Original Article

Adrenomedullin Promotes Proliferation and Migration of Cultured Endothelial Cells

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Adrenomedullin (AM) is a vasoactive hormone which exerts its action through cyclic adenosine monophosphate(cAMP) /cAMP-dependent protein kinase (PKA) cascade and intracellular Ca^{2+} mobilization. Recently, evidence has accumulated that AM plays a critical role in the regulation of vascular tone, remodeling and morphogenesis. And although numerous reports have examined the action of AM on cultured vascular cells, the results have not been consistent and have depended on the experimental conditions used. Accordingly, the purpose of this study was to clarify the effect of AM on the proliferation and migration of cultured endothelial cells. Our results revealed that AM promoted the growth and migration of endothelial cells (ECs). AM significantly promoted the proliferation of human umbilical vein endothelial cells (HUVECs) (56.0±8.7% over the controls at 10^{-9} mol/l) and this stimulative effect was inhibited by two AM antagonists, AM(22−52) and calcitonin gene-related peptide (CGRP) (8−37). The number of HUVECs migrated to the lower surface of the transwell apparatus was also increased dose-dependently in the AM group (30.4±4.2% over the controls at 10^{-7} mol/l), and this increase was suppressed by the two AM antagonists and by two PKA antagonists, adenosine 3′,5′-cyclic monophosphorothioate Rp-isomer and myristoylated protein kinase A inhibitor amide 14−22. The promoting action of AM on endothelial migration was also suppressed by LY294002, an inhibitor for phosphatidylinositol 3-kinase, but not by N^G-nitro-L-arginine-methyl ester (L-NAME), an antagonist for nitric oxide synthase (NOS). These results indicate that AM promotes proliferation and migration of ECs *via* a cAMP/PKA dependent pathway and lend support to the idea that AM exerts beneficial effects on vascular regeneration and might be used as a novel therapeutic strategy for patients with vascular disease. (*Hypertens Res* 2003; 26 (Suppl): S93−S98)

***Key Words*: adrenomedullin, endothelial cells, proliferation, migration, vascular regeneration**

Introduction

Regeneration of the endothelium after vascular injury is a protective mechanism that limits the development of atherosclerosis (*1*). Postnatal angiogenesis from pre-existing vessels is an important process to alleviate tissue ischemia (*2*). In both processes, proliferation and migration of vascular endothelial cells play critical roles and are regulated by many vasoactive agents.

We have reported the significance of natriuretic peptides (NPs) for the regulation of vascular tone and remodeling. We previously demonstrated that C-type natriuretic peptide (CNP) is secreted from endothelial cells (ECs) to act as a local regulator of vascular tone and growth (*3*). We have also revealed that the endothelial secretion of CNP is stimulated by various cytokines and growth factors that are activated in proliferative vascular lesions and modulate vascular remodeling, especially by transforming growth factor-β and tumor necrosis factor-α (*4*). Furthermore, we revealed that adenovirus-mediated gene transfer of the CNP gene promoted endothelial regeneration accompanied with re-differentiation and

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growth suppression of vascular smooth muscle cells (VSMCs) *in vitro* and *in vivo via* an NPs/cyclic guanosine monophosphate (cGMP)/cGMP-dependent kinase (cGK) pathway (*5*, *6*).

Adrenomedullin (AM) is a potent vasorelaxant peptide that was originally isolated from human pheochromocytoma cells on the basis of its ability to elevate cyclic adenosine monophosphate (cAMP) levels in rat platelets (*7*). AM was shown to be secreted from ECs (*8*) and VSMCs (*9*) to act as a local vasorelaxing hormone. AM has also been shown to be present in atherosclerotic lesions, and the AM gene has been identified in macrophages of such lesions (*10*). Its secretion has been augmented by several cytokines, such as interleukin-1, tumor necrosis factor (TNF)-α and lipopolysaccharide (*11*). Furthermore, hypoxia responsive elements (HREs) have been identified in the AM gene, and hypoxic conditions have been shown to induce its expression and secretion from human umbilical vein endothelial cells (HUVECs) (*12*). These findings suggest the significance of AM for atherogenesis and angiogenesis. Recently, mice genetically engineered to overexpress or underexpress the AM gene were developed to determine the *in vivo* significance of AM. Mice overexpressing the AM gene in their vasculature showed reduced blood pressure, and this effect was abolished by administration of N^G-nitro-L-arginine-methyl ester (L-NAME) (*13*), suggesting that AM-induced vasorelaxation was mediated by an nitric oxide (NO)/cGMP dependent pathway. On the other hand, mice lacking the AM gene did not survive the embryonic stage and showed abnormal vascular structure and subcutaneous hemorrhage (*14*). Heterozygote mice with a disrupted AM gene showed an increase in blood pressure. The plasma AM levels are known to be increased in human patients with congestive heart failure (*15*), renal diseases (*16*) and hypertensive disorders (*17–19*). These observations suggested the significance of AM in vascular morphogenesis and regulation of vascular tone *in vivo.*

There have been more than a few reports on the effect of AM on proliferation and migration of several cell types. AM inhibited the proliferation of rat VSMCs cultured in the presence of 5% serum (*20*), and it also inhibited migration of VSMCs stimulated by 5–10% serum, platelet-derived growth factor (PDGF), or angiotensin II through a cAMP-dependent pathway (*21*, *22*). However, in rat quiescent VSMCs, AM promoted cell proliferation independent of the cAMP/cAMP-dependent protein kinase (PKA) pathway (*23*). Thus AM might show dual activities on the growth of cultured VSMCs depending on the experimental conditions.

The effect of AM on proliferation also depends on cell types. For example, AM has been reported to stimulate proliferation of Swiss 3T3 fibroblasts (*24*), human oral keratinocytes (*25*), and certain tumor cell lines (*26*). On the other hand, it inhibited the proliferation of rat mesangial cells (*27*) and cardiac fibroblasts (*28*).

In cultured ECs prepared from rat aorta and incubated in medium containing 0.25% serum, AM was demonstrated to significantly suppress apoptosis without inducing cell proliferation (*29*). In another report, however, it was shown that AM promoted proliferation of HUVECs (*30*). Thus, there is much room for clarification with respect to the effect of AM on the proliferation and migration of ECs.

Based on these findings, the objective of this study was to determine whether AM promotes proliferation and migration of cultured human endothelial cells, and if so, to determine the possible mechanism of these effects.

Methods

Materials

Human AM, AM(22–52), and calcitonin gene-related peptide (8–37) (CGRP(8–37)) were obtained from the Peptide Institute (Osaka, Japan). Adenosine 3′,5′-cyclic monophosphorothioate Rp-isomer (Rp-cAMP) and myristoylated protein kinase A inhibitor amide 14–22 (PKI), both of which are PKA antagonists, and LY294002, which is an inhibitor for phosphatidylinositol 3-kinase (PI3K), were purchased from Calbiochem (San Diego, USA). L-NAME, an antagonist for nitric oxide synthase (NOS), was purchased from Nakalai Tesque (Kyoto, Japan). Hoechst 33342 was purchased from Molecular Probes (Eugene, USA).

Cell Culture

HUVECs (Clonetics, Waltersville, USA) were grown in the basic medium containing 2% fetal bovine serum (FBS) and growth supplements (EGM-2; Clonetics), as previously reported (*31*). Subconfluent cell cultures between passages 4 and 6 were used for each experiment.

Cell Proliferation Assay

The increase of cell number was assessed by modified MTT assay, as previously reported (*32*). After 48 h of incubation in the basic medium containing 0.5 or 2.0% FBS, Cell Count Reagent SF (Nacalai Tesque) was added and the incubation was continued for another 4 h. The reaction was terminated by cooling the cells on ice, and the absorbance at a wavelength of 450 nm was measured to examine the cell number. The real cell number was confirmed to be linearly proportional to the OD_{450} value.

Cell Migration Assay

Migration assays were performed using a transwell apparatus containing a light-opaque membranous insert (6.4 mm diameter, 3 μm pores) designed to absorb fluorescence (FALCON HTS FluoroBlok Multiwell Insert System; Becton Dickinson, Franklin Lakes, USA). Cells pre-labeled with Hoechst 33342, a membrane-permeable nuclear staining fluorescent agent binding to the AT-rich regions of DNA, were added to the upper chamber at a density of 1×10^5 cells/cm^2 and al-

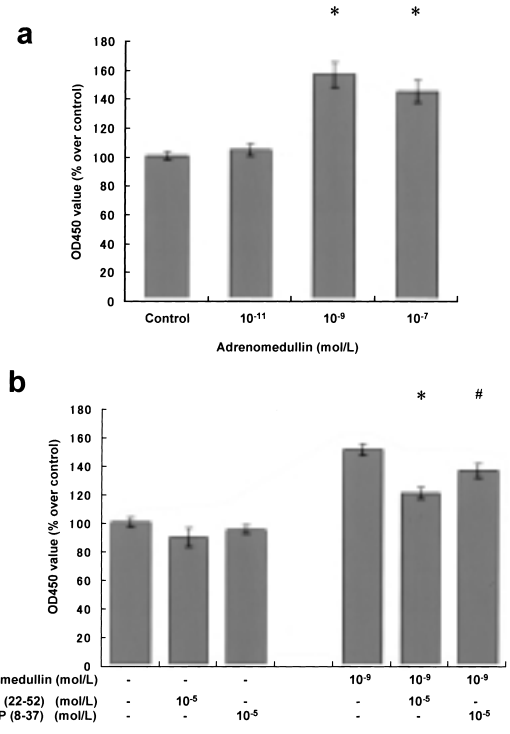

Fig. 1. *AM promoted the proliferation of HUVECs. (a) AM promoted proliferation of HUVECs in medium containing 0.5% serum. The cells were incubated for 48 h and the number of cells was estimated by modified MTT assay.* *p$<$*0.01* vs. *controls* (n$=$*18). (b) 10*$^{-9}$*mol/l AM-induced proliferation was blocked significantly by two AM antagonists, AM(22–52) (10*$^{-5}$*mol/l) and CGRP(8–37) (10*$^{-5}$*mol/l).* *p$<$*0.01* vs. *controls,* $^{\#}$p$<$*0.05* vs. *controls* (n$=$*18).*

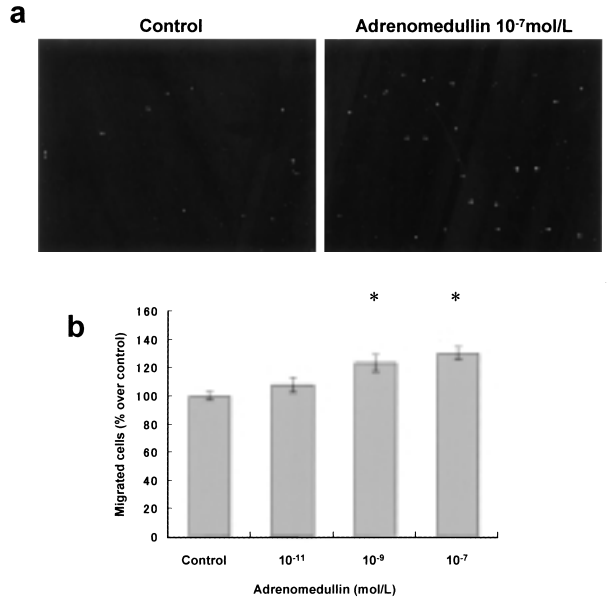

Fig. 2. *AM promoted the migration of HUVECs. (a) The representative images of HUVECs migrated to the lower surface of the transwell apparatus. The cells were pretreated with Hoechst 33342 and allowed to migrate for 4 h. The cells migrated beyond the membranous insert were photographed, and the number of cells was counted with a fluorescent microscope and quantifiable software. (b) AM dose-dependently promoted the migration of HUVECs in the range of 10*$^{-11}$*–10*$^{-7}$*mol/l. The cells were placed in the upper chamber and AM was added to the lower chamber for chemotaxis. The number of HUVECs migrated to the lower surface of the insert was increased in the AM-treated groups.* *p$<$*0.01* vs. *controls* (n$=$*6).*

lowed to migrate for 4 h at 37°C in the serum-free condition. The number of cells that had migrated through the insert was counted using a fluorescent microscope (Axiovert S100 and AxioCam MRc; Carl Zeiss, Oberkochen, Germany) and software for quantification (Scion Image 0.4.0.2; Scion Corp., Fredevick, USA) (*33*). Four fields ($\times$100 magnification) were photographed for each well. In some experiments, cells were pretreated for 30 min with several inhibitors of the AM/cAMP/PKA pathway to confirm the specificity of the migratory effect of AM.

Statistics

All data are expressed as the mean$\pm$SEM. Statistical analysis was performed with Student's *t* test or analysis of variance (ANOVA). Values of $p<0.05$ were considered to indicate statistical significance.

Results

AM Promoted Proliferation of the Cultured Endothelium

AM dose-dependently increased the number of HUVECs incubated for 48 h in the medium containing 0.5% FBS (Fig. 1a). The proliferative effect of 10^{-9} mol/l AM was 56.0$\pm$8.7% over the control value ($p<0.01$ *vs*. controls), and no further increase in cell number was observed at a concentration of 10^{-7} mol/l AM. The proliferative effect of AM on ECs was reduced by the higher concentration (2.0%) of FBS (11.9$\pm$3.3% over the control value at 10^{-11} mol/l, $p<0.05$; 13.9$\pm$2.9% at 10^{-9} mol/l, $p<0.05$; 13.0$\pm$2.8% at 10^{-7} mol/l, $p<0.05$ *vs.* controls: $n=6$ in each group) AM-induced proliferation was blocked significantly with two AM antagonists, AM(22–52) (10^{-5} mol/l) and CGRP(8–37) (10^{-5} mol/l), when ECs were stimulated by 10^{-9} mol/l AM in 0.5% serum (Fig. 1b).

AM Promoted Migration of the Cultured Endothelium

Figure 2a shows the effect of 10^{-7} mol/l AM on the migration of cultured HUVECs compared with the control value. As shown in the photographs, the number of HUVECs migrated to the lower surface of the insert was increased in the AM-treated group. Figure 2b demonstrates the dose-dependent effect of AM on the migration of HUVECs.

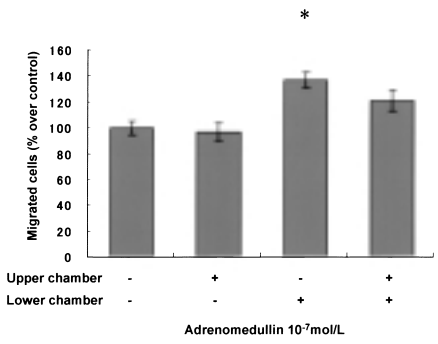

Fig. 3. *AM-induced migration was chemotactic. AM (10^{-7} mol/l) was added to either the upper, lower or both chambers. The number of migrated cells was significantly increased in the group in which AM was added to the lower chamber only.* * $p<0.01$ vs. *controls (the group without AM) (*n=*6).*

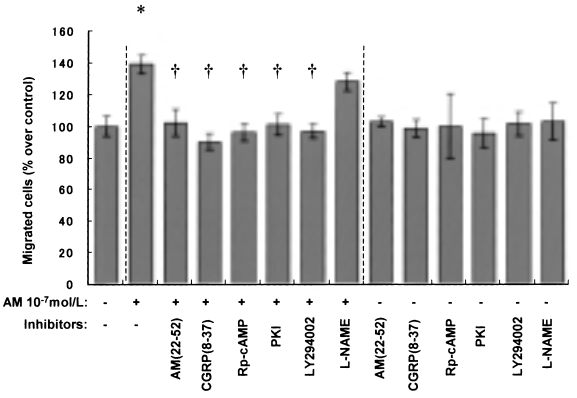

Fig. 4. *AM-induced migration was inhibited by blockade of the AM/cAMP/PKA cascade. AM-induced migration was blocked significantly by pretreatment with AM(22–52) (10^{-5} mol/l) or CGRP(8–37) (10^{-5} mol/l), and with Rp-cAMP (5×10^{-6} mol/l) or PKI (5×10^{-6} mol/l). It was also suppressed with LY294002 (2×10^{-5} mol/l). However, L-NAME (10^{-5} mol/l) did not significantly affect the AM-induced migration.* * $p<0.01$ vs. *controls (the group in which neither AM nor inhibitor was added),* † $p<0.01$ vs. *AM alone (the group in which AM (10^{-7} mol/l) was added in the lower chamber without inhibitors) (*n=*6).*

AM Promoted Migration of the Endothelium in a Chemotactic Manner

The migratory process contains two components: chemotaxis and chemokinesis. We therefore evaluated whether AM exerts a chemotactic or chemokinetic influence (or both) on ECs. The chemotactic property was determined by the migratory effect of AM (10^{-7} mol/l) when it was added to the lower chamber only, and the chemokinetic property was determined by adding AM to both the lower and upper chambers. As shown in Fig. 3, AM significantly promoted endothelial migration when it was added to the lower chamber only. This result indicates that AM-induced migration was chemotactic for cultured HUVECs.

AM-Induced Migration Was Mediated by the cAMP/PKA Pathway

As shown in Fig. 4, AM (10^{-7} mol/l in the lower chamber)-induced migration was blocked significantly by pretreatment with the two AM antagonists, AM(22–52) (10^{-5} mol/l) and CGRP(8–37) (10^{-5} mol/l). The migratory response induced by AM was also significantly suppressed by the PKA inhibitors, Rp-cAMP (5×10^{-6} mol/l) and PKI (5×10^{-6} mol/l). In addition, the inhibitor of PI3K, LY294002 (2×10^{-5} mol/l), significantly suppressed AM-induced migration of EC, but in contrast, L-NAME (10^{-5} mol/l) had no significant effect. The inhibitors at the doses used in the present study had no apparent effect on basal endothelial migration in the serum-free medium.

Discussion

In this study, we demonstrated that the proliferation and migration of HUVECs were augmented by AM in a dose-dependent manner, and that the AM antagonists (AM(22–52), CGRP(8–37)) blocked these effects. The AM-induced migration was predominantly chemotactic and was inhibited by the PKA antagonists (Rp-cAMP, PKI) and also the PI3K inhibitor (LY294002), but was not inhibited by the NOS antagonist (L-NAME).

In previous reports, the effect of AM on proliferation and migration varied according to the cell types and experimental conditions used. The dependency on the cAMP/PKA pathway of AM-induced biological effects on vascular cells was also different in each report. AM exerted an anti-proliferative effect on VSMCs explanted from rat aorta and incubated in medium with 5% serum (*20*). In this report, the inhibitory process was thought to be cAMP-dependent. AM was also reported to inhibit proliferation of rat glomerular mesangial cells (*27*) and rat cardiac fibroblasts (*28*) in a cAMP/PKA-dependent manner. On the other hand, in quiescent VSMCs prepared from rat aorta and made quiescent by incubation with serum-free medium for 2–3 days, AM was shown to stimulate DNA synthesis and cell proliferation with an increase in mitogen-activated protein kinase (MAP kinase) activity (*23*). In this case, the proliferative process was thought to be independent of the cAMP/PKA pathway. Furthermore, AM receptor antagonism with CGRP(8–37) inhibited rat carotid artery neointimal hyperplasia (*34*), which implies the significance of endogenous AM in balloon injury-induced proliferation of VSMCs, although the anti-proliferative effect could be caused by the blockade of the CGRP activity because CGRP(8–37) might antagonize both AM and CGRP receptors. With regard to migration of cultured VSMCs, it has been reported that AM inhibited the migration of VSMCs stimulated by 5–10% serum, PDGF, or

angiotensin II (*21*, *22*), and the inhibitory process was thought to be dependent on the cAMP/PKA pathway.

In the case of the vascular endothelium, reports have shown that AM had an anti-apoptotic effect on ECs incubated in 0.25% serum or in the absence of serum, and that this effect appeared to be mediated by a mechanism not related to the cAMP/PKA pathway (*29*, *35*). In this report, AM neither induced cell proliferation nor stimulated [^{3}H]thymidine incorporation of rat ECs in medium containing 0.25% serum (*29*). In another report, however, it was shown that AM promoted the proliferation of HUVECs cultured in medium containing 10% serum (*30*). With regard to the effect of AM on migration of ECs, there has been no previous report as far as we know.

In this study, we revealed that AM promoted the proliferation and migration of HUVECs incubated in medium with 0.5−2.0% serum and in the absence of serum, respectively. Furthermore, the migratory effect appeared to be dependent on the cAMP/PKA cascade. We previously reported that AM increased cAMP production and exerted an antiproliferative action on bovine aortic endothelial cells (BAECs) (*36*). In that report, we showed that exogenously administered AM inhibited PDGF-stimulated DNA synthesis in a dose-dependent manner and that neutralization of endogenously secreted AM by the monoclonal antibody against AM increased [^{3}H]thymidine uptake. The experimental conditions of that study differed from those of the present study in several points: the former study employed a higher concentration of serum, showed a potent stimulation of cellular proliferation by PDGF, and employed endothelial cells from different animal species (BAECs). Together with previous reports, these results lead us to assume that one of the critical factors which determine the effect of AM on proliferation of VSMCs and ECs is the serum concentration used in the experiments. In the present study, we tried to determine the effects of different serum concentrations (0.5% and 2.0%) on AM-induced proliferation, and we found that the higher concentration of serum (2.0%) reduced the proliferative effect of AM on ECs.

Recently, there has been accumulating evidence that the NO and NPs/cGMP/cGK pathways are significant for regulation of not only vascular tone but also vascular remodeling and postnatal angiogenesis (*37*). NO is also known to mediate vascular endothelial growth factor (VEGF)-induced proliferation of ECs and angiogenesis (*38*) by activating the cGMP/cGK pathway. As for AM-induced vasodilatation, it has already been reported that activation of the NO/cGMP pathway plays a significant role (*39*). However, it remains to be clarified how significant this pathway is for AM-induced cell proliferation and migration of VSMCs and ECs. It was previously shown that the anti-apoptotic effect of AM was mediated by NO, but this effect of NO was thought to be independent of the cGMP/cGK pathway (*35*). In this study, we tried to examine the involvement of NO in the migratory effect of AM on ECs. The administration of L-NAME, however, had no significant effect on the AM-induced migration. This result suggests that AM-induced migration is not mediated by the NO pathway.

Recent studies support the significance of the PI3K/Akt pathway in endothelial regeneration and postnatal angiogenesis (*40*). Furthermore, it has been shown that AM promoted Akt phosphorylation of rat aorta in a time- and dose-dependent manner and induced NO production and vasodilation (*41*). Accordingly, we here examined the involvement of the PI3K/Akt pathway in AM-induced migration of ECs. The PI3K inhibitor, LY294002, significantly suppressed AM-induced migration, which suggests involvement of the PI3K/Akt pathway in AM-induced migration of ECs. We have also confirmed the augmentation of Akt phosphorylation by administration of AM to HUVECs (data not shown). We are further investigating the relationship and molecular mechanism of Akt phosphorylation and cell migration induced by AM.

In summary, we revealed that the AM/cAMP/PKA cascade promotes the proliferation and migration of the cultured endothelium. Thus the AM/cAMP/PKA cascade or its downstream molecules might be useful as a therapeutic target to modulate vascular remodeling or support vascular regeneration.

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