Effects of adrenomedullin on acute ischaemia-induced collateral development and mobilization of bone-marrow-derived cells

Minami ABE⁎, Masataka SATA⁎, Etsu SUZUKI†, Ryo TAKEDA⁎, Masao TAKAHASHI⁎, Hiroaki NISHIMATSU‡, Daisuke NAGATA⁎, Kenji KANGAWA§, Hisayuki MATSUO§, Ryozo NAGAI⁎ and Yasunobu HIRATA⁎

⁎Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Tokyo 113-8655, Japan, †Department of Nephrology and Endocrinology, University of Tokyo Graduate School of Medicine, Tokyo 113-8655, Japan, ‡Department of Urology, University of Tokyo Graduate School of Medicine, Tokyo 113-8655, Japan, and §National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

ABSTRACT

Adrenomedullin exerts not only vasodilatory effects, but also angiogenic effects. In the present study, we investigated the effects of adrenomedullin on collateral formation and circulating bone-marrow-derived cells after acute tissue ischaemia. Bone marrow of 8–10-week-old female C57BL/6J mice was replaced with that from GFP (green fluorescent protein) transgenic mice (GFP mice). At 8 weeks after transplantation, hindlimb ischaemia was induced by resecting the right femoral artery and a plasmid expressing human adrenomedullin (50 µg) was injected into the ischaemic muscle, followed by in vivo electroporation on a weekly basis. Overexpression of adrenomedullin significantly enhanced the blood flow recovery compared with controls (blood flow ratio, 1.0 ± 0.2 compared with 0.6 ± 0.3 respectively, at week 4; P < 0.05) and increased capillary density in the ischaemic leg as determined by anti-CD31 immunostaining of the ischaemic muscle (567 ± 40 compared with 338 ± 65 capillaries/mm² respectively, at week 5; P < 0.05). There were more GFP-positive cells in the thigh muscle of the mice injected with adrenomedullin than in that of the control mice (29.6 ± 4.5 compared with 16.5 ± 3.3 capillaries/mm² respectively, at week 5; P < 0.05). We repeated the same experiments using LacZ-knock-in mice instead of GFP mice, and obtained similar results. These findings suggest that adrenomedullin may augment ischaemia-induced collateral formation with some effects on circulating bone-marrow-derived cells.

INTRODUCTION

AM (adrenomedullin) was isolated as a vasodilatory peptide from pheochromocytoma [1]. The major source of circulating AM is the vascular wall [2–4], suggesting an important role in the regulation of vascular tone. However, AM has been found to exert many other vascular actions, including inducing the release of NO (nitric oxide) from vascular endothelial cells [5] and exerting an anti-apoptotic effect on endothelial cells [6]. The mechanism of these effects seems to involve the activation of the PI3K (phosphoinositide 3-kinase)/Akt pathway [7,8]. Furthermore, AM expression is markedly up-regulated in response to hypoxia [9,10]. These findings

Key words: adrenomedullin, angiogenesis, bone-marrow-derived cell, collateral development, endothelium, ischaemia.

Abbreviations: AM, adrenomedullin; APC, allophycocyanin; EPC, endothelial progenitor cell; ERK1/2, extracellular-signal-regulated kinase 1/2; GFP mice, transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP (green fluorescent protein); NO, nitric oxide; eNOS, endothelial NO synthase; PE, phycoerythrin; PI3K, phosphoinositide 3-kinase; X-Gal, 5-bromo-4-chloroindol-3-yl β-d-galactopyranoside.

Correspondence: Dr Yasunobu Hirata (email hirata-2im@h.u-tokyo.ac.jp).
suggest that AM may play a physiological role in tissue ischaemia. We have reported previously [11] that AM augments collateral development in the ischaemic hindlimb tissues of mice and that this effect is NO-dependent; however, the detailed mechanism remains unclear.

It has been clarified that bone-marrow-derived stem cells participate in tissue repair and remodelling of remote organs [12]. Bone-marrow-derived EPCs (endothelial progenitor cells) have been shown to participate in angiogenesis in tumours [13] and tissue ischaemia [14]. EPCs are mobilized into the systemic circulation by several angiogenic substances via NO-related mechanisms [15–20].

We hypothesized that AM may promote the mobilization of EPCs after acute tissue ischaemia. Thus we studied the effect of AM on collateral development in the hindlimbs of a mouse model of ischaemia. Furthermore, we investigated the origin of the cells in the newly formed collateral vessels.

**MATERIALS AND METHODS**

**Animals**

C57BL/6J mice were purchased from SLC Japan (Shizuoka). GFP mice (transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP (green fluorescent protein) were generously given by Dr Masaru Okabe (Genome Information Research Center, Osaka University, Osaka, Japan). ROSA26 mice, which are knock-in mice expressing the LacZ gene in essentially all tissues (C57BL/6 × 129S background), were originally purchased from Jackson Laboratory (B6; 129S-Gtrosa26). ROSA26 mice were maintained in our animal facility and intercrossed with C57BL/6J mice. The resulting littermates were used for this study. All protocols involving animals were in accordance with the Institutional Guidelines for Animal Care established by the University of Tokyo, and complied with the Guide for the Care and Use of Laboratory Animals.

**Bone marrow reconstitution**

Female wild-type mice (8–10 weeks of age; C57BL/6J mice) were lethally X-ray-irradiated with a total dose of 9 Gy (MRB-1520RB; Hitachi). Bone marrow cells were harvested from femurs and tibias of donor mice. The recipient mice received unfractionated bone marrow cells (3 × 10^6) suspended in 0.3 ml of PBS by tail vein injection. At 8 weeks after bone marrow transplantation, hindlimb ischaemia was induced in the recipient mice.

**Mouse hindlimb ischaemia model**

Unilateral hindlimb ischaemia was induced in approx. 17-week-old wild-type female mice (C57BL/6J mice), which had received bone marrow from GFP mice. Animals were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg of body weight). Human AM cDNA was subcloned into pcDNA3 at EcoRI and XbaI sites (hAMpcDNA3). We injected either hAMpcDNA3 or pcDNA3 (50 µg of naked plasmid/50 µl of PBS) into the ischaemic muscle, followed by in vivo electroporation (100 V for 50 ms, six times). The plasma concentration of human AM at 1 week after the last injection was much higher in mice treated with AM than in mice treated with pcDNA, suggesting a marked elevation of local concentrations of AM. The proximal and distal portions of the femoral artery and the distal portion of the saphenous artery were ligated. Subsequently, arteries and all side branches were dissected free and excised. The skin was closed with 5-0 surgical suture.

**Monitoring of hindlimb blood flow**

Hindlimb blood flow was measured with a Laser Doppler Perfusion Imager system (Moor Instruments). Excess hair was removed from the limbs, and anaesthetized mice were placed on to a heating plate at 40°C. Results are expressed as the ratio of perfusion in the right (ischaemic) compared with the left (untreated) limb.

**FACS analysis**

At 5 weeks after hindlimb ischaemic surgery, all of the mice were killed and their peripheral blood and bone marrow were harvested. Bone-marrow-derived cells were detected and analysed by FACS. Peripheral blood monocytes were isolated by centrifugation against a Histopaque-1083 density gradient. Bone marrow from hindlimb femurs was washed with physiological salt solution containing heparin. Peripheral blood and bone marrow were mixed with 1% (w/v) albumin/PBS, incubated with an APC (allophycocyanin)-conjugated anti-(c-Kit) antibody (BD Biosciences) and a PE (phycoerythrin)-conjugated anti-(Sca-1) antibody (BD Biosciences), and analysed by flow cytometry.

**Detection of GFP-positive cells in bone-marrow-chimaeric mice**

At 5 weeks after surgery, mice were killed with an overdose of pentobarbital and perfused with 0.9% NaCl at a constant pressure via the left ventricle, followed by perfusion fixation with 4% paraformaldehyde/PBS. The ischaemic muscles were fixed further in 4% paraformaldehyde overnight at 4°C. To preserve the GFP signal for histological analyses, muscles were embedded in plastic resin (Technovit 8100; Heraeus Kulzer), according to the manufacturer’s instructions. Briefly, muscles were washed overnight in PBS/6.8% (w/v) sucrose at 4°C, dehydrated in 100% ace tone and embedded. The polymerized block was cut using a rotary microtome (HM335E; MICROM International) with a disposable knife (Histoknife; Heraeus Kulzer). Thin sections (3–4 µm) were stretched in a water bath, mounted on
silanized slides (Matsunami) and dried for 2 h at 37 °C. The sections were washed in PBS and used for immunofluorescence studies.

**Detection of LacZ-positive cells**

To confirm the effect of AM on bone-marrow-derived cells and angiogenesis, we also induced hindlimb ischemia in bone-marrow-chimaeric mice which had received bone marrow transplantation from LacZ transgenic mice and injected with AM. LacZ was detected by X-Gal(5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) staining. Ischaemic muscles were excised and stained with X-Gal solution [1 mg/ml X-Gal, 2 mmol/l MgCl2, 5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 0.01 % sodium deoxycholate and 0.02 % Nonidet 40 in PBS] at 37 °C overnight, fixed further in 4 % paraformaldehyde overnight at 4 °C, and embedded in paraffin. Thin sections (5 µm) were de-paraffinized and used for histological analyses.

**Measurement of capillary density in the ischaemic leg**

At 5 weeks after surgery, mice were killed by intraperitoneal injection of an overdose of pentobarbital. Whole limbs were fixed in methanol overnight. Ischaemic thigh muscles were embedded in paraffin. Sections (5 µm) were de-paraffinized and incubated with a rat-monoclonal antibody against murine CD31 (clone MEC13.1; BD Biosciences). Antibody distribution was visualized using the avidin–biotin complex technique and Vector Red Chromogenic substrate (Vector Laboratories), followed by counterstaining with haematoxylin. Capillaries were identified by positive staining for CD31 and morphology. Ten different fields from each tissue preparation were selected, randomly and capillaries were counted. Capillary density was expressed as the number of capillaries/mm².

**Statistics**

All values are means ± S.E.M. Statistical comparisons of the means were performed by ANOVA, followed by the Student–Neumann–Keuls test. A P value of < 0.05 was considered to be statistically significant.

**RESULTS**

**Effects of AM on blood flow recovery**

Hindlimb ischaemia was induced in bone-marrow-chimaeric mice. Mice were treated with pcDNA or AM (n = 12 in each group), and the blood flow in the ischaemic and non-ischaemic legs was monitored weekly by laser Doppler imaging. In the control mice treated with pcDNA, blood flow in the ischaemic leg recovered gradually, reaching half of the blood flow in the untreated leg at 4 weeks. AM enhanced the blood flow recovery as shown in Figure 1. Collateral formation was evaluated as the capillary density of the ischaemic hindlimb muscle at 4 weeks after surgery. Consistent with blood flow recovery, anti-CD31 immunostaining (Figure 2) revealed that AM significantly increased the number of detectable capillaries in the ischaemic leg (567 ± 40 capillaries/mm² in mice treated with AM compared with 338 ± 65 capillaries/mm² in control at week 5; P < 0.05).

**Detection of bone-marrow-derived cells**

At 8 weeks after bone marrow transplantation, we examined the peripheral blood of wild-type mice that had received bone marrow cells from GFP mice. FACS analysis revealed that more than 90 % of the peripheral leucocytes in the recipient mice had already been replaced by GFP-positive cells from the donor mice. Bone-marrow-derived cells in the ischaemic tissue were detected by fluorescence
microscopy. There were significantly ($P < 0.05$) more GFP-positive cells in ischaemic muscle of mice treated with AM (29.6 $\pm$ 4.5 cells/mm$^2$) compared with control mice injected with pcDNA (16.5 $\pm$ 3.3 cells/mm$^2$; $n = 6$ per group; Figure 3A). No GFP-positive cells were found in non-ischaemic leg muscles (0.5 $\pm$ 0.02 cells/mm$^2$). The section of ischaemic muscle embedded in plastic resin was immunostained using a fluorescent-conjugated anti-CD31 antibody, an endothelial cell marker. As shown in Figure 3(B), some of the GFP-positive cells in ischaemic muscle expressed CD31.

Detection of LacZ-positive cells in bone-marrow-transplanted mice

As shown in Figure 4, AM significantly increased blood flow recovery in ischaemic muscle of LacZ-bone-marrow-chimaeric mice compared with control mice (1.05 $\pm$ 0.18 compared with 0.56 $\pm$ 0.14 respectively, at week 5; $P < 0.01$). Capillary density was also significantly increased in ischaemic muscle of LacZ-bone-marrow-chimaeric mice compared with control mice (568 $\pm$ 18 compared with 358 $\pm$ 46 capillaries/mm$^2$ respectively; $P < 0.05$). There were more LacZ-positive cells in ischaemic muscles of mice in the AM group than in the control group (3.2 $\pm$ 0.7 compared with 0.3 $\pm$ 0.4 cells/mm$^2$ respectively; $n = 12$ per group; $P = 0.001$). There was a negligible number of LacZ-positive cells in
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Figure 5  FACS analysis of circulating peripheral blood at 5 weeks after surgery
Peripheral blood monocytes were isolated and were mixed with 1% (w/v) albumin/PBS. Cells were then incubated with an APC-conjugated anti-(c-Kit) antibody and a PE-conjugated anti-(Sca-1) antibody, and analysed by FACS. Representative analysis of Sca1-positive cells (A) and c-Kit-positive cells (B) in circulating peripheral blood are shown.

It has been suggested that eNOS (endothelial NO synthase)/NO plays an important role in the mobilization of EPCs. Previous studies support this notion: physical training increases angiogenesis in mice [23]; oestrogen was found to promote re-endothelialization after arterial injury and to increase the number of circulating EPCs [24]; and atorvastatin also increased angiogenesis in infarcted myocardium in mice [15]. All of the three treatments increased circulatory EPCs. Furthermore, these effects were suppressed by inhibiting eNOS. We have reported [11] that AM activates eNOS via the P13K/Akt pathway and that the angiogenic effect of AM was diminished in eNOS-knockout mice. This suggests that AM-induced EPC mobilization may also be related to eNOS activation. In fact, inhibition of P13K by wortmannin decreased the angiogenic effects of AM. Iwase et al. [25] introduced a cocktail therapy of bone-marrow-derived mononuclear cells and AM infusion for hindlimb ischaemia. This therapy was more effective than treatment with bone-marrow-derived mononuclear cells or with AM alone. They showed that AM inhibited apoptosis of bone-marrow-derived cells and enhanced the differentiation of bone-marrow-derived cells into EPCs. These findings support further the important role of AM in the mobilization of EPCs.

The role of these EPCs on new vessel formation in ischaemic tissues has been explored. Transplantation of peripheral or bone-marrow-derived EPCs augments collateral blood flow and prevents hindlimb autoamputation [20]. In the same way, after acute myocardial infarction, transplantation of bone-marrow-derived EPCs or CD34-positive cells resulted in angiogenesis and improved cardiac function [26,27]. CD34-positive cells augment blood flow recovery in ischaemic lesions in a diabetic mouse model [28]. These studies indicate that EPCs potentiate blood flow recovery in impaired vessels.

There is a possibility that the mobilization of EPCs is modulated by NO. In eNOS-deficient mice, bone marrow transplantation itself does not work well [29]; therefore it seems that eNOS of stromal cells modulates the mobilization of EPCs. It would be plausible that EPC mobilization by AM-induced NO release via activation of the P13K/Akt pathway might be impaired in eNOS-deficient mice, in which AM failed to augment collateral development. AM activates not only the P13K/Akt pathway and guanylate cyclase, but also adenylate cyclase, ERK1/2 (extracellular-signal-regulated kinase1/2) and p125FAK (focal adhesion kinase). It has been reported that in addition to P13K inhibition, inhibitors of protein kinase A and ERK1/2 also suppress migration and tube formation in HUVECs (human umbilical veins endothelial cells) [8,30]. Although it seems likely that NO plays a critical role in AM-induced collateral formation in response to ischaemia, various factors may be involved in this effect.

non-ischaemic muscles. In addition, as shown in Figure 5, FACS analysis of peripheral blood revealed an increase in Sca-1 and c-Kit double-positive cells in mice treated with AM compared with controls (7.3 ± 2.1 compared with 3.9 ± 1.1 % respectively; n = 12 per group; P = 0.007).

DISCUSSION

In the present study, we have demonstrated that AM enhanced collateral development in response to acute ischaemia. This angiogenic effect of AM has been confirmed both in vivo and in vitro by other investigators [21,22], although the methods of AM administration were different. Our results suggest that AM-induced angiogenesis might be associated with mobilization of bone-marrow-derived cells. We visualized bone-marrow-derived cells using marker gene transgenic mice, i.e. GFP and LacZ mice. The capillaries in hindlimb ischaemic muscle contained these cells. Therefore ischaemia itself is a stimulus for the mobilization of bone-marrow-derived cells, because these cells were not observed in non-ischaemic muscle. AM substantially increased bone-marrow-derived cells in the ischaemic thigh. Moreover, some of the cells expressed the endothelial-cell-specific protein CD31. FACS analysis revealed that treatment with AM increased the number of Sca1 and c-kit double-positive cells in peripheral blood and bone marrow. Although the identification or definition of EPCs is controversial, these findings suggest that AM may promote the mobilization of EPCs.
The results of the present study were not conclusive as to whether local or systemic AM was the cause of the responses seen. In fact, local delivery of the AM gene increased the circulating AM level to a level higher (approx. 5 × 10^{-12} M) than that observed in control mice. However, Western blot analysis detected human AM in the thigh muscle only in the injected side [11]. The vasodilatory action of AM requires at least 10^{-12} M [7]. Moreover, bone-marrow-derived cells were not detected in non-ischaemic legs. Although we did not examine the effect of transfected AM on the non-ischaemic side, these findings suggest that locally expressed AM, rather than circulating AM, may exert the angiogenic effect. Iimuro et al. [21] reported that in heterozygotic AM-knockout mice blood flow recovery after experimental ischaemia was significantly less than in their wild-type control. Furthermore, mice treated with AM22-52, a competitive inhibitor of AM, had reduced capillary development. Thus not only exogenous AM, but also endogenous AM, may be involved in collateral formation in response to acute leg ischaemia.

In conclusion, AM augmented collateral development after acute tissue ischaemia. AM increased Sca-1- and c-Kit-positive cells in the peripheral blood. In the ischaemic muscle, more bone-marrow-derived cells were observed, some of which expressed CD31. Therefore AM appears to mobilize EPCs and increase collateral development. AM may have a therapeutic potential in treating ischaemic diseases.

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**REFERENCES**


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