Vascular Abnormalities and Elevated Blood Pressure in Mice Lacking Adrenomedullin Gene

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Background—Adrenomedullin (AM) is a vasodilating peptide involved in the regulation of circulatory homeostasis and in the pathophysiology of certain cardiovascular diseases. Levels of AM are markedly increased in the fetoplacental circulation during pregnancy, although its function there remains unknown. To clarify the physiological functions of AM, we chose a gene-targeting strategy in mice.

Methods and Results—Targeted null mutation of the AM gene is lethal in utero: the mortality rate among AM−/− embryos was >80% at E13.5. The most apparent abnormality in surviving AM−/− embryos at E13.5 to E14.0 was severe hemorrhage, readily observable under the skin and in visceral organs. Hemorrhage was not detectable at E12.5 to E13.0, although the yolk sac lacked well-developed vessels. Electron microscopic examination showed endothelial cells to be partially detached from the basement structure at E12.5 in vitelline vessels and hepatic capillaries, which allowed efflux of protoerythrocytes through the disrupted barrier. The basement membrane was not clearly recognizable in the aorta and cervical artery, and the endothelial cells stood out from the wall of the lumen, only partially adhering to the basement structure. AM+/− mice survived to adulthood but exhibited elevated blood pressures with diminished nitric oxide production.

Conclusions—AM is indispensable for the vascular morphogenesis during embryonic development and for postnatal regulation of blood pressure by stimulating nitric oxide production. (Circulation. 2001;104:1964-1971.)

Key Words: vasculature ■ endothelium ■ blood pressure

Adrenomedullin (AM) is a novel vasodilating peptide originally isolated from human pheochromocytoma.1 AM consists of 52 amino acid residues with a ring structure stabilized by a disulfide bridge and C-terminal amide and has partial structural homology with calcitonin gene-related peptide (CGRP). High levels of AM are found in the adrenal medulla, heart, lung, and kidney.2 In addition, AM has been shown to circulate in plasma,3 and circulating AM is secreted mainly from vascular cells.4 Apart from its vasodilatory effect, AM has diuretic properties,5 has bronchodilatory effects,6 inhibits the release of aldosterone and renin,6–10 Moreover, plasma AM is elevated by such pathological conditions as hypertension, renal failure, heart failure, and shock,11–13 although the precise meaning of the elevated AM production still remains to be clarified.

It was recently reported that plasma AM is elevated during pregnancy,14 that AM levels are elevated in the amniotic fluid and in fetal membranes,15 and that placental tissues express high levels of AM.16 In particular, AM is highly expressed in trophoblast giant cells.17 Still, the physiological functions of AM in the fetoplacental circulation remain unknown. Given the diversity of its effects, AM may also act as an autocrine and/or paracrine factor affecting fetoplacental function and morphogenesis during development.
To uncover the physiological roles played by AM, we chose a gene-targeting strategy in mice. Here, we show that targeted null mutation of the AM gene is lethal to mice in utero because of abnormalities of the vascular structure. Furthermore, adult heterozygotes exhibit elevated blood pressure with decreased nitric oxide (NO) production.

Methods

Generation of AM-Knockout Mice

A targeting vector was constructed to replace the 2.4-kb fragment encompassing the 1.3-kb 5′-flanking region, exons 1 to 3, and part of exon 4 of proadrenomedullin with the neomycin resistance gene (Figure 1a). The plasmid was linearized and then introduced into 129/Sv-derived SM-1 embryonic stem cells by electroporation. Homologous recombinants were identified by Southern blot analysis (Figure 1b), and 2 independently targeted clones were injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeras were crossbred with C57BL/6 females, and germ-line transmission was verified by Southern blot analysis (Figure 1c). All experiments were performed in accordance with the Declaration of Helsinki and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was prepared from embryonic day (E) 10.5 embryos by use of RNAzol (Biotex) and reverse-transcribed (RT). Polymerase chain reaction (PCR) was carried out on the resultant cDNA samples with primers 5′-CAATCAGAGCGAAGCCCACA-3′ (sense) and 5′-GGAGAGTATCAGCCTGTAAG-3′ (antisense).

Radioimmunoassays for Adrenomedullin

Tissue AM levels were measured by radioimmunoassay with an anti-AM antibody that recognized the C-terminal amide structure. The protocols for this assay were reported previously.

Exogenous Administration of AM

Pregnant female AM+/− mice crossbred with AM−/− males were given recombinant AM by use of a subcutaneous micro-osmotic pump.
Histological Examination
Whole E12.5 to E13.5 embryos, along with the yolk sac and placenta, were fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and cut into 4-μm sections. The sections were stained with hematoxylin-eosin.

Whole-Mount Immunostaining
Immunohistochemical staining with anti-platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Mec13.3, PharMingen) was performed, essentially as described by Sato et al. Briefly, embryos were fixed with 4% paraformaldehyde. The fixed embryos were treated with 5% H2O2, rinsed with PBS, and incubated overnight in PBS containing 2% goat serum. Embryos were then incubated with Mec13.3 overnight, rinsed, incubated with goat anti-rat IgG horse-radish peroxidase, and subjected to color development.

Transmission Electron Microscopy
Specimens were fixed with 2.5% glutaraldehyde and 4% osmium tetroxide, embedded in epoxy resin (Epok) 812 (Oken Shoji Co), cut into ultrathin sections, double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 electron microscope.

Vascular Casting and Scanning Electron Microscopy
The method for making vascular corrosion casts of mouse embryos was described by Kondo et al. Casts were cut into small pieces suitable for observation, gold-coated with an ion sputter, and examined under the skin (Figure 2, a and b) and in the lung and liver (Figure 2, c through h). Hemorrhage was not yet detectable at E12.5 to E13.0, although the embryos showed abnormal vitelline vessels on the yolk sac (Figure 3, a and b); histological examination showed only the presence of poorly developed vitelline vessels (Figure 3, c and d). The umbilical artery was abnormally constricted (Figure 3, e and f). These embryos also exhibited accumulation of pericardial effusion (Figure 3, g through j). The placental weight at E13.0 was significantly lower in AM−/− than wild-type embryos (0.057 ± 0.005 g [n = 7] versus 0.073 ± 0.005 g [n = 10], P < 0.05). In AM−/−, the fetal side of the placenta had an ischemic appearance (Figure 3, k and l), and the number of embryonic protoerythrocytes was reduced in the capillaries, suggesting poor chorionic circulation (Figure 3, m and n).

Statistical Analysis
Values were expressed as mean ± SEM. Student’s t test was used to determine significant differences between means. Values of P < 0.05 were considered significant.

Results
Generation of AM-Null Mice
AM−/− mice, in which AM levels in the heart and lung were reduced to $\approx$50% of those of wild-type mice (Figure 1d), were normal in appearance. No AM−/− newborns were obtained from the AM+/− intercrosses, however, indicating that the AM null mutation was lethal in utero. Southern blot (Figure 1c) and RT-PCR (Figure 1e) analyses confirmed the AM−/− genotype and the lack of AM gene expression in AM−/− embryos, respectively.

Mortality of AM−/− Embryos
We analyzed embryos from timed AM−/− intercrosses. The mortality rate among AM−/− embryos at E13.5 was 83% (20 deaths among 24 homozygotic embryos), and none survived to E14.5 (Table). Administration of recombinant AM to crossbred AM−/− females decreased the mortality rate among AM−/− embryos at E13.5 to 21.4% and increased the survival period to E14.5, confirming that the deaths of the AM−/− embryos were caused by AM deficiency.

Macroscopic and Histological Observation of AM−/− Embryos
The most apparent abnormality in AM−/− embryos at E13.5 to E14.0 was severe hemorrhage, which was observable under the skin (Figure 2, a and b) and in the lung and liver (Figure 2, c through h). Hemorrhage was not yet detectable at E12.5 to E13.0, although the embryos showed abnormal vitelline vessels on the yolk sac (Figure 3, a and b); histological examination showed only the presence of poorly developed vitelline vessels (Figure 3, c and d). The umbilical artery was abnormally constricted (Figure 3, e and f). These embryos also exhibited accumulation of pericardial effusion (Figure 3, g through j). The placental weight at E13.0 was significantly lower in AM−/− than wild-type embryos (0.057 ± 0.005 g [n = 7] versus 0.073 ± 0.005 g [n = 10], P < 0.05). In AM−/−, the fetal side of the placenta had an ischemic appearance (Figure 3, k and l), and the number of embryonic protoerythrocytes was reduced in the capillaries, suggesting poor chorionic circulation (Figure 3, m and n).

Abnormalities of Vasculature in AM−/− Embryos
We examined the formation of the vasculature in AM−/− embryos. No abnormalities were observed in whole-mount E10.5 specimens immunostained for PECAM-1, a marker of vascular endothelial cells (Figure 4, a and b). Electron microscopic examination, however, revealed that at E12.5, before hemorrhagic changes were detectable macroscopically, endothelial cells had partially detached from the basement structure in vitelline vessels (Figure 4, c and d) and in hepatic capillaries (Figure 4, e and f), allowing efflux of protoerythrocytes through the disrupted barrier (Figure 4, e and f).

The endothelial cells in AM−/− embryos were cuboid, rather than flat as in wild-type, and thus stood out from the wall of the lumen, leaving gaps where they were not adhering to the

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<th>Genotype of Embryos From AM−/− Intercrosses</th>
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(Alzet, model 1007D), which infused AM-containing solution (27 μg/mL) at a rate of 0.52 μL/h for 1 week (E7 to E14).
basement membrane (Figure 4, g and h). This change in endothelial cell shape reflected abnormalities in structure of the underlying basement membrane. The 3-layer structure of the basal membrane formed by the lamina rara interna, lamina densa, and lamina rara externa was not clearly recognizable in AM/H11002/H11002 embryos (Figure 4, i and j). In addition, abnormal cytoplasmic projections of the endothelial cells into the basement structure were observed. Despite the abnormal and incomplete adhesion of endothelial cells to the basement structure, tight junctions between endothelial cells remained intact in AM/H11002/H11002 embryos (Figure 4j).

Scanning Electron Microscopy of the Corrosion Casts
Scanning electron microscopic observation of the corrosion casts of the AM/H11002/H11002 embryonic hepatic vasculature showed a complex mesh-like network of fused vessels, which may be the product of diffuse shunt formation among the vessels (Figure 5, a and b). The usual coral-like appearance of the vessel structure was therefore not apparent. In addition, the casts of the aorta and cervical artery showed numerous holes (Figure 5, c and d), which most likely correspond to protruding or detaching endothelial cells.

Vasoregulatory Function of AM
We measured arterial blood pressures in conscious, unrestrained AM/H11002/H11002 mice and found them to be significantly higher than in their wild-type littermates (mean blood pressure 128.4±2.2 versus 118.7±2.4 mm Hg, P<0.01, n=20 each) (Figure 6a). When L-NMMA, an NO synthase inhibitor, was administered to determine whether this elevation in blood pressure was related to diminished AM-induced NO production,23 we found that it elicited a rise in blood pressure that was significantly smaller in AM/H11002/H11002 mice than in wild-type mice (18.5±2.0 versus 27.7±2.5 mm Hg, P<0.01, n=10 each) (Figure 6b). Moreover, direct estimation of NO concentrations in perfusate from isolated kidneys revealed that basal NO production in AM/H11002/H11002 mice was significantly lower than in wild-type mice (132.7±14.2 versus 275.8±20.6 fmol · min⁻¹ · g⁻¹, P<0.01, n=7 each) (Figure 6c).

Discussion
Although a number of studies have implicated AM in circulatory regulation and fetal growth, there is as yet no direct evidence of its function. Here, we showed that targeted null mutation of the AM gene is lethal to mice in utero because of disruption of the vasculature, with corresponding intraorgan and subcutaneous hemorrhage and circulatory collapse. Furthermore, adult heterozygotes exhibit increased blood pressure with decreased NO production. These observations clarify 2 aspects of the physiological roles of AM: before birth, AM is indispensable to the vascular morphogen-
esis; after birth, AM exerts an effect on blood pressure via an NO-dependent pathway.

The development of endothelial and smooth muscle cells themselves seemed to proceed normally in $AM^{-/-}$ embryos: no clear abnormality was detected in the vascular structure before E10.5, and immunohistochemical analysis showed PECAM-1 and smooth muscle α-actin to be unaffected before E10.5. Conversely, hemorrhage was the outstanding feature of $AM^{-/-}$ embryos at later stages of development, which may suggest the fragility of vascular structure once it was formed. Caron and Smithies\textsuperscript{24} reported that $AM^{-/-}$ embryos showed extreme hydrops fetalis. Typical hydrops fetalis was sometimes detected in our mice, but the severity was different between embryos. Many $AM^{-/-}$ embryos showed hemorrhage without severe edema (see Figure 2b).

We speculated that increased permeability of the vasculature was the primary cause of both edema and hemorrhage. To uncover the cause of hemorrhage, we carried out a detailed electron microscopic examination of $AM^{-/-}$ embryos, which revealed an abnormal basement membrane structure. The

**Figure 3.** Appearance of E13.0 wild-type (a, c, e, g, i, k, m) and $AM^{-/-}$ (b, d, f, h, j, l, n) embryos. Well-developed vitelline vessels were detected on yolk sac of wild-type (a) but were poorly developed in $AM^{-/-}$ (b) embryos; yolk sac sections also showed poorly developed vitelline vessels in $AM^{-/-}$ embryos (d). Umbilical artery and vein of $AM^{-/-}$ embryos showed abnormal constriction (f), and pericardial space was dilated (h, j). Fetal side of placenta showed ischemic appearance in $AM^{-/-}$ embryos (l). Sections of placenta showed reduced numbers of embryonic protoerythrocytes (n). c, d, m, n, Bars=100 μm; i, j, 200 μm.
3-layer structure of basal membrane was not clearly recognizable in major vessels. Moreover, fibrous structures were detected in the basement portion, most of which consisted of amorphic depositions. These abnormalities most likely disrupt the stable adhesion of endothelial cells to the basement membrane. Normal basement membrane contains such N-glycosylated protein constituents as fibronectin, vitronectin, laminin, nidogen, and type IV collagen. Because AM is reported to be involved in the regulation of the synthesis of the extracellular matrix,25,26 one explanation for the disrupted basement structure is that AM deficiency leads to abnormal production and incorporation of these molecules.

Another abnormality of the vasculature was found in the vitelline vessels on the yolk sacs of AM−/− embryos at E12.5. Because smaller yolk sac vessels appear first and then combine to form larger vessels in normal development, poorly developed vitelline vessels may be a marker of impaired vascular remodeling.27 In AM−/− embryos, improper interaction among blood vessels once they are formed may thus result in aberrant vascular remodeling. Electron microscopic examination also revealed detachment of endothelial cells from the basement structure in vitelline vessels. Accumulating evidence indicates that cell-matrix adhesive interactions are integral elements of vascular development; indeed, the basement membrane has been shown to be crucially involved in cell proliferation, migration, and differentiation.28,29 Moreover, differentiation of endothelial cells into capillary-like structures is markedly accelerated by culture on a reconstituted gel composed of basement membrane proteins,30 suggesting that the abnormal formation of the basement structure may also explain the abnormal vascular remodeling seen in the yolk sac. Exogenous administration of AM improved the appearance of the yolk sac vessels and reduced the death rate at least in E13.5 embryos. Nevertheless, AM−/− embryos could not survive beyond E15.0; finally, severe hemorrhage occurred. One possibility is that paracrine or autocrine functions of AM may be more essential for normal vascular development and exogenous administration might not be sufficient to replace the AM deficiency. The present findings demonstrate that AM is an essential factor in vascular development and that characterization of the vascular defects in AM−/− mice presents an opportunity to further investigate the controlling mechanisms in vascular development.

Figure 4. Vascular structure of wild-type (a, c, e, g, i) and AM−/− (b, d, f, h, j) embryos. a and b, Whole-mount immunostaining of PECAM-1 in E10.5 wild-type (a) and AM−/− (b) embryos showed no abnormalities. c through j, Transmission electron micrographs of endothelial cells in vitelline vessels (c, d), hepatic capillaries (e, f), and cervical arteries (g through j) from E12.5 wild-type (c, e, g, i) and AM−/− (d, f, h, j) embryos. Endothelial cells in AM−/− mice were often detached from basement structure (d, arrows), allowing efflux of protoerythrocytes through disrupted barrier (f, arrow). E indicates endothelial cell; H, hepatocyte. In cervical arteries of AM−/− embryos, endothelial cells stood out from wall of lumen, leaving partial adhesion to basement (h). Endothelial cells of AM−/− embryos were cuboid (h), whereas those of wild-type embryos were flat (g). High-density band representing lamina densa was detected in wild-type (i, arrowheads) but was unclear in AM−/− embryos (j, *). Tight junctions between endothelial cells were intact in AM−/− (j, arrowhead). c through j, Bars = 2 μm.
A complex mesh-like network of fused vessels was observed in livers of AM−/− embryos (b). Numerous holes were detected on surfaces of casts of cervical arteries from AM−/− embryos (d). a through d, Bars=100 μm.

Figure 5. Scanning electron micrographs of corrosion casts of vasculature from wild-type (a, c) and AM−/− (b, d) embryos. A complex mesh-like network of fused vessels was observed in livers of AM−/− embryos (b). Numerous holes were detected on surfaces of casts of cervical arteries from AM−/− embryos (d).

Unlike the homozygotes, heterozygotes survived until adulthood. One notable phenotypic characteristic of AM−/− adult mice was their comparatively high blood pressure. In our earlier study, transgenic mice overexpressing AM exhibited lower blood pressures than wild-type mice. In that context, our present finding confirms that steady-state AM can regulate blood pressure.

AM probably acts to modulate NO production, thereby regulating vascular tone. Blockade of NO synthase with L-NMMA elevated blood pressure to a smaller degree in AM−/− mice, suggesting that diminished NO synthesis is partially responsible for the elevated blood pressure in AM−/− mice. In fact, direct measurement of NO concentrations in perfusate from isolated kidneys of AM−/− and wild-type mice confirmed the changes in NO production in the former.

From a clinical viewpoint, our results raise a very important issue. It was recently confirmed that AM is markedly increased in the fetoplacental circulation. Although its physiological significance is still unclear, the administration of AM decreases pup mortality in the gestosis model, and physiological significance is still unclear, the administration of AM decreases pup mortality in the gestosis model, and we hope, allows development of new approaches to the treatment for such ailments.

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References


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