Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene

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Adrenomedullin, a recently identified potent vasodilator, is expressed widely and has been suggested to have functions ranging from reproduction to blood pressure regulation. To elucidate these functions and define more precisely sites of Adm expression, we replaced the coding region of the Adm gene in mice with a sequence encoding enhanced green fluorescent protein while leaving the Adm promoter intact. We find that Adm 

embryos die at midgestation with extreme hydrops fetalis and cardiovascular abnormalities, including overdeveloped ventricular trabeculae and underdeveloped arterial walls. These data suggest that the absence of Adm may be one cause of nonimmune hydrops fetalis in humans.

Materials and Methods

Gene Disruption by Homologous Recombination. Mice lacking Adm were generated with standard gene targeting methods (4). The targeting construct was made (Fig. 1a) with (i) a 3.7-kb BamHI/SpeI genomic fragment that includes the Adm promoter; (ii) a 600-bp PCR-generated fragment that begins 5' with the endogenous SpeI site, contains exon 1, intron 1, part of exon 2, and ends 3' at the endogenous initiator methionine; (iii) a 700-bp cDNA encoding EGFP (CLONTECH); (iv) a 300-bp bovine growth hormone poly(A) addition region; and (v) a 1.3-kb XhoI/Sacl genomic fragment from downstream of the Adm gene. 129-derived SvEv embryonic stem cells (TC-1, a gift from Phil Leder, Harvard University, Boston, MA) were electroporated: G418/ganciclovir-resistant colonies, initially identified by the PCR-based assay depicted in Fig. 1 a and c, were expanded; and homologous recombination was confirmed by Southern blot analysis with the use of an 800-bp SacI/AvrII probe external to the targeting construct sequence (Fig. 1 a and b). Male chimeras were mated to wild-type SvEv females to establish an isogenic Adm line, and all experiments were conducted on the resulting isogenic line.

To generate Adm 

embryos, timed heterozygous matings were set up, and the morning of vaginal plug detection was considered to be embryonic day 0.5 (E0.5). To genotype the embryos, DNA was isolated from yolk sac tissue (E8.5–E12.5) or from a small piece of tail tissue (E12.5–E14.5) and analyzed by the PCR-based strategy described above.

Expression Analysis by Using Real-Time Quantitative Reverse Transcription–PCR. Expression of the Adm gene was characterized by real-time quantitative reverse transcription–PCR with the Sequence detection system (Perkin–Elmer). Primers for Adm amplification were 5'-GAGCGGAGGCCCACTTCTG-3' and 5'-GAAGGGCATCCATTTGCT-3'. The probe for Adm detection was 5'-FAM-CTACCGCCAGAGCATGAACCAGGG-Tamra-3'. All reactions included a β-actin internal standard. The primers used for β-actin amplification were 5'-CTGCTCGA-CGCGCAAGTC-3' and 5'-CAAGAAGGAAAGGCTG-GAAAAGA-3'. The probe for β-actin detection was 5'-TET-

Abbreviations: Adm, Adrenomedullin; EGFP, enhanced green fluorescent protein; CGRP, calcitonin gene-related peptide; En, embryonic day n; H&E, hematoxylin/eosin.

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CACTATTGGCAACGAGCGTTCGG-Tamra-3'. RNA was isolated from E9.5 embryos with the use of the TRIzol Reagent (GIBCO/BRL). The reactions were performed with 5 μg total RNA with minor differences from ABI 7700 manufacturer’s instructions. Relative levels of Adm expression were determined by the ΔΔCt method and expressed as a percentage of wild type (Fig. 1d). All assays were repeated twice, each with duplicates.

Expression Analysis by Using GFP Fluorescence. Adult or embryonic heterozygote animals were euthanized, and organs or whole embryos were fixed in 4% paraformaldehyde. Tissues were embedded in Tissue-Tek OCT media (Sakura Finetek, Torrance, CA), and 8-μm frozen sections were analyzed under fluorescent microscopy. The slides were then stained with hematoxylin/eosin (H&E) to histologically confirm sites of expression. To visualize Adm expression during development, heterozygote embryos were fixed in 4% paraformaldehyde and analyzed by fluorescent confocal microscopy.

Histological Analysis. Embryos, dissected from the uterus at the desired gestational times, were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five-micrometer-thick sections were mounted on slides and stained with H&E.

Results

Targeted disruption of the Adm gene replaced the protein-coding region of the gene with a cDNA coding for EGFP but left the 5′ regions of the gene intact, so that EGFP expression is Adm specific (Fig. 1a). The disrupted allele was detected by Southern blot analysis (Fig. 1b) and by PCR (Fig. 1c). To confirm that the targeting inactivated the Adm gene, quantitative reverse transcription–PCR was performed on embryonic RNA, with the use of an Adm TaqMan probe and the ABI7700 Sequence Detection system. Adm-/- embryos express Adm mRNA at levels indistinguishable from zero, thus confirming complete loss of Adm production; heterozygotes have half-normal expression (Fig. 1d).

Mice heterozygous for Adm showed no overt differences from wild-type mice and were used to determine the pattern of Adm expression by observing fluorescence from its surrogate, EGFP. As expected from previous studies (5, 6), the adult adrenal medulla showed strong fluorescence, but no fluorescence was detected in the zona fasciculata/reticularis of the adrenal cortex (Fig. 2a). Confocal microscopy of E9.5 Adm+/-/ embryos showed moderate expression in the heart and strong expression in the developing vasculature (Fig. 2b). E13.5 Adm+/-/ embryos express Adm in the cardiac myocytes of the developing ventricle (Fig. 2c). The vascular smooth muscle cells of the aorta and esophagus and the endothelial cells of the aorta and thoracic duct strongly express Adm (Fig. 2d).
Mice homozygous for the targeted allele die in utero, as shown by our finding that among the progeny of Adm<sup>+/−</sup> × Adm<sup>+/−</sup> matings 32% were Adm<sup>+/+</sup>, 68% were Adm<sup>+/−</sup>, but none were Adm<sup>−/−</sup>. Timed matings between Adm<sup>+/−</sup> mice showed that at E12.5 the Adm<sup>−/−</sup> embryos are indistinguishable from their Adm<sup>+/−</sup> and Adm<sup>+/+</sup> littermates. However, the Adm<sup>−/−</sup> embryos die between E13.5 and E14.5, as evidenced by increased numbers of resorbing Adm<sup>−/−</sup> embryos (Table 1). Early in this period, the yolk sac cavity becomes distended (Fig. 3a), and Adm<sup>−/−</sup> embryos exhibit modest general edema with a notable increase in the size of their thoracic cavities relative to control littermates (Fig. 3b). By E14.5, Adm<sup>−/−</sup> embryos suffer from extreme hydrops fetalis (Fig. 3c) that encompasses the entire body and neural structures (Fig. 3d). Note that the yolk sacs have been removed from the embryos illustrated in Fig. 3 b−d. Histological examination of the Adm<sup>−/−</sup> embryos (Fig. 3f) confirms the enlargement of their thoracic cavities and their extreme and generalized hydrops compared with control littermates (Fig. 3e).

Edema in mice embryos has previously been described in association with cardiovascular defects, although we find no reports of edema of this severity (7, 8). We therefore examined the condition of the heart in transverse sections of E13.5 and E14.5 Adm<sup>−/−</sup> embryos. Fig. 4 illustrates that the E13.5 Adm<sup>−/−</sup> heart (Fig. 4d) is typically only two-thirds the size of the wild-type heart (Fig. 4a) but shows signs of increased left ventricular trabecular development leading to smaller chamber size. By E14.5, this pathological overdevelopment becomes more marked (Fig. 4b and e): the compact zone appears thin and convoluted, the number of trabeculae is increased, and the myocardium has a generally disorganized appearance (Fig. 4c and f). The mitral and tricuspid valves, intraventricular septum, and atria of Adm<sup>−/−</sup> embryos show no obvious abnormalities at any time.

The aorta and carotid artery of the Adm<sup>−/−</sup> embryos are also abnormal (Fig. 5 c and d); they have markedly thinner walls and are irregular in shape when compared with wild type (Fig. 5a and b). However, we observed no significant differences in the external appearance of the smaller vessels of the paws and tail (Fig. 2b and d). The thinner major arteries and the cardiac pathology of the Adm<sup>−/−</sup> embryos demonstrate that cardiovascular development is abnormal in the absence of Adm.

**Discussion**

Because our Adm<sup>−/−</sup> animals express EGFP in place of Adm, we comment on a recent report that extremely high levels of a GFP transgene expressed in the heart of adult mice causes dilated cardiomyopathy (9). For several reasons, it is highly unlikely that the abnormal trabecular development in the ventricles of our Adm<sup>−/−</sup> embryos is caused by EGFP toxicity. First, the level of EGFP reported to be toxic appears much greater than in our mice, as judged by fluorescence. Second, our phenotype manifests itself over 24−48 h during embryonic development, as opposed to a several-month progression in the adult transgenic mice. Third, other investigators have previously shown that GFP is nontoxic in mice (10−12), and that moderately expressed cardiac-specific GFP transgenes do not cause adverse effects during embryogenesis or adulthood (13, 14).

Nonimmune hydrops fetalis has an incidence of 1:3,000 births and a high fetal mortality rate. One-quarter of these cases are associated with cardiovascular defects such as arrhythmias and malformations. Fifteen percent are of unknown etiology. Here

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**Table 1. Genotype of embryos from Adm<sup>+/−</sup> × Adm<sup>+/−</sup> matings**

<table>
<thead>
<tr>
<th>Age</th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
<th>Total</th>
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<td>53</td>
<td>23</td>
<td>113</td>
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<td>11</td>
<td>15</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
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<td>9</td>
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<td>20</td>
</tr>
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<td>14</td>
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<tr>
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*Dying or resorbed embryos.*
we describe a type of nonimmune hydrops fetalis that is associated with cardiovascular defects and is caused by a single gene defect. Cardiovascular defects have been reported in mice having a wide variety of disrupted genes [RXR α (8, 15), α 4 integrin (16), PDGF α (17), BARK 1 (18), Hand-1 (19, 20), PPAR γ (21), calreticulin (22), jumonji (23), etc.] likely reflecting the many ways in which cardiac anomalies can be induced. However, the consequences of these gene deficiencies differ in two fundamental ways in which cardiac anomalies can be induced. However, the consequences of these gene deficiencies differ in two fundamental ways. First, any observed edema tends to be mild, localized, and subcutaneous. Second, the heart defects are generally characterized as involving hypoplasia indicative of impaired myocardial growth and subsequent ventricular dilation. In contrast, Adm−/− hearts show evidence of trabecular hyperplasia that can progress sufficiently to cause ventricular occlusion. Adm, secreted by neonatal rat cardiomyocytes, inhibits protein synthesis and collagen production when it is added to cultured cardiomyocytes (24) or cardiac fibroblasts (25). In line with these findings, our observations suggest that absence of Adm in the developing heart leads to abnormally enhanced growth. However, in apparent contrast, our studies also show that the absence of Adm results in less than normal vascular smooth muscle cell development in large arteries, which is unlikely to be secondary to low blood pressure caused by a poorly functioning heart because it does not occur in other mice with cardiac failure.

The extreme hydrops of Adm−/− embryos requires comment. For the above reasons, it is unlikely to be solely because of cardiac or vascular defects. Likewise, it is unlikely that abnormal vascular permeability could account for the hydrops, because gene disruption models with vascular permeability defects do not show the same degree of edema (26–28). Abnormalities in lymphatic vessel development could theoretically cause the severe edema, and lymphatic vessels sprout from veins around E12.5 in mice, which is the time of the hydrops onset in our mutants. Furthermore, we find that Adm is expressed in the thoracic duct of Adm−/− embryos (Fig. 2d). However, current data are not yet sufficient to allow a chain of events to be specified that leads to the hydrops of Adm−/− embryos. Nevertheless, our overall data suggest that a genetically determined absence of Adm could be one cause of nonimmune hydrops fetalis in humans.

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