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Expression of Adrenomedullin, a Hypotensive Peptide, in the Trophoblast Giant Cells at the Embryo Implantation Site in Mouse

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Adrenomedullin (AM) is a newly discovered hypotensive peptide which is believed to play an important role for blood pressure control in the adult. Although it has been well established that a major production site of AM is vascular endothelial cells, we now show that AM is most highly expressed in trophoblast giant cells, which are derived from the conceptus and are directly in contact with maternal tissues at the implantation site. Northern blot and *in situ* hybridization analyses show that the AM mRNA begins to be detected just after implantation and its level peaks at 9.5 days postconception (d.p.c.) in those cells. Expression then falls dramatically after 10.5 d.p.c., coincident with the completion of the mature chorioallantoic placenta. Immunohistochemical analyses show that the AM peptide is secreted from the trophoblast giant cells into the surrounding tissues, i.e., embryo, decidua, and maternal circulation. In contrast, the expression of an AM receptor was not detected by Northern blot analyses in either embryo or trophoblast giant cells at 7 d.p.c., when the AM gene is most highly expressed in the trophoblast giant cells. This suggests that the AM produced and secreted from the embryo's trophoblast giant cells acts on the maternal tissues rather than on the embryonic tissues. Based on these results, we propose that the high production of AM may be the mechanism by which the embryos survive at the early postimplantation period by pooling maternal blood in the implantation site in order to secure nutrition and oxygen before the establishment of efficient embryo-maternal circulation through the mature placenta.

Key Words: **adrenomedullin; mouse; implantation; gene expression; trophoblast giant cells.**

INTRODUCTION

The rapidly growing mammalian embryo must interface with its mother for nourishment and respiration. The failure to establish this critical contact results in the loss of the embryo *in utero* (Copp, 1995; Cross *et al.*, 1994). For survival through the peri-implantation stage, one of the key players is the trophoblast giant cell. These cells are derived from the conceptus and are directly in contact with maternal tissues at the implantation site (Hoffman and Wooding, 1993; Rossant, 1986). To understand the functions of trophoblast cells as well as other cells at the implantation site, we are constructing a catalog of genes expressed in those cells by sequencing randomly chosen cDNA clones made from the ectoplacental cone (EPC) of 7.5 days postconception (d.p.c.) mouse embryos. While working on this sequencing project, we encountered cDNA clones which were clearly identified as the mouse adrenomedullin (AM) gene.

AM is a hypotensive peptide that reduces blood pressure without changing the heart rate (Kitamura *et al.*, 1993a,b; Richards *et al.*, 1996; Sakata *et al.*, 1993). The human AM peptide, consisting of 52 amino acids, was originally isolated from an adrenal pheochromocytoma (Kitamura *et al.*, 1993a). The AM peptide is processed from a precursor which is 185 amino acids in length and shows a slight homology to the calcitonin gene related peptide (CGRP). The AM gene is expressed in a wide variety of tissues, such as the adrenal gland, lung, kidney, and heart (Ichiki *et al.*, 1994; Kitamura *et al.*, 1993b; Sakata *et al.*, 1993, 1994). The highest expression of the gene has been observed in vascular endothelial cells, which secrete the peptide (Sugo *et al.*, 1994); this has suggested paracrine and/or autocrine control of blood pressure by AM (Ishizaka *et al.*, 1994; Richards *et*

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al., 1996). In fact, concentrations of the AM peptide in human plasma are elevated in patients with essential hypertension, congestive heart failure, and renal failure (Ishimitsu *et al.*, 1994b; Jougasaki *et al.*, 1995b). Also, the level of AM mRNA is significantly induced in ischemic brain cortex (Wang *et al.*, 1995). These findings suggest that AM plays important physiological and pathophysiological roles in cardiovascular systems.

It has been reported recently that the AM peptide plays more diversified roles such as natriuretic (Jougasaki *et al.*, 1995a) and bronchodilatory functions (Kanazawa *et al.*, 1994) and regulation of insulin (Martinez *et al.*, 1996) and pituitary adrenocorticotropin secretion (Samson *et al.*, 1995). In addition, the involvement of AM in the regulation of cellular growth (Withers *et al.*, 1996) and elevated expression of the AM gene in tumor cell lines (Miller *et al.*, 1996) have been reported.

We were intrigued by the unexpected expression of the AM gene at the peri-implantation stage and decided to examine this in more detail. Here we report the cloning, sequencing, genetic mapping, and expression analyses of the mouse AM and its receptor genes. We show expression of the mouse AM gene in the trophoblast giant cells during the peri-implantation period by *in situ* hybridization, and we discuss the new role of trophoblast giant cells in controlling blood flow at the implantation site.

MATERIALS AND METHODS

Cloning and sequencing. Construction of the cDNA library and the associated large-scale cDNA sequencing project will be described elsewhere. The longest cDNA clone (C0010H07) was selected for this analysis from the eight cDNA clones showing strong similarity to the human AM gene. Cycle sequencing reactions were carried out using *Taq* DyeDeoxy terminators (Applied Biosystems), and the samples were run on a Model 377 DNA sequencer (Applied Biosystems). A total 1381-bp cDNA sequence was obtained and deposited to the GenBank public sequence database (Accession No. U77630). The sequence revealed $(GT)_{20}$ repeats in the 3′-UTR. To exclude this repeat sequence for Southern, Northern, and *in situ* hybridization analyses, a 684-bp DNA fragment was amplified by a PCR primer pair (AM5-1, 5′-CGCTGATGAGACGACAGTTC-3′; AM3-1, 5′-CATTGCACGTTCCTCGCTAGG-3′), cloned into pCR-ScriptSK(+) (Stratagene), and named H0201A08. Sequence similarity searches were performed by the BLAST program against the nonredundant sequence database in the NCBI (NIH) (Altschul *et al.*, 1990). The amino acid sequence was deduced from the nucleotide sequence and analyzed by Genetyx-Mac 7.3 program (Software Development Co.).

Whole-mount and section in situ hybridization. Both sense and antisense single-stranded cRNA probes were synthesized from the linearized plasmid (clone H0201A08) in the presence of digoxigenin–UTP (Boehringer-Mannheim). Whole-mount *in situ* hybridization was performed according to a published protocol (Wilkinson, 1992). *In situ* hybridization of tissue sections was carried out as described (Yotsumoto *et al.*, 1996) with some modifications, i.e., hybridization and washing were done at 68°C.

Immunohistochemistry. Immunohistochemical staining was done as described. Briefly, sections were incubated with rabbit anti-rat AM 1-50 serum (Peninsula Laboratories, Inc.) at 4°C overnight. The serum was diluted 1:800 in PBS before use. Bound polyclonal antibody was detected using the LSAB kit (DAKO Corp.) using aminoethylcarbazole as a peroxidase substrate (red color in the photographs). Control staining with either PBS or nonimmune rabbit serum (DAKO Corp.) did not show any signals. To clearly identify the trophoblast giant cells at the implantation site, some of the serial sections were stained with rabbit anti-human keratin serum (DAKO Corp.) according to the manufacturer's instructions. The sections were counterstained with Meyer's hematoxylin.

Immunoblot analysis. The fresh embryo and placenta obtained from a 12.5-day p.c. mouse were immediately minced and homogenized in RIPA buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml of PMSF, and 0.27 TIU/ml of aprotinin) in a conical glass homogenizer and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant fractions were subjected to SDS–PAGE with a 17.5% gel, transferred to Immobilon (Millipore), and allowed to react with rabbit anti-rat AM 1-50 serum (Peninsula Laboratories, Inc.) diluted at 1:800 for overnight at 4°C. As a negative control for the first antibody we used a normal rabbit IgG (Sigma) diluted at 1:800. The secondary antibody was a peroxidase-linked donkey anti-rabbit IgG (Amersham) diluted at 1:1000, which was detected by H_2O_2 and diaminobenzidine.

Northern blot analysis. Total RNAs were isolated from decidua and extraembryonic tissue with the embryo proper at 6.5 and 7.5 d.p.c.; decidua and extraembryonic tissue without the embryo proper at 8.5, 9.5, and 10.5 d.p.c.; yolk sac and placenta at 12.5, 14.5, 16.5, and 18.5 d.p.c.; and adult adrenal glands and lungs by Micro RNA Isolation kit (Stratagene). Poly(A)$^+$ RNA for Northern analysis was isolated from the total RNAs with an Oligotex mRNA Midi kit (Qiagen). Four micrograms of poly(A)$^+$ RNA [2 μg of poly(A)$^+$ RNA for lung] was electrophoresed on a 1.2% agarose gel containing 6.3% formaldehyde. RNAs were transferred to Hybond N+ membrane (Amersham) using 20× SSC and the membrane was hybridized for 1 h with an ExpressHyb hybridization solution (Clontech) first to AM, then rehybridized to proliferin (PLF; clone C0002G11), and finally rehybridized to β-actin (Clontech) probes. The filter was washed with solution 1 (2× SSC, 0.05% SDS) at room temperature for 40 min and then washed with solution 2 (0.1× SSC, 0.1% SDS) at 65°C for 40 min. The filter was then exposed to phosphor screens to allow subsequent quantification and then to autoradiographic film (Kodak X-OMAT AR). The phosphor screens were scanned on a Storm 860 (Molecular Dynamics) and individual bands were quantified using ImageQuant software. After each hybridization, filters were washed in boiled 0.5% SDS solution for 10 min to remove the labeled probe. Membranes for staged mouse embryos and adult organs were purchased from Clontech. Hybridization was performed according to the manufacturer's instructions.

Mapping. Gene mapping by polymerase chain reaction (PCR) was performed as described (Ko *et al.*, 1994). For AM gene mapping, the PCR primer pair used for genotyping was AM-up2, 5′-CGTGAATGTCTCAGCAAGGTG-3′; and AM-dn1, 5′-CGATAATCAGGCGCTCTCCAC-3′. The length of polymorphism which distinguishes the PCR product (102 bp) of the C57BL/6J allele from that (106 bp) of the *Mus spretus* allele was used to type The Jackson Laboratory BSS Backcross DNA Panel [(C57BL/6JEi × SPRET/Ei) F1 × SPRET/Ei] (Rowe *et al.*, 1994). The polymorphism was due to the $(GT)_n$ repeats in the 3′-UTR of the AM cDNA.

For AM-R gene mapping, a primer pair for PCR was designed

from the 3′-end of the mouse expressed sequence tag (EST) (W75568). Primer sequences are AMR-up2, 5′-TCCATGCTG-CACTGTGTGGC-3′; and AMR-dn1, 5′-CGATTC-CCAACCCCACACGC-3′. The annealing temperature for PCR was 68°C and the size of the PCR product is 526 bp. The PCR products amplified from the genomic DNAs of C57BL/6J and *M. spretus*, respectively, were sequenced by an ABI 377 DNA sequencer. The sequence of the PCR product from *M. spretus* genomic DNAs was deposited in GenBank (Accession No. AF001229). The sequence comparison revealed a restriction fragment length polymorphism (RFLP) at an *Ava*II site; the C57BL/6J allele produced two fragments (81 and 445 bp) and the *M. spretus* allele produced one 526-bp fragment. The RFLP was used to type The Jackson Laboratory BSS Backcross DNA Panel [(C57BL/6JEi × SPRET/Ei) F1 × SPRET/Ei] (Rowe *et al.*, 1994).

The raw mapping data for the cross are accessible through The Jackson Laboratory World Wide Web server [http://www.jax.org/resources/documents/cmdata] and the Mouse Genome Database (Accession Nos. MGD-JNUM-40224 and MGD-JNUM-38173).

RESULTS

cDNA cloning and sequence analysis of mouse AM. While working on a single-pass sequencing project on our cDNA library made from the ectoplacental cone (EPC) of 7.5 d.p.c. mouse embryos, we found 8 cDNA clones with strong sequence similarity to human AM (Kitamura *et al.*, 1993b) of 2103 cDNA clones sequenced. Because the frequency of the cDNA clones in the library roughly corresponds to the expression levels of the genes in this tissue, these data suggest that the mouse homolog of AM is highly expressed in the EPC. Complete analysis of the cDNA showed that its sequence agreed with an independently determined mouse strain 129/SvJ AM cDNA sequence that was reported while this paper was being prepared (Okazaki *et al.*, 1996). One amino acid of 184 was different, but Southern blot analysis indicates that the gene is single copy (data not shown), and the difference is most likely explained by a sequence polymorphism between C57BL/6J mice, which we employed, and 129/SvJ.

Expression analyses of mouse AM by Northern blot. The high levels of AM mRNA at the implantation site prompted us to investigate further the temporal, level, and spatial distribution of its expression as indices of its possible involvement in the implantation phase. To our surprise, Northern blot analysis of staged embryos from 7, 11, 15, and 17 d.p.c. mice showed that AM is most highly expressed in the 7 d.p.c. mouse embryo (Fig. 1a). Then, the expression level of AM became undetectably low at 11 d.p.c. The low level of AM gene expression was detected at 15 d.p.c. and its expression was slightly increased by 17 d.p.c. In controls with adult mouse RNA preparations, Northern analyses showed high expression of AM in heart, lung, and kidney and weak expression in liver and skeletal muscle (Fig. 1b). This pattern of tissue-specific expression confirms results reported for rat (Sakata *et al.*, 1993; Sugo *et al.*, 1994), porcine (Kitamura *et al.*, 1994), and human (Kitamura *et al.*, 1993b) AM. In addition to a single major transcript of about 1.5 kb, we observed a few minor transcripts, which were also noted in rat (Sakata *et al.*, 1993; Sugo *et al.*, 1994) and human studies (Kitamura *et al.*, 1993b).

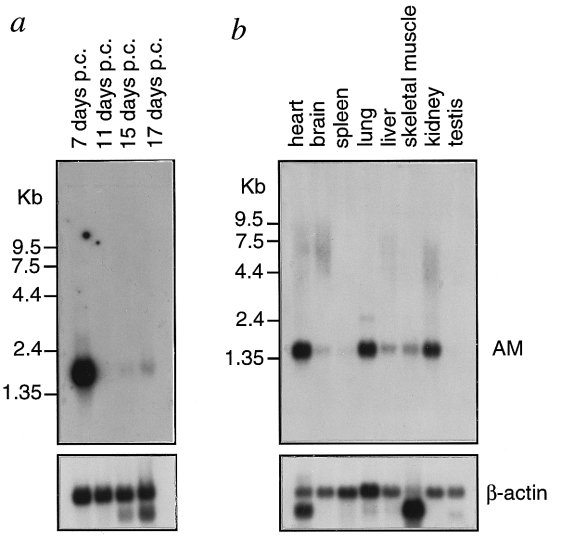


FIG. 1. Stage-specific and tissue-specific expression of the AM gene by Northern blot analyses. (a) A membrane (Clontech) containing 2 μg of poly(A)$^+$ RNA from 7, 11, 15, and 17 d.p.c. embryo was hybridized with a ^{32}P-labeled AM cDNA probe (clone H0201A08) (top) and with a β-actin probe (bottom). The RNA preparation from the 7 d.p.c. embryo contains extraembryonic tissues, while those from embryos at other stages do not. (b) A membrane (Clontech) containing 2 μg of poly(A)$^+$ RNA from various adult organs was hybridized with a ^{32}P-labeled AM cDNA probe (clone H0201A08) (top) and with a β-actin probe (bottom).

Expression analyses of mouse AM by in situ hybridization. Because the RNA preparation from the 7 d.p.c. embryo contains both extraembryonic and embryonic tissues and those from 11, 15, and 17 d.p.c. embryos contain only the embryonic tissues, it is possible that this high expression of the AM gene at 7 d.p.c. was contributed by the expression of this gene in the extraembryonic tissues. To localize better the site of expression, a digoxigenin-labeled cRNA probe was hybridized to whole and sectioned embryos from the implantation period of development. In controls for all experiments, a sense probe invariably showed no signal, as expected (e.g., Figs. 2b and 2d). In contrast, an antisense probe showed very high expression of the AM gene in relatively large cells on most of the embryo surface at 7.5 and 8.5 d.p.c. (Figs. 2a and 2c).

More detailed analyses were done on sections of mouse embryos from various stages. Expression of the AM gene was first seen, though weakly, at 6.5 d.p.c. (Fig. 3a). At this stage the expression was mainly localized to cells at the border between maternal and fetal tissues, especially in the antimesometrial region and the EPC (Fig. 3a). At 7.5 d.p.c., very strong and distinctive signals were observed in a ring of

cells lying at the interface between maternal and fetal tissues (Fig. 3b). Histologic features and location identify those cells unequivocally as trophoblast giant cells and trophoblast cells in the EPC (Copp, 1995; Cross *et al.*, 1994; Hoffman and Wooding, 1993; Rossant, 1986). Identification of the trophoblast giant cells was also confirmed by staining the sections with rabbit anti-human keratin serum, which is a well-known sensitive marker for the trophoblast cells (Daya and Sabet, 1991) (data not shown). At 8.5 d.p.c., a strong signal was still observed in the trophoblast giant cells, but had declined sharply in the EPC (Figs. 2c and 3c). Furthermore, the trophoblast giant cells at the antimesometrial region showed stronger signals than those at the mesometrial region (Fig. 3c); the same cells account for most of the observed expression at 9.5 d.p.c. (Figs. 3d–3f). At 12.5 d.p.c., weak expression of the AM gene was detected in the limited number of trophoblast giant cells in the placenta and yolk sac by *in situ* hybridization (data not shown).

Expression of the mouse AM gene in extraembryonic tissues. Because expression of the AM gene is limited to extraembryonic cells during early postimplantation development, the expression of the gene in the trophoblast giant cells could be quantitated further by Northern blot analyses using poly(A)$^+$ RNA extracted from the decidua and extraembryonic parts of the staged mouse embryos. AM expression was again first observed at about 6.5 d.p.c. and then increased to a peak level at 9.5 d.p.c. (Fig. 4). Thereafter, by 12.5 d.p.c., expression dropped precipitously. Consistent with the results by *in situ* hybridization, a low level of AM gene expression was detected by Northern blot analyses throughout the postimplantation period (Fig. 4). In contrast, the expression of proliferin, an angiogenic placental hormone (Jackson *et al.*, 1994) primarily expressed in the trophoblast giant cells (Lee *et al.*, 1988), started at about 8.5 d.p.c., peaked at 10.5 d.p.c., and was then continuously expressed at a relatively high level until the end of gestation (Fig. 4).

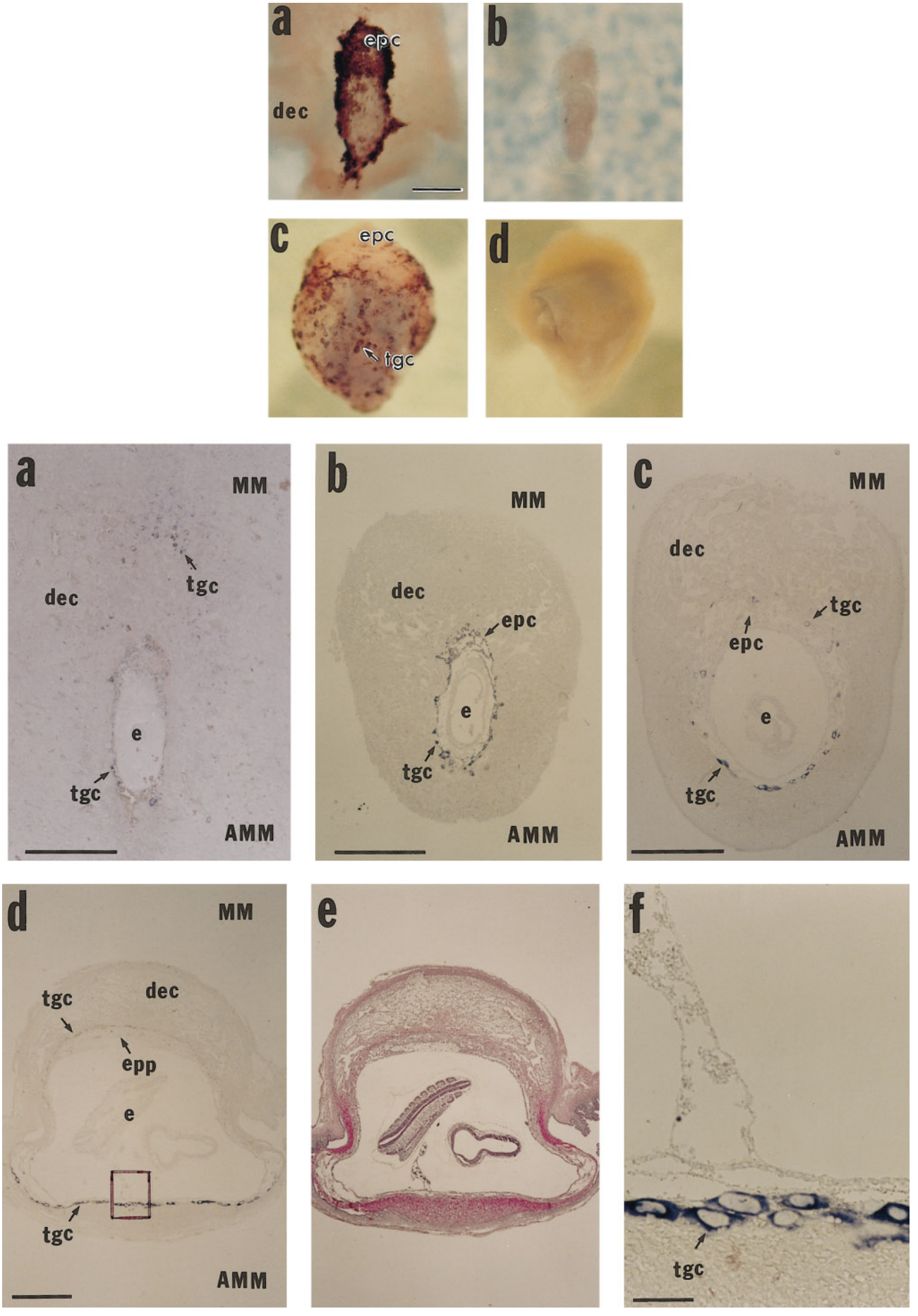
Expression of AM peptide examined by immunohistochemistry. It has been known that, as with other biologically active peptides affecting circulation such as endothelin and atrial natriuretic peptide, AM in the adult heart, lung, and kidney is continuously secreted without storage in the cytoplasm of synthesizing cells (Washimine *et al.*, 1995). To test the possibility of AM peptide secretion from trophoblast giant cells, the distribution of AM peptide was directly observed by immunohistochemistry with a rabbit anti-rat AM 1-50 serum.

At 6.5 d.p.c., a weak signal was observed in the EPC and surrounding maternal decidua in the mesometrial region and in the yolk cavity in the antimesometrial region (Fig. 5a). Consistent with a paracrine and/or autocrine role, the AM peptide was localized not only in the trophoblast giant cells, along with its mRNA, but also in the decidua, embryo proper, ectoplacental cavity, exocoelemic cavity, and yolk sac cavity at 7.5 d.p.c. (Fig. 5b). At 8.5 d.p.c., strong signals were observed particularly in the embryo proper, extraembryonic tissues, and surrounding decidua at the antimesometrial region (Fig. 5c). In a magnified view of the yolk sac area, a strong signal was observed in the yolk sac cavity with a weaker signal in the trophoblast giant cells (Figs. 5d and 5e). Interestingly, the AM peptide was concentrated in the yolk cavity, where maternal blood cells and nascent embryonic blood cells were very close to each other (Figs. 5d and 5e). In the mature placenta at 12.5 d.p.c., a weak signal was observed in the mesometrial side of the spongiotrophoblast layer and the trophoblast giant cells, but the decidual region, especially loose decidua, was much more intensely stained (Fig. 5f). In the magnified view it was clear that the AM peptide was localized in the subregion of the placenta, which was lined with trophoblast giant cells (Fig. 5g). Thus, the AM peptide consistently diffused primarily outside of the trophoblast giant cells where it was produced. This is in contrast to other secretory proteins such as proliferin, whose both protein product and mRNA are present at high levels in the trophoblast giant cells (Lee *et al.*, 1988).

To confirm that the rabbit anti-rat AM 1-50 serum used in this study recognizes mouse AM in these particular tissue sections, immunoblots of 12.5 d.p.c. embryo and placenta extracts were performed (Fig. 6). Two proteins with approximately 14.5- and 7.3-kDa molecular weights, which correspond to those of pro-AM and AM, respectively, were clearly detected in the placenta extract (Fig. 6, lane a). The results were consistent with those reported previously (Miller *et al.*, 1996; Montuenga *et al.*, 1997). Similar immunoreactive proteins were also detected in the embryo ex-

FIG. 2. Localization of mouse AM in the mouse embryo by whole-mount *in situ* hybridization. (a, b) Whole-mount *in situ* hybridization of an embryo *in utero* at 7.5 d.p.c. by antisense probe (a) and sense probe (b). The decidua were cut to expose embryos. (c, d) Whole-mount *in situ* hybridization of an embryo *in utero* at 8.5 d.p.c. by antisense probe (c) and sense probe (d). The embryo was cut out from the deciduum. epc, ectoplacental cone; dec, decidua; tgc, trophoblast giant cell. Scale bar: 500 μm, all panels.

FIG. 3. Localization of mouse AM in the mouse embryo by *in situ* hybridization. (a) *In situ* hybridization of a section of embryo *in utero* at 6.5 d.p.c. To enhance the signal intensity, a color expression time of this sample was extended to approximately threefold longer than those of other stages. (b) *In situ* hybridization of a section of embryo *in utero* at 7.5 d.p.c. (c) *In situ* hybridization of a section of embryo *in utero* at 8.5 d.p.c. (d, e) A pair of serial sections of embryos *in utero* at 9.5 d.p.c. One section is hybridized with antisense probe (d). The other section is stained with hematoxylin and eosin (e). (f) Magnified view of antimesometrial region. MM, mesometrial; AMM, antimesometrial; dec, decidua; e, embryo; epc, ectoplacental cone; tgc, trophoblast giant cells. Scale bars: 500 μm in a, 1 mm in b–e, and 100 μm in f.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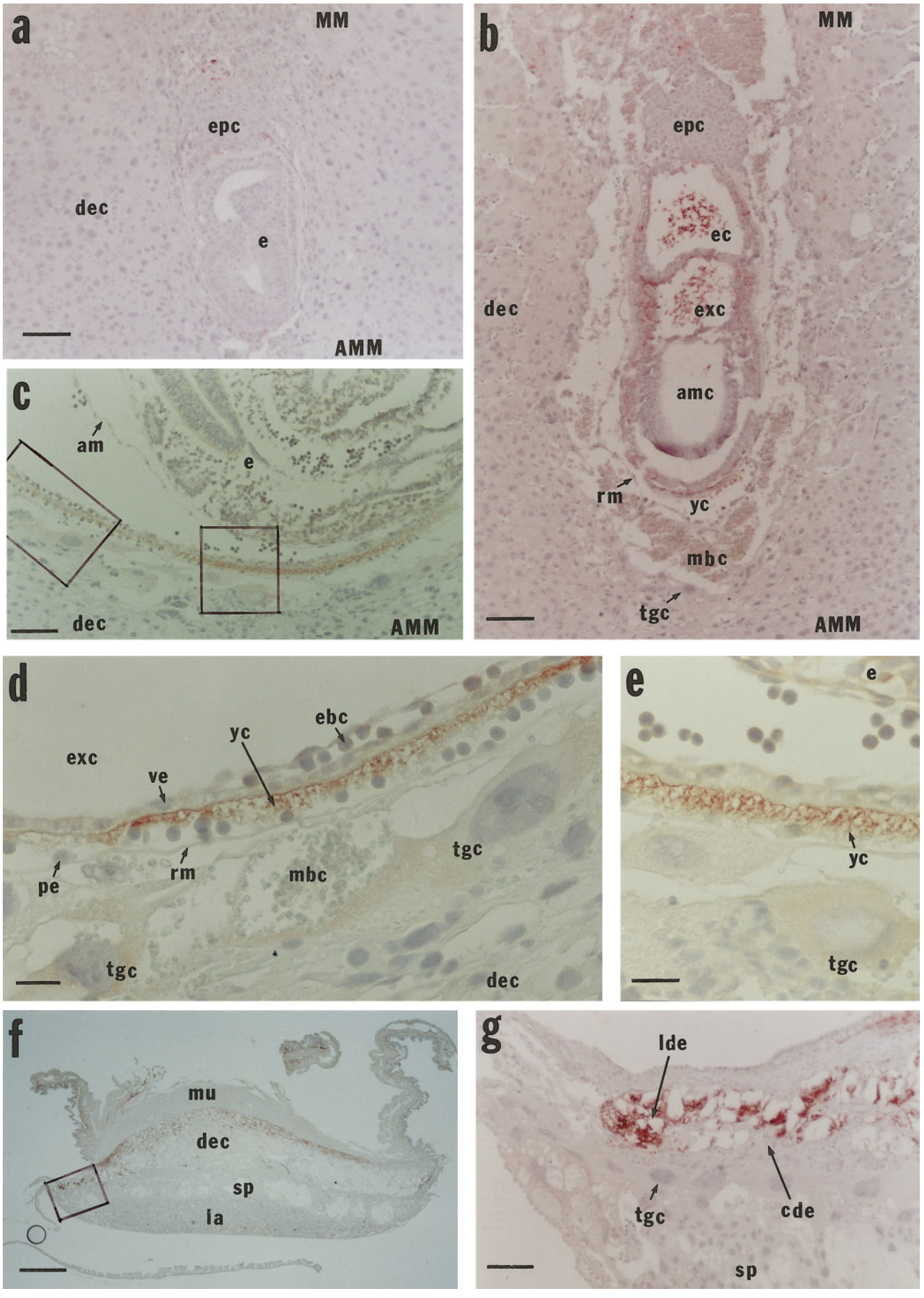
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tract, although they were very faint (Fig. 6, lane b). The controls using normal rabbit IgG did not detect any immunoreactive protein (Fig. 6, lanes c and d).

Identification of mouse AM-R cDNA. To investigate the target tissues of the AM peptide secreted from the trophoblast giant cells, we performed expression analyses of the adrenomedullin receptor (AM-R) gene. AM-R was originally isolated as an orphan receptor from rat lung [L09249 (Harrison *et al.,* 1993), L04672 (Eva and Sprengel, 1993)]. The cDNA encodes a protein that contains seven transmembrane domains and is a member of the G protein-linked receptor superfamily. Later, Clark and associates identified the cDNA clone for the AM-R based on two criteria (Kapas *et al.,* 1995). First, the expression pattern of the gene is consistent with that of the AM gene. Both genes are expressed predominantly in the lung, adrenal gland, heart, and spleen. Second, they have demonstrated that when the full-length cDNA is expressed in COS-7 cultured cells, the receptor binds to adrenomedullin and mediates a cAMP response in a manner similar to that observed in cardiovascular and other target tissues.

By searching the public database (NCBI, NIH) with this rat gene sequence, we found two mouse sequences showing strong similarity to the rat AM-R gene. One mouse cDNA sequence (D17292 [BALB/c]) was originally identified as a putative G protein-coupled receptor with unknown function and deposited in the database (Saeki *et al.,* 1993). The other clone was an EST from the HHMI-Washington University Mouse EST Project (W75568 [C57BL/6J]; (Marra *et al.,* 1998)).

To determine whether the mouse gene (D17292) is the ortholog of the rat AM-R gene, we have performed a detailed sequence comparison between these genes. The comparison was made by the Higgins–Sharp method (Higgins and Sharp, 1988) using MacDNASIS sequence analyses programs (Hitachi). The results shown in Fig. 7 clearly demonstrate the high degree of sequence similarity between these genes and establish the mouse gene (D17292, W75568) as the ortholog of the rat AM-R gene. The EST clone (W75568) was purchased from Research Genetics and resequenced to confirm the identity. Although there are some sequence variations between the EST (W75568) and the cDNA (D17292), this is probably due to the slight sequence difference between the mouse strain C57BL/6J and BALB/c. The uniqueness of the EST clone was confirmed by Southern blot analysis (data not shown).

Expression analyses of AM-R by Northern blot. Northern blot analysis using the mouse EST cDNA clone (W75568) as a probe showed one major band at about 1.8 kb and some minor bands at about 2.2, 3.0, and 4.4 kb. The gene was highly expressed in lung, liver, heart, spleen, and kidney (Fig. 8a). The size of transcripts and tissue-specific expression pattern are similar to those reported for rat AM-R (Kapas *et al.,* 1995). The tissues expressing the AM-R gene corresponded well to those expressing the AM gene (Figs. 1b and 8a). The results were consistent with a paracrine mechanism for the action of AM peptide.

To our surprise, the expression pattern of the AM-R gene during early mouse development showed a sharp contrast with that of the AM gene (Figs. 1a and 8b). The expression level of the AM-R gene was undetectably low at 7 and 11 d.p.c., but dramatically increased by 15 d.p.c. and stayed relatively high until 17 d.p.c. Further analyses of AM-R gene expression in the extraembryonic tissues using the same membrane as used in Fig. 4 also showed very low expression throughout the postimplantation period (data not shown). These data indicate that the AM-R gene is not expressed in either embryonic or extraembryonic tissues at 7.5 d.p.c., while the AM gene is highly expressed in the extraembryonic tissues, especially in the trophoblast giant cells at this stage. This suggests an intriguing possibility that the maternal tissues, especially maternal decidua and/or mother's body, are the target of the AM peptide that is synthesized and secreted from the embryo's trophoblast giant cells at the implantation site. Histological observations (e.g., Figs. 5d and 5e) showed that the trophoblast giant cells are in direct contact with maternal blood, suggesting the possibility that the AM peptide is also secreted into the maternal circulation.

Genetic mapping of mouse AM and AM-R on the mouse genome. Since the primary function of the AM peptide is the control of blood pressure, it is now reasonable to suspect that AM is a candidate gene in the causation of preeclampsia, the gestational proteinuric hypertension (Sibai, 1996) that is believed to be caused by insufficient invasion of trophoblasts into the uterine stroma (Cross *et al.,* 1994; Wegner and Carson, 1994). To examine this possibility, we have mapped both AM and AM-R on the mouse genetic map by using The Jackson Laboratory's Interspecific Backcross Mouse Panel (Rowe *et al.,* 1994).

Analysis of the genotype using the MapManager Program (Manly, 1993) has mapped the mouse AM gene (locus

FIG. 5. Immunohistochemical analysis by affinity-purified antibody raised against rat AM. (a) Embryo *in utero* at 6.5 d.p.c. (b) Embryo *in utero* at 7.5 d.p.c. (c) Embryo and deciduum in the antimesometrial side at 8.5 d.p.c. (d, e) Magnified view of boundary between the trophoblast giant cells and maternal deciduum at 8.5 d.p.c. in the antimesometrial side. (f) Placenta at 12.5 d.p.c. (g) Magnified view of a part of placenta. MM, mesometrial; AMM, antimesometrial; dec, decidua; ec, ectoplacental cavity; exc, exocoelemic cavity; amc, amniotic cavity; rm, Reichert's membrane; yc, yolk cavity; mbc, maternal blood cells; pe, parietal endoderm; ve, visceral endoderm; ebc, embryo blood cells; mu, muscle layer of uterus; e, embryo; epc, ectoplacental cone; la, labyrinthine trophoblast; sp, spongiotrophoblast; tgc, trophoblast giant cells; lde, loose decidua; cde, compact decidua. Scale bars: 200 μm in a–c, 50 μm in d and e, 1 mm in f, and 100 μm in g.

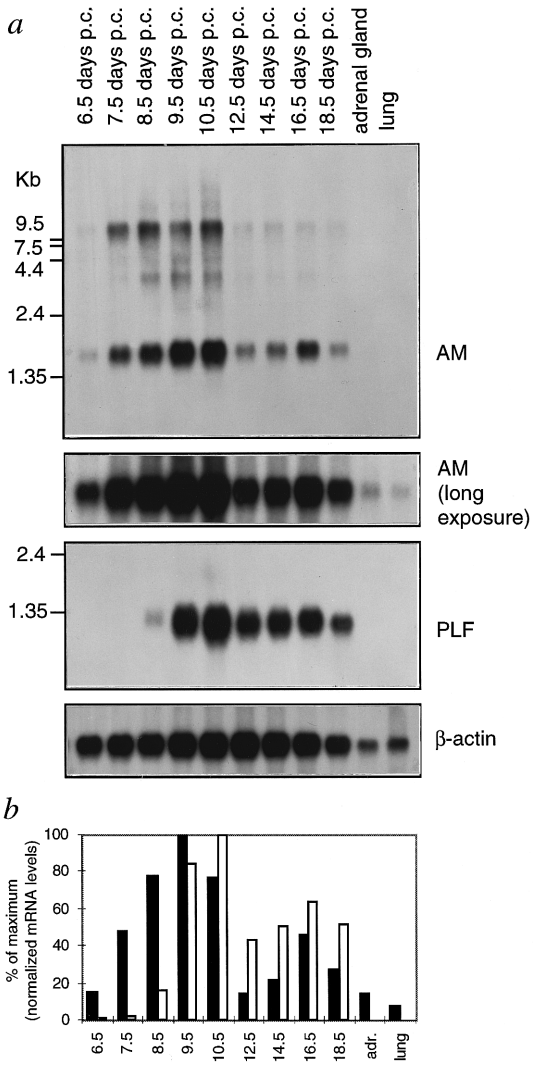

FIG. 4. Expression of the AM gene in the extraembryonic tissues during mouse development. (a) Four micrograms of poly(A)$^+$ RNA from each tissue except for lung (2 μg) was hybridized with a ^{32}P-labeled cDNA probe from the AM (clone H0201A08) (first panel), proliferin (PLF) (clone C0002G11) (third panel), and β-actin (fourth panel). The second panel shows the result of the AM probe after 6 days exposure with intensifying screen at -80°C. Poly(A)$^+$ RNAs were purified from the following tissues at each developmental stage: whole decidua including extraembryonic tissue and embryo proper (6.5 and 7.5 d.p.c.); decidua and extraembryonic tissue, but without embryo proper (8.5, 9.5, and 10.5 d.p.c.); yolk sac and placenta (12.5, 14.5, 16.5, and 18.5 d.p.c.); adult adrenal glands and lungs. (b) Relative changes in mRNA level of AM (black bars) and PLF (white bars; normalized against β-actin) are shown as percentage of maximum.

symbol *Adm*) on chromosome 7: two crossovers of 94 backcross animals distal to *D7Mit37* and the *Hbb* locus; and the mouse AM-R gene (locus symbol *Admr*) to the distal end of mouse chromosome 10, *D10Mit35*-(1.06 ± 1.06)-*D10Wsu93e*-(1.06 ± 1.06)-*Dagk1*-(1.06 ± 1.06)-*Admr*, *Atoh3*, *Cd63*-(1.06 ± 1.06)-*Apof*. The hormone and its receptor were not linked on the mouse genome in this case. The region where the AM gene (*Adm*) was mapped is syntenic with human chromosome 11p15.5, where the human AM is already mapped (Brilliant *et al.*, 1996; Ishimitsu *et al.*, 1994a; Okazaki *et al.*, 1996). The human ortholog of the AM-R gene may map to 12q13 based on the syntenic correlation between this region of the mouse genome and human chromosome 12q13.3. Because the linkage studies have limited the location of the preeclampsia gene in humans to chromosome 1, 3, 9, or 18 (Hayward *et al.*, 1992), this makes it unlikely that both AM and AM-R are candidate genes for preeclampsia. We also could not find any known loci with phenotypes that affect blood pressure by searching the Online Mendelian Inheritance of Man (OMIM).

DISCUSSION

In this report, we have shown that mouse AM is most highly expressed in the early postimplantation stage by Northern blot analyses. It is unlikely that the AM gene is expressed at the stages before implantation, because a large-scale cDNA sequencing analysis of 3.5 d.p.c. mouse blastocysts, which are at a stage immediately before implantation, did not show any hits to the AM gene in the collection of more than 6000 ESTs (M.S.H.K., unpublished observation). Therefore, we can conclude that the AM gene shows a spike-like expression pattern with peaks at 7–9 d.p.c. during mouse development, although a relatively lower level of expression was detected throughout the postimplantation period. Extensive examination of whole mounts and sections observed by *in situ* hybridization (6.5–9.5 d.p.c.) evidenced AM expression only in the trophoblast cells. Although at later stages such as 12.5 d.p.c. the expression of the AM gene became evident in the embryo proper, e.g., in the neuroepithelium (data not shown), these expression levels were much less than those in the trophoblast giant cells at its peak expression. This indicates that the peak production of AM mRNAs was exclusively from the trophoblast giant cells at these stages.

It has been known that the vascular endothelial cells show the highest expression level of AM gene in adult (Sugo *et al.*, 1994), suggesting that the tissue is a primary production site of AM peptide. The expression of AM in mouse trophoblast giant cells can be compared to that in cultured rat vascular endothelial cells, which have been reported to express AM at levels 20- to 40-fold higher than those of adult lung and adrenal gland (Sugo *et al.*, 1994). In the mouse embryonic tissues used in our analyses the expression level of AM at 9.5 d.p.c. is 7- to 8-fold higher than that of the adrenal gland and lung of the mature animal (Figs. 4a and 4b). Because the trophoblast giant cells only occupy a tiny fraction of the cells in the tissue mass used for RNA

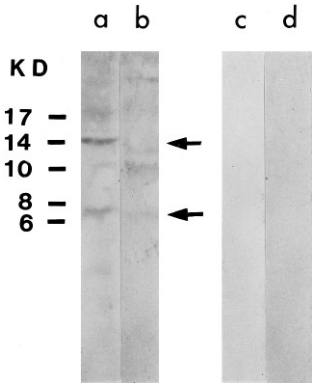

FIG. 6. Immunoblot analysis. The homogenates of 12.5 d.p.c. mouse placenta and embryo were separated by SDS–PAGE, transferred to Immobilon membrane, and reacted with rabbit anti-rat AM 1-50 serum (a and b) or normal rabbit IgG (c and d). Molecular weight markers are indicated on the left. Lanes a and c represent protein fractions of mouse placenta, and lanes b and d represent protein fractions of mouse embryo. Arrows indicate immunoreactive proteins.

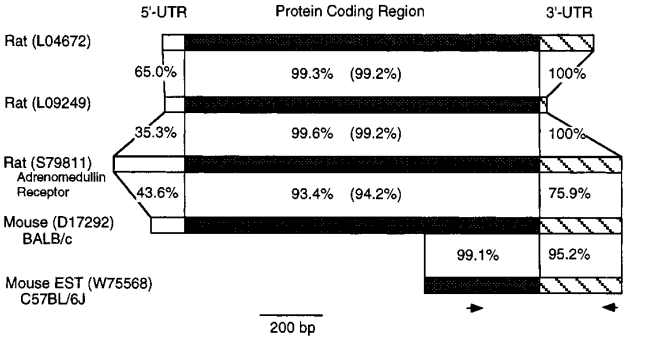

FIG. 7. Sequence comparison of the adrenomedullin receptor gene. Percentage similarities between cDNA sequences at the nucleotide level are shown for the 5′-UTR, protein coding region, and 3′-UTR, respectively. For protein coding regions, percentage similarities between cDNA sequences in the amino acid level are shown in parentheses. The locations of PCR primer pairs for gene mapping are shown by arrows.

extraction (Figs. 2 and 3), one can estimate that the expression of AM in trophoblast giant cells reaches levels at least an order of magnitude higher than in cultured vascular endothelial cells. One can therefore infer that AM is expressed most in the trophoblast giant cells during embryogenesis and consequently may have a primary function soon after implantation.

While preparing this paper, a developmental study of the expression patterns of AM and AM-R in rat and mouse was published (Montuenga *et al.*, 1997). Although the authors emphasize mainly the role of AM in late postimplantation development, they also point out that the AM gene is expressed in trophoblast cells as well as many other cell types at the implantation site. However, there are some discrepancies between the current report and their report. First, the authors reported that the AM gene is expressed rather ubiquitously and expressed in both decidual cells and trophoblast giant cells at 8–9 d.p.c. (Montuenga *et al.*, 1997), while we observed the expression of the AM gene predominantly in the trophoblast giant cells at these stages. Second, the authors reported that the AM-R gene is also expressed at high levels in the trophoblast giant cells at 8–9 d.p.c. (Montuenga *et al.*, 1997), while we could not detect the expression of the AM-R gene in the trophoblast giant cells by Northern blot analyses. Although we do not yet know the reason for these discrepancies, the different observations in the expression patterns of AM and AM-R at the implantation site led two groups to different suggestions about the role of the AM system at the implantation site. In contrast to our ideas discussed in this paper, Montuenga and associates suggest the involvement of the AM system in the invasion of the trophoblast giant cells into the maternal decidua by autocrine growth-promoting mechanisms based on their observation that both AM and AM-R genes are expressed in the trophoblast giant cells. However, this idea does not seem to be consistent particularly with one of our results, which shows that at 8.5 and

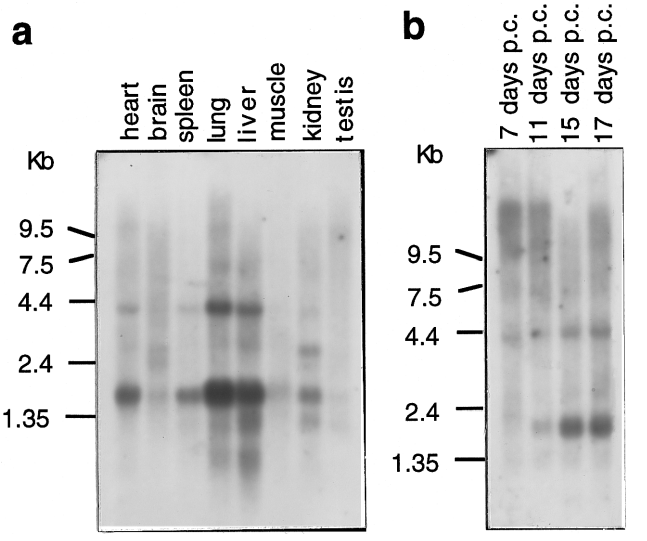

FIG. 8. Stage-specific and tissue-specific expression of the adrenomedullin receptor gene by Northern blot analysis. (a) A membrane (Clontech) containing 2 μg of poly(A)$^+$ RNA from 7, 11, 15, and 17 d.p.c. embryo was hybridized with a ^{32}P-labeled adrenomedullin receptor cDNA probe (W75568). The RNA preparation from 7 d.p.c. embryo contains extraembryonic tissues, while those from embryos at other stages do not. (b) A membrane (Clontech) containing 2 μg of poly(A)$^+$ RNA from various adult organs was hybridized with a ^{32}P-labeled mouse adrenomedullin cDNA probe (W75568).

9.5 d.p.c. the expression of AM is mainly in the antimesometrial side and its expression in the mesometrial side is very weak (Figs. 3c and 3d). If the AM is functioning for the invasion of trophoblast and embryo into the maternal decidua, we should have seen a stronger expression of AM in the mesometrial side than in the antimesometrial side. Indeed, such an expression pattern has been observed for gelatinase B, which is directly involved in implantation and the invasion of trophoblast cells into the maternal decidua (Alexander *et al.*, 1996).

The unique expression pattern of AM corresponds very well with the dramatic changes in the support mechanism for embryos during the peri-implantation stage (Copp, 1995; Cross *et al.*, 1994). Just after implantation (5.5–7.5 d.p.c.), the embryo relies on surrounding maternal blood for life support. Then, from 7.5 to 9.5 d.p.c., the rapidly developing EPC and allantois—that is, the nascent precursor of the chorioallantoic placenta—become the means for life support, especially on the mesometrial side of embryonic tissues. For the rest of the embryo, especially in the antimesometrial side, the major route of transport for nutrients is through the yolk sac. This is the exact period when AM production sharply begins and peaks. Around 10 d.p.c., when the mature chorioallantoic placenta is completed, there is a simultaneous sharp decline in AM production. At this point the chorioallantoic placenta begins to provide much more efficient nourishment through the umbilical cord and into the embryonic circulation. Both Northern blot and *in situ* hybridization analyses show high levels of AM gene expression in trophoblast cells during the period when the embryo relies on inefficient modes of obtaining nourishment; the decline of AM production occurs as the chorioallantoic placenta is established. Especially suggestive are the *in situ* hybridization results showing high levels of AM gene expression in the trophoblast giant cells of the antimesometrial region at 8.5 and 9.5 d.p.c. (Figs. 3c, 3d, and 3f). Of course, residual AM may also function in the mature placenta, where many vascular control mechanisms are known (Myatt, 1992). For example, AM has recently been detected in human cytotrophoblasts cultured from term placenta (Morrish *et al.*, 1996) and in human second-trimester amniotic fluid as well as amniotic membrane (Macri *et al.*, 1996). It has also been reported that the serum AM level of pregnant woman at 36–41 weeks gestation is higher than that of nonpregnant woman (Di Iorio *et al.*, 1997).

The specific expression of the AM gene in the trophoblast giant cells coincides with the known critical function of the cells, which act (i) as the leading edge of embryo invasion of the maternal endometrium; (ii) as an immunological barrier protecting the embryo from the maternal immune response; and (iii) as an endocrine organ, synthesizing and secreting steroid and peptide hormones (Cross *et al.*, 1994; Hoffman and Wooding, 1993; Rossant, 1986). The results presented in this report suggest that AM secretion is a significant feature of the endocrine function of trophoblast giant cells. This new feature highlights a similarity of the trophoblast giant cells to vascular endothelial cells. A speculative similarity has been suggested earlier, based on the anatomical juxtaposition of both cell types in direct contact with blood (Cross *et al.*, 1994), although the embryological origins of the two cell types are completely different (Risau, 1995; Rossant, 1986). In this sense, our results further support the recent report showing a striking similarity between human cytotrophoblast cells and vascular endothelial cells by examining the cell adhesion phenotype switch (Zhou *et al.*, 1997).

Concerning AM function as a hypotensive peptide, in adult animals the targets of AM are believed to be both vascular smooth muscle cells and endothelial cells in the aorta (Shimekake *et al.*, 1995). Because there are no vascular smooth muscle cells underlying the trophoblast giant cells, the target of AM at the implantation site could be the trophoblast giant cells themselves. However, the undetectable level of AM receptor gene expression in both embryo proper and extraembryonic tissues including the trophoblast giant cells at the implantation site strongly suggests that the AM peptide produced and secreted from the trophoblast giant cells at the implantation site has a target in the maternal tissues. According to the commonly accepted paracrine/autocrine paradigm of the AM peptide function, we propose that AM acts on the blood vessels in the maternal decidua and enhances local circulation by pooling maternal blood at the implantation site. Alternatively, AM could influence maternal systemic blood pressure at the very early postimplantation period.

Whatever the mode of AM action, its involvement in embryonic metabolism and survival has implications for maternal–fetal medicine. They range from possible side effects of AM administered as an antihypertensive drug for pregnant women to a potential therapeutic use to control the implantation process. Obviously, the production of an AM knock-out mouse will provide a useful model to test these ideas.

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