzerland), and expanded in medium (EBM-2, Lonza) with growth supplements (EGM-2MV, SingleQuots, Lonza). HUVEC and HLMVEC were cultured on collagen I-coated dishes

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(Iwaki, Chiba, Japan) and uncoated dishes, respectively. All the cells were used within passages 5–8.

2.2 Characterization of cultured lymphatic endothelial cells

To confirm the purity of expanded HLMVEC, we stained these cells with a LEC-specific marker Prox1.[20] The cells were stained with anti-Prox1 antibody (Acris, Hiddenhausen, Germany) followed by Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA), and counterstained with 4′,6-diamidino-2-phenylindole (Dojindo, Kumamoto, Japan). The images were obtained with a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

To examine the expression of AM receptor messenger ribonucleic acid (mRNA) in HLMVEC, reverse transcription polymerase chain reaction (RT-PCR) was performed. Total RNA were prepared from cultured HLMVEC using an RNeasy mini kit (Qiagen, Hilden, Germany). PCR was carried out on a thermocycler (ASTEC PC-818, Fukuoka, Japan) using the following protocol: an initial denaturation step at 95°C for 5 min, followed by 40 cycles at 95°C for 60 s, 60°C for 30 s, and 72°C for 60 s. The specific primer pairs were: calcitonin-receptor-like receptor (CRLR), 5′-CTCCTCTACATTATCC ATGG-3′, and 5′-CCTCCTCTGCAATCTTTCC-3′; receptor-activity-modifying proteins (RAMP) 1, 5′-AGTTCCAGGTAGACATGG-3′ and 5′-GCCTACACAATGCCCTCA-3′; RAMP2 5′-AAAGGATTGGTGCGACTG-3′ and 5′-GGAAGTGGAGTAACATGG-3′; RAMP3 5′-AGACAGGCATGTT GGAGA-3′ and 5′-TTCCAGCTTGCCAGGTGT-3′.[21] A set of β-actin primers was used as a Control for the RT-PCR.

2.3 Cell proliferation assay

HLMVEC were cultured for 36 h in EBM-2 medium containing 5% foetal bovine serum (FBS, Invitrogen) with (10^{-7} M) or without AM (Sionogi, Osaka, Japan). The cells were stained with Diff-Quik (Sysmex Internal Reagents, Kobe, Japan), photographed (BZ-9000, Keyence), and the number of cells was counted. In addition, HLMVEC were cultured in 96-well plates (5000 cells/well) with AM (10^{-9}–10^{-7} M) or 3′, 5′-cyclic adenosine monophosphate 8-bromo sodium salt (8-Br-cAMP) (10^{-6}–10^{-4} M) (Calbiochem, San Diego, CA, USA), a cell-permeable cAMP analogue, and cell proliferation were measured by MTS [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay (Promega, Madison, WI, USA). The reagent was added and plates were incubated for 4 h, and absorbance was measured at 490 nm (Bio-Rad, Hercules, CA, USA).

2.4 Cell migration and network formation assay

Migration assay was performed using a Transwell permeable support (Corning, NY, USA) containing a membranous insert (6.4 mm diameter, pore size 8.0 μm, collagen matrix uncoated). HLMVEC in EBM-2 with 5% FBS were added to the upper chamber at a density of 10^5 cells/cm^2, AM (10^{-9}–10^{-7} M) in EBM-2 medium containing 5% FBS was added to both the lower and upper chambers or to the lower chamber only, and HLMVECs were allowed to migrate for 12 h at 37°C. The cells remaining at the upper surface of the membrane were scraped off, and the cells on the lower side of the membrane were stained with Diff-Quik (Sysmex Internal Reagents), then the number of migrated cells was counted under a microscope (BZ-9000, Keyence).

Network formation assay was performed using Matrigel tissue culture plates (12-well plates, Becton Dickinson, San Jose, CA, USA). HLMVEC (10^5 cells/well) were seeded into each well and incubated for 18 h in EBM-2 medium containing 5% FBS with (10^{-7} M) or without AM. During this period, the morphologic changes of the cells were observed under a microscope (BZ-9000, Keyence).

2.5 Analysis of intracellular signalling

Intracellular cAMP was measured as reported previously.[22] Briefly, following treatment of HLMVEC (2×10^5 cells) with various concentrations of AM for 5 min, the medium was removed and washed twice with PBS (phosphate buffered saline). The cellular extract was obtained by addition of cold 70% ethanol. Each ethanol sample was evaporated in a vacuum until dry and dissolved in radioimmunoassay (RIA) buffer. cAMP was measured using an RIA kit (cAMP assay kit, Yamasa Shoyu, Chiba, Japan). Radioactivity was measured with a gamma counter (Aloka, Tokyo, Japan).

HLMVEC (5000 cells/well in 96-well plates) were pretreated for 30 min with 3′, 5′-cyclic adenosine monophosphorothioate Rp-isomer (Rp-cAMP) (10^{-3} M, Calbiochem), an antagonist of cAMP, PD98059, and U0126 (5×10^{-6} and 10^{-6} M, respectively, Calbiochem), inhibitors of mitogen-activated protein kinase kinase (MEK). The cells were further incubated for 36 h with AM (10^{-7} M) or 8-Br-cAMP (10^{-4} M) then MTS assay was performed as described previously.

2.6 Animal model and adrenomedullin administration

Male BALB/c mice (8–10 weeks, Japan SLC, Hamamatsu, Japan) were randomly divided into two groups: an AM treatment group and a Control group ($n = 10$ in each group). Tail lymphoedema was created as described previously with modification.[23] In brief, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg, Dainippon Sumitomo Pharma, Osaka, Japan). A 1.5–2 mm-wide ring of skin was removed 1 cm distal to the tail base, leaving the underlying bone, muscle and major blood vessels intact, and then the tail was wrapped with adhesive tape to protect the surgical site from interference. The AM group received continuous subcutaneous injection of recombinant human AM (Shionogi) dissolved in 0.9% saline at a rate of 0.05 μg/kg/min for 14 days from the day of operation, using an osmotic minipump (Alzet, Cupertino, CA, USA).[24,25] The Control group received 0.9% saline instead of AM.

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.7 Measurements of plasma adrenomedullin concentration and tail lymphoedema

Twenty-four hours after the start of AM administration, blood samples were collected into ice-cooled tubes with 70 μg/mL aprotinin and 1.5 mg/mL ethylenediaminetetraacetic acid 2Na (Bayer, Leverkusen, Germany), immediately centrifuged at 4°C, and stored at −80°C until use. Plasma human AM level was measured using an immunoradiometric assay kit (Shionogi), as described previously.[26] In brief, 200 μL of standard or plasma sample was placed in a polystyrene tube, and a mixture of biotinylated anti-AM and iodolabelled anti-AM was added. One bead coated with anti-biotin antibody was added (total volume = 300 μL), and the mixture was incubated at 4°C for 20 h. After removal of the incubation mixture, the bead was washed twice with 2 mL distilled water and radioactivity was measured with a gamma counter (Aloka).

Two days after operation, the tape wrapping the injury site was removed, and tail diameter at 1 cm distal to the distal edge of the injury site was measured by a calliper. Measurement was performed every other day until the mice were sacrificed on day 16 postoperation.

2.8 Fluorescence microlymphangiography

Lymphatic vessels in the dermis of the tail were evaluated by fluorescence microlymphangiography as previously described.[23] In brief, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) on day 16 postoperation. Fluorescein isothiocyanate (FITC)-dextran (70 kDa, Sigma-Aldrich) (2 mg/mL) was infused into the tip of the mouse tail. Then, images of superficial capillaries of the tail were photographed (MZ16FA, Leica Microsystems, Wetzlar, Germany).

2.9 Immunohistochemical study

On day 16 postoperation, the injury sites were removed, fixed in 4% paraformaldehyde, embedded with paraffin, and cut into 5 μm sections. For antigen retrieval, the sections were incubated with citrate buffer (DAKO, Glostrup, Denmark) at 120 C for 10 min. After treatment with protein block (DAKO), the samples were incubated with anti-lymphatic endothelial hyaluronan receptor-1 (LYVE-1) antibody (R&D Systems, Minneapolis, MN, USA), anti-Podoplanin antibody (Acris) or anti-von Willebrand factor (vWF) antibody (AB7356, Chemicon, Temecula, CA, USA) for 1 h at room temperature, and further incubated with horseradish peroxidase-conjugated antibodies (DAKO), then visualized with a DAB kit (Wako, Osaka, Japan). Cell nuclei were counterstained with haematoxylin (Muto, Tokyo, Japan).

2.10 Western blot analysis

HLMVEC were incubated for 12 h in basal medium containing 5% FBS and stimulated with (10^{-7} M) or without AM, then lysed in sample buffer with protease inhibitor (Complete, Roche, IN, USA). The lysates were loaded on 7.5% sodium dodecylsulphate-polyacrylamide gels (Bio-Rad, Hercules, CA, USA), transferred to membranes (Millipore, MA, USA) and probed with anti-ERK and anti-phosphorylated ERK (p-ERK) (Thr202/Thr204) antibodies (Cell Signaling, Boston, MA, USA) or anti-Akt and anti-phosphorylated Akt (p-Akt) (Ser473) antibodies (Cell Signaling). An anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA) served as a loading control. The membranes were then incubated with horseradish peroxidase-conjugated antibodies (Cell Signaling), and visualized by enhanced chemiluminescence reaction (GE Healthcare, Piscataway, NJ, USA).

HLMVEC were stimulated with (10^{-7} M) or without AM for 12 h, then lysed in sample buffer. Tail tissue of the injury site was obtained on day 8 postoperation and also lysed in sample buffer. Concentrations of VEGF-C and VEGF-A in each lysate were measured by western blot analysis. An anti-VEGF-C (H-190, Santa Cruz Biotech, Santa Cruz, CA, USA) antibody or an anti-VEGF-A (VEGF147, Santa Cruz, for HLMVEC lysate; AB1442, Chemicon, for tail tissue lysate) antibody was used as the probe. A rabbit polyclonal antibody against α-tubulin (Abcam, Cambridge, MA, USA) was used as an internal control.

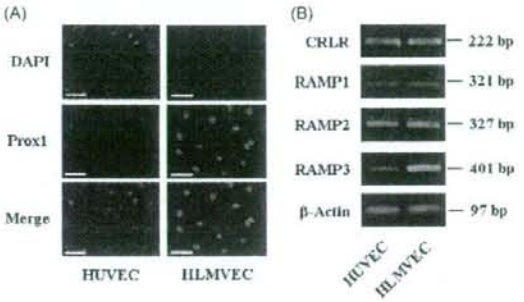

Figure 1 Characteristics of cultured human lymphatic microvascular endothelial cells (HLMVEC). (A) Fluorescent images of human umbilical vein endothelial cells (HUVEC) and HLMVEC, stained with anti-Prox1 antibody (green) and 4',6-diamidino-2-phenylindole (blue). Scale bars: 50 μm. All HLMVEC stained positive for Prox1. (B) HLMVEC expressed CRLR (calcitonin-receptor-like receptor), RAMP1 (receptor-activity-modifying protein), RAMP2, and RAMP3 mRNA (messenger ribonucleic acid). HUVEC were used as a control.

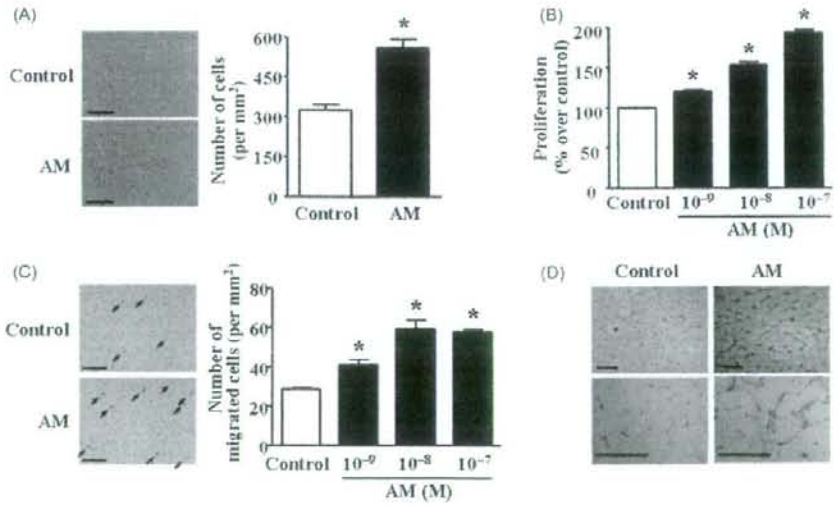

Figure 2 Effects of adrenomedullin (AM) on lymphatic endothelial cells (LEC). (A) Left panels, representative photographs of cultured human lymphatic microvascular endothelial cells (HLMVEC), stimulated with AM (10^{-7} M) for 36 h and stained with Diff-Quik. Scale bars: 200 μm. Right panel, quantitative analysis of proliferated HLMVEC ($n = 4$). (B) MTS [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay demonstrated dose-dependent promotion of proliferation in cultured HLMVEC by AM ($n = 8$). (C) Left panels, representative photographs of HLMVEC in migration assay, stimulated with AM (10^{-7} M) for 12 h and stained with Diff-Quik. Arrows indicate migrated cells. Scale bars: 100 μm. Right panel, quantitative analysis of migrated cells ($n = 6$). (D) Representative photographs of HLMVEC in network formation assay, stimulated with AM (10^{-7} M) for 18 h. Scale bars: 500 μm. Data are mean ± SE. *$P < 0.05$ vs. Control.

2.11 Statistical analysis

All data are expressed as mean ± SE. Comparisons of parameters among the groups were made by one-way ANOVA (analysis of variance), followed by Newman–Keul's test. Comparisons of parameters between two groups were made by Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Expression of calcitonin-receptor-like receptor and receptor-activity-modifying proteins mRNA in lymphatic endothelial cells

To determine whether LEC express AM receptors, we analysed commercially available HLMVEC which were positive for lymphatic maker Prox1 (*Figure 1A*). HLMVEC expressed CRLR, RAMP1, RAMP2, and RAMP3 mRNA (*Figure 1B*).

3.2 Enhancement of proliferation, migration, and network formation in cultured lymphatic endothelial cells by adrenomedullin

AM (10^{-7} M) significantly increased the number of cultured HLMVEC (*Figure 2A*). MTS assay also demonstrated that AM enhanced proliferation of cultured HLMVEC in a dose-dependent manner (*Figure 2B*). The number of migrated cells was significantly increased by AM (10^{-9}–10^{-7} M) when it was added to the lower chamber only (*Figure 2C*). This result indicates that AM is chemotactic for LEC. In addition, a marked increase in network formation was observed in the AM (10^{-7} M) group as compared with the Control group (*Figure 2D*). These results suggest that AM promotes proliferation, migration, and network formation of LEC.

3.3 Activation of cAMP/MEK/ERK pathway in lymphatic endothelial cells by adrenomedullin

We examined whether AM increases intracellular cAMP, the major second messenger of AM,[27,28] in cultured HLMVEC. AM dose-dependently increased the cAMP level in these cells (*Figure 3A*). Cell proliferation induced by AM was inhibited by a cAMP antagonist, Rp-cAMP (*Figure 3B*). In addition, a cAMP analogue, 8-Br-cAMP induced proliferation of HLMVEC in a dose-dependent manner (*Figure 3C*).

ERK and Akt are known to regulate cell proliferation and these signalling pathways were reported to function downstream of the AM/cAMP pathway.[29,30] Therefore, we investigated the activity (phosphorylation) of ERK and Akt in cultured LEC after stimulation with AM. The level of p-ERK in HLMVEC was significantly increased as early as 5 min after the start of AM (10^{-7} M) stimulation (*Figure 4A*). In contrast, the level of p-Akt in HLMVEC was not markedly increased by AM (*Figure 4B*). In addition, the cell proliferation induced by AM or 8-Br-cAMP was significantly attenuated by MEK inhibitors, PD98059, and U0126 (*Figure 4C* and *D*). These results suggest that AM-induced cell proliferation is mediated at least in part by the cAMP/MEK/ERK pathway.

3.4 Improvement of mouse tail lymphoedema by adrenomedullin

To evaluate the effect of AM on lymphoedema, a mouse model of tail lymphoedema was developed in BALB/c mice, and human AM was administered to the mice at a rate of 0.05 μg/kg/min (*Figure 5A*). Plasma human AM level was 5.4 ± 0.9 fM in AM-treated mice, whereas human AM was not detected in Control mice. The tail became oedematous after the surgical procedure and this change peaked on day 8 postoperation then gradually recovered in the AM and Control groups. However, AM treatment promoted the recovery of the injury site (*Figure 5B*). A significant difference in tail thickness was observed on days 14 and 16 postoperation (*Figure 5C*). These results suggest that AM improves secondary lymphoedema of the tail in mice.

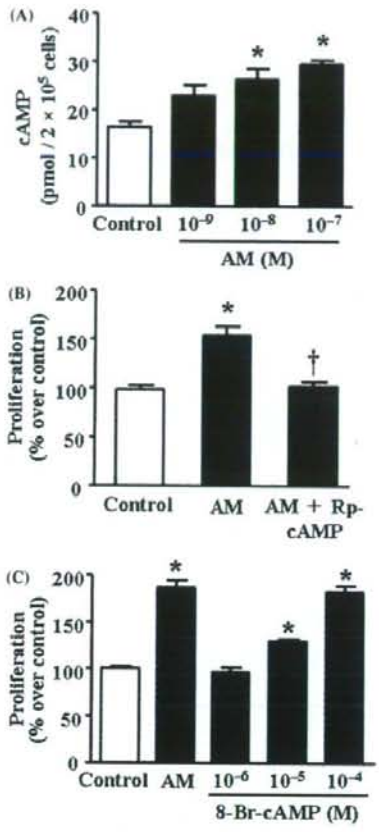

Figure 3 Relation between adrenomedullin (AM) and cyclic adenosine monophosphate (cAMP) in lymphatic endothelial cells (LEC) proliferation. (*A*) Following stimulation of human lymphatic microvascular endothelial cells (HLMVEC) with AM (10^{-9}–10^{-7} M) for 5 min, cAMP concentration was dose-dependently increased ($n = 4$). (*B*) MTS assay demonstrated that AM (10^{-7} M)-induced proliferation of HLMVEC was inhibited by a cAMP antagonist, Rp-cAMP (10^{-3} M) ($n = 8$). (*C*) MTS [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay demonstrated that a cAMP analogue, 8-Br-cAMP enhanced the proliferation of cultured HLMVEC in a dose-dependent manner ($n = 8$). Data are mean ± SE. *$P < 0.05$ vs. Control, †$P < 0.05$ vs. AM.

3.5 Promotion of lymphangiogenesis and angiogenesis by adrenomedullin

Lymphatic flow in the tail was evaluated by fluorescence microlymphangiography on day 16 postoperation

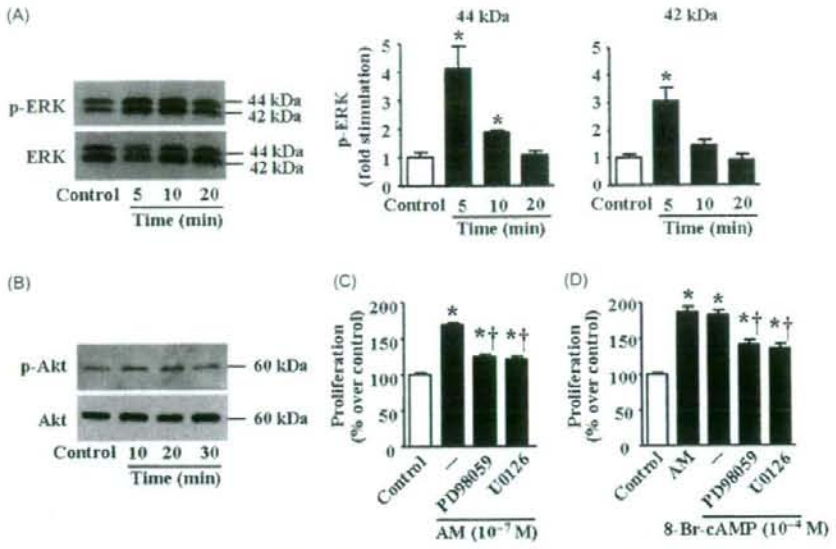

Figure 4 Intracellular signalling pathway induced by adrenomedullin (AM) in lymphatic endothelial cells (LEC). (*A*) Left panels, western blot analysis of phosphorylated extracellular signal-regulated kinase (p-ERK) and ERK in human lymphatic microvascular endothelial cells (HLMVEC), stimulated with AM (10^{-7} M) for 5, 10, and 20 min. Middle and right panel, quantitative analysis of p-ERK (44 and 42 kDa) ($n = 4$). (*B*) Western blot analysis of phosphorylated Akt (p-Akt) and Akt in HLMVEC, stimulated with AM (10^{-7} M) for 10, 20, and 30 min. (*C* and *D*) AM (10^{-7} M) or 8-Br-cAMP (cyclic adenosine monophosphate) (10^{-4} M)-induced proliferation of HLMVEC was inhibited by mitogen-activated protein kinase kinase (MEK) inhibitors, PD98059 and U0126 ($n = 6$). Data are mean ± SE. *$P < 0.05$ vs. Control, †$P < 0.05$ vs. AM or 8-Br-cAMP.

(*Figure 6A*). After infusion of FITC-dextran, fluorescence was soon observed in the distal part of the injury site. Although slow drainage of FITC-dextran was seen in Control group, rapid movement of fluorescence to the proximal part of the injury site was observed in the AM group.

Lymphatic vessels in the injury site were revealed by staining with anti-LYVE-1 antibody or anti-Podoplanin antibody. Blood vessels in the injury site were visualized by staining with anti-vWF antibody (*Figure 6B*). A large number of LYVE-1-, Podoplanin-, and vWF-positive vessels were observed on day 16 postoperation. The density of lymphatic vessels and blood vessels in the injury site was higher in the AM group than in the Control group. We determined the expression of VEGF-C and VEGF-A in the injury site after AM administration. Western blot analysis demonstrated that expression of VEGF-C or VEGF-A was not notably affected by AM (0.05 μg/kg/min) administration. *In vitro* study also demonstrated that the expression of VEGF-C or VEGF-A in HLMVEC was not significantly changed by AM (10^{-7} M) stimulation. These results suggest that AM directly promotes lymphangiogenesis and angiogenesis in a mouse model of tail lymphoedema.

4. Discussion

In this study, we demonstrated that (i) AM augmented proliferation and migration of LEC, and that the proliferation induced by AM was mediated at least in part by the cAMP/MEK/ERK pathway and (ii) administration of AM promoted lymphangiogenesis and improved mouse secondary lymphoedema.

AM has a variety of biological effects including angiogenic properties.[31–33] However, whether AM induces lymphangiogenesis and what is the underling mechanism responsible for the process remain unknown. In the present study, HLMVEC expressed CRLR, RAMP1, RAMP2, and RAMP3 mRNA. This suggests that AM could stimulate LEC through a complex of CRLR and one of the three RAMPs. Treatment of cultured HLMVEC with AM enhanced cell proliferation. Migration assay and network formation assay also demonstrated that AM enhanced cell migration and network formation. The process of lymphangiogenesis is known to include proliferation and migration of LEC,[9,34] therefore, AM-induced lymphangiogenesis is mediated by promotion of proliferation and migration of LEC.

Earlier studies have shown that cAMP plays an important role in proliferation of vascular endothelial cells.[35] In the present study, AM increased intracellular cAMP level in HLMVEC, and the proliferation induced by AM was inhibited by a cAMP antagonist Rp-cAMP. ERK in cultured LEC was markedly activated by treatment with AM. In addition, AM- or 8-Br-cAMP-induced cell proliferation was attenuated by MEK inhibitors, PD98069 and U0126. These results suggest that AM-induced cell proliferation is mediated by cAMP and at least in part through its downstream MEK/ERK pathway. In fact, cAMP-dependent ERK activation has been shown in the proliferation of HUVEC following adenosine receptor stimulation.[36]

We demonstrated the therapeutic potential of AM for lymphoedema using a mouse model of tail lymphoedema. The mouse tail exhibits a highly regular hexagonal network of lymphatic vessels in the skin. Therefore, depletion of circumferential skin in the tail obstructs lymphatic flow, resulting in acute lymphoedema, so this system could serve as a model of secondary lymphoedema to examine lymphangiogenesis *in vivo*.[7,23] Mature AM peptide consists of 52

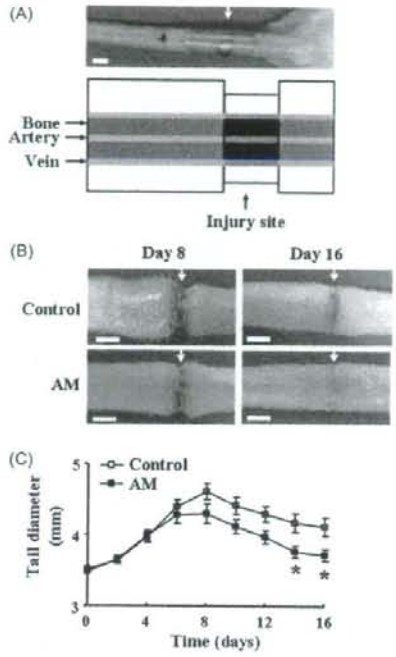

Figure 5 Effect of adrenomedullin (AM) infusion on mouse tail lymphoedema. (A) Upper panel, an operative model of mouse tail lymphoedema was created by removing tail skin circumferentially 1 cm distal to the tail base. Arrow indicates injury site. Scale bar: 2 mm. Lower panel, schematic diagram of injury site. (B) Representative photographs of tail on days 8 and 16 postoperation. Arrows indicate injury sites. Scale bars: 2 mm. (C) Quantitative analysis demonstrated a significant decrease in tail thickness in AM-treated mice vs. Control mice on days 14 and 16 postoperation ($n = 10$). Data are mean ± SE. $^*P < 0.05$ vs. Control.

amino acids in humans and 50 amino acids in the mouse. Mouse AM is 88% identical to human AM.[37,38] Administration of human AM to the mouse exerts biological effects *in vivo*.[32,39] In the present study, continuous administration of human recombinant AM at a rate of 0.05 μg/kg/min promoted recovery of the injury site and promoted a decrease in tail thickness. The number of LYVE-1- or Podoplanin-positive lymphatic vessels and vWF-positive blood vessels in the injury site were significantly increased in AM-treated mice. These findings suggest that AM improves lymphoedema and induces both lymphangiogenesis and angiogenesis. The mechanism of accelerated healing of the tail injury in AM-treated mice is unclear. However, previous studies showed that angiogenesis as well as lymphangiogenesis is crucial in the wound-healing process.[40,41] Therefore, the accelerated healing of the tail injury may be explained in part by increased blood vessels and lymphatic vessels in AM-treated mice.

VEGF-C and VEGF-A are key factors in lymphangiogenesis and angiogenesis.[42,43] However, in the present study, AM did not affect the expression of VEGF-C or VEGF-A in the tail tissue or in cultured HLMVEC. These results suggest that AM may directly stimulate LEC and endothelial cells to promote lymphangiogenesis and angiogenesis in our animal model.

In conclusion, AM promoted LEC proliferation at least in part through the cAMP/MEK/ERK pathway. Administration of AM improved secondary lymphoedema, and promoted lymphangiogenesis and angiogenesis. Thus, administration of AM may be a novel therapeutic strategy for patient with lymphoedema.

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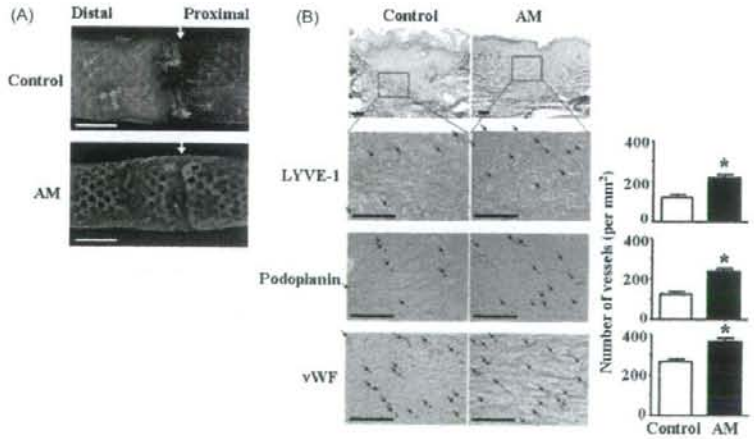

Figure 6 Histological analysis of lymphangiogenesis and angiogenesis. (A) Representative photographs of lymphatic flow in mouse tail. Fluorescence was mainly observed in the distal part of the injury site in Control mice, but was observed in the whole area of the tail in adrenomedullin (AM)-treated mice. Arrows indicate injury sites. Scale bars: 2 mm. (B) Left panels, representative photographs of tail sections on day 16 postoperation, immunostained with anti-LYVE-1 (lymphatic endothelial hyaluronan receptor-1), anti-Podoplanin and anti-vWF (von Willebrand factor) antibodies. Arrows indicate LYVE-1-, Podoplanin- or vWF-positive vessels. Left upper panels show the whole area of the injury site. Scale bars: 100 μm. Right panels, quantitative analysis demonstrated significant increases in LYVE-1- and Podoplanin-positive lymphatic vessels, and vWF-positive blood vessels in AM-treated mice vs. Control mice ($n = 5$). Data are mean ± SE. $^*P < 0.05$ vs. Control.