Impaired placental neovascularization in mice with pregnancy-associated hypertension

Mitsuko Furuya^{1,*}, Junji Ishida^{2,3,*}, Saki Inaba^{2,3}, Yoshitoshi Kasuya⁴, Sadao Kimura⁴, Ryoichi Nemori⁵ and Akiyoshi Fukamizu^{2,3}

Preeclampsia is a serious disorder that may result in severe morbidity and mortality for mother and fetus, and it is thought that the placental dysfunction is important in the pathogenesis of preeclampsia. As the model of preeclampsia, we previously generated a transgenic mouse model that developed pregnancy-associated hypertension (PAH) by mating females expressing human angiotensinogen with males expressing human renin. In PAH mice, maternal blood pressure started to rise from days 12 to 13 of gestation (E12–13) to term (E19–20), which is accompanied by the fetal intrauterine growth retardation and systemic maternal disorders including proteinuria and convulsion. To understand the pathology of the complications in PAH mice that overlap with those in human preeclampsia, we analyzed the PAH placenta sequentially from the onset of hypertension to the term of delivery. In PAH placenta, histological analysis revealed that the microvessel densities of fetal vasculature at term were significantly lower than those of normal placenta, and the majority of terminal vessels of PAH placenta were lacking for pericytes and basement membrane. The interaction between fetal vasculature and maternal blood canal at labyrinth of PAH placenta was morphologically distorted, and the expression patterns of key molecules in neovascularization of PAH placenta were distinct from those of normal placenta during pregnancy. In addition, maternal plasma level of soluble form of vascular endothelial growth factor receptor-1 (sVEGFR-1) was significantly increased in PAH at E19. Furthermore, in uteroplacental site, *in situ* proteolytic activity of PAH mice was suppressed from E16 to term compared to that of normal pregnancy, and the expression of matrix metalloproteinase-2 mRNA was strikingly downregulated at E16 in PAH mice. Collective data suggest that the impairments of fetoplacental neovascularization and uteroplacental remodeling contribute to the development of complications in PAH.

Laboratory Investigation (2008) **88**, 416–429; doi:10.1038/labinvest.2008.7; published online 11 February 2008

KEYWORDS: neovascularization; placenta; preeclampsia; transgenic mice; tissue remodeling; angiotensin

Preeclampsia is a life-threatening complication of pregnancy for both mother and fetus during gestation. It is characterized by blood pressure (BP) elevation and proteinuria after 20 weeks of gestation in human.¹ Although the etiology and pathology of preeclampsia remain enigmatic, it is thought that placental dysfunction is one of the major causes of this disease, and that several uteroplacental events are suggested to be involved in the course. During the early stage of gestation, extravillous trophoblasts invade endometrium to establish blood flow by performing epithelial–endothelial transformation and replacing the walls of spiral arteries with the help of activated decidual natural killer (NK) cells.² The

studies on uteroplacental immunologic microenvironments in preeclampsia have suggested that abnormal interaction between extravillous trophoblasts and decidual NK cells might explain in part the impaired pseudovasculogenesis.^{3,4} Genetic studies on familial preeclampsia have also demonstrated that specific patterns of genetic variants or quantitative trait loci (QTL) such as AGT, STOX1 and 5q/13q QTL are involved in the matrilineal predisposition to this disease.^{5–7}

The studies on pathogenesis of preeclampsia including those listed above have contributed to our understanding of genetic susceptibilities and uteroplacental microenvironments

¹Department of Molecular Pathology, Chiba University Graduate School of Medicine, Chiba, Japan; ²Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan; ³Center for Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba, Japan; ⁴Department of Biochemistry and Molecular Pharmacology, Chiba University Graduate School of Medicine, Chiba, Japan and ⁵Fuji Film Life Science Laboratory, Kanagawa, Japan

Correspondence: Professor Dr A Fukamizu, PhD, Graduate School of Life and Environmental Sciences, Center for Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Ten-nodai Tsukuba, Ibaraki 305-8577, Japan.

E-mail: akif@tara.tsukuba.ac.jp

*These authors contributed equally to this work.

Received 11 July 2007; revised 26 November 2007; accepted 28 December 2007

in poor placentation. On the other hand, it has not been well documented the placental pathology during the last half of gestation as maternal systemic disorders are getting worse. Although placental organogenesis is established for the most part during the first half of gestation, its function increases dramatically in the last half to supply enough oxygen and nutrients for exponential fetal growth.⁸ Since maternal hypertension starts and accelerates during the last half of gestation in preeclampsia, it is requisite to monitor the pathological events of the placenta under maternal hypertension. Currently, very limited information is available about the pathological changes in preeclamptic placenta from the onset of hypertension to delivery.

We had generated mice with pregnancy-associated hypertension (PAH) by mating females expressing human angiotensinogen (hANG) with males expressing human renin (hRN).⁹ In PAH mice, maternal hypertension starts from 13 days of gestation (E13) until delivery (E19–20) due to the generation of excessive angiotensin-I, the precursor of angiotensin-II, by hRN secretion from the fetal side to the maternal circulation.⁹ Systolic BP at E19 in PAH mother reaches 160 mmHg, whereas that in normal pregnant mouse remains around 100 mmHg.⁹ In addition to hypertension, PAH mother shows proteinuria, cardiac hypertrophy and often convulsions. The fetus at term in PAH pregnancy shows severe intrauterine growth retardation (IUGR), and the mean body weight of PAH fetus at E19 is about 65% of that of wild-type (WT) fetus.^{10,11} In our preliminary histological investigation on PAH placenta, PAS-positive uterine–NK cells infiltrated the spiral arteries as sufficiently as those in WT at E10, and cytokeratin (CK)-positive trophoblasts replaced spiral arteries at E13 (Supplementary Figure 1A–D). These observations indicate that this model probably does not have the history of poor placentation or abnormal immune responses in the early stage of pregnancy. However, biological and physiological data demonstrated that renin–angiotensin system (RAS)-mediated maternal hypertension beginning in the second half of gestation leads to the pathological condition that meets the criteria of preeclampsia.

In this study, we investigated pathological changes in the placenta under the condition of accelerating maternal hypertension. In fetoplacental region, microvessel densities were significantly low, and fetal-derived endothelial cells (ECs) were lacking for appropriate pericytes coverage and basement membrane support. On the basis of quantitative reverse transcriptase-mediated PCR (RT-PCR) analysis, we identified the changes in the expression of key molecules that are involved in pathological neovascularization in PAH. We also demonstrated that maternal plasma levels of soluble form of vascular endothelial growth factor receptor-1 (sVEGFR-1, also named sFlt-1) at E19 were significantly higher in PAH than those in WT. In addition, in uteroplacental junction, proteolytic activities and the expression of matrix metalloproteinases (MMPs) in PAH mice were suppressed, and trophoblast progenitor cell population was

reduced. Our results indicated the impairments of fetoplacental neovascularization and uteroplacental remodeling in PAH placenta, and suggested its possible involvement in the development of preeclamptic complications in the latter half of pregnancy.

MATERIALS AND METHODS

Animals

Mice homozygous for hANG transgene and mice homozygous for hRN transgene with a C57BL/6J genetic background were reported previously.⁹ Animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba, and in accordance with the Regulation for Animal Experiments in our university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. Transgenic mice and age-matched WT mice (C57BL/6J) at 8–16 weeks old were used for cross-mating.

Sample Preparation

Placental tissues were obtained from PAH and WT mice at E13, E16 and E19 of gestation. The definition of embryonic day is as follows. When unplugged condition of the female is confirmed, it is noted as E0.5. Pups of WT mice (C57BL/6J) are delivered from E19.5–E20, thus E19 is defined as the day before parturition. Some of placentas were immediately frozen in liquid nitrogen and the others were fixed by 4% paraformaldehyde and embedded in paraffin. For RT-PCR analysis, the placenta was carefully divided into uteroplacental part that contained decidua and junctional zone, and labyrinth that contained fetal-derived vessels and maternal blood canals. In addition, fetal hearts, brains, kidneys and lungs were obtained from PAH and WT fetus at E19.

RT-PCR and Real-Time RT-PCR

Total RNAs from the frozen tissues were obtained using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Expression levels of MMP-2, MMP-9, tissue inhibitor of MMP (TIMP)-1, TIMP-2, APJ, apelin, Claudin-5, endothelial nitric oxide synthase (eNOS), Ang-1, Ang-2, Tie-1, Tie-2, endothelin-1 (ET-1) and GAPDH were examined by RT-PCR with the following primers: 1637F (CCTCCCCCG ATGCTGATACT) and 1768R (CGCCAAATAAACCGGTCTT) for MMP-2, 1003F (GTGGACGCGACCGTAGTTG) and 1131R (TGTCAGTGTCTGAAGTTTCATGTG) for MMP-9, 298F (AAGTCCCAGAACCGCAGTGA) and 379R (AGAG TACGCCAGGGAACCAA) for TIMP-1, 623F (CGCTGGACG TTGGAGGAA) and 704R (GTCCCAGGGCACAATGAAGT) for TIMP-2, 454F (TGGCTGACTTGACCTTTGTG) and 1020R (TTCACCAGGTGTAAGGCAT) for APJ,¹² 321F (GTTG CAGCATGAATCTGAGG) and 548R (CTGCTTTAGAAAGG CATGGG) for apelin,¹² 180F (TCTGGTGCTGTGTCTGG

TAGGAT) and 265R (TGCGCCGTCACGATGTT) for Claudin-5, 1221F (AATTAATGTGGCCGTGTTGCA) and 1301R (GCTCATTTTCCA- GGTGCTTCA) for eNOS, 1639F (TATG TGCAAATGCGCTCTCAT) and 1769R (GGAGTAACTGGG CCCTTTGAA) for Ang-1, 1418F (AGGCGCATTCGCT GTATGAT) and 1547R (TTGTTCATTGTCCGAATCCTTTG) for Ang-2, 2352F (CTCCGTCTGGGCCTATATCCA) and 2482R (CGCACACGGAAAAGATATCGT) for Tie-1, 2492F (CTGAG AACACATAGGATCAAGCAA) and 2622R (AACAGC ACGGTGATGCAAGTC) for Tie-2, 663F (CCGTATGGACTGG GAGGTCT) and 744R (TGGTGAGCGCACTGACATCT) for ET-1, 442F (CTGCACCACCAACTGCTTAGC) and 637R (CAAAGTTGTCATGGATGACC) for GAPDH.¹³ cDNA synthesis was performed using QuantiTect RT (Qiagen). RT-PCR amplification was performed using AmpliTaq Gold PCR Master Mix (PE Applied Biosystems) and PCR thermocycler (PE 9700, PE Applied Biosystems). Conditions for PCR were as follows: at 95°C for 5 min, 30 cycles at 95°C for 15 s, 58°C for 15 s, 72°C for 1 min, with an extension step of 7 min at 72°C at the end of the last cycle. RT-PCR was performed in the homogenized samples from three different mating pairs in each gestational period, respectively. For real-time RT-PCR, QuantiTect SYBR Green PCR kit (Qiagen) and PCR amplifications in ABI-PE Prism 7000 sequence detection system (PE Applied Biosystems) were used according to the protocol provided by the manufacturer. The conditions for PCR were as follows: at 50°C for 2 min, at 95°C for 15 min, 40 cycles at 95°C for 30 s, at 60°C for 30 s. GAPDH was used as internal control genes. mRNA levels of each sample normalized against GAPDH were examined triplicate and showed as mean $\pm$ S.E.

Immunohistochemistry

Rabbit polyclonal antibody against α -smooth muscle actin (α -SMA) was from Abcam (Cambridge, UK), CK was from Dakocytomation (Carpinteria, CA, USA). Rat monoclonal antibody against Sca-1 was from R&D (Minneapolis, MN, USA), and CD31 (PECAM-1) was from BD (San Jose, CA, USA). Immunohistochemistry was carried using labeled streptavidin-biotin-peroxidase method and microwave antigen retrieval technique. Working dilution in these antibodies was 1:100. As negative controls, the primary antibody was replaced by the serum of appropriate species with dilution of 1:100. Positive controls known to contain the antigen in question were processed simultaneously. Microvessel counting was carried out by selecting two center areas in the labyrinth (1 mm²) from 5 different samples in each group, that is, 10 areas. For fluorescence double staining, Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) were used and observed using fluorescence microscopy (Zeiss Axio Imager). Double stainings were performed according to the manufacturer's instructions. The specimens were incubated with the primary antibody for 60 min, and then reacted with Alexa Fluor 488 goat anti-rat IgG for 60 min. After the detection of primary antigen, specimens were incubated with 1.5% H₂O₂ to

quench endogenous peroxidase activity for 30 min, and successively incubated with blocking reagent for 30 min. Then the secondary target was detected by conventional secondary immunofluorescence labeling. The specimens were incubated with the primary antibody for 60 min, and then reacted with Alexa Fluor 555 goat anti-rabbit IgG for 60 min. In each case, serial sections were prepared and one was stained with hematoxylin and eosin (H&E) and the others were stained with periodic acid silver-methenamine (PAM) and phosphotungstic acid hematoxylin (PTAH). PAM stainings were performed as follows. Deparaffinized tissues were immersed in 0.5% periodic acid for 15 min, and washed. Successively, they were placed in Gomori's silver-methenamine solution at 60°C for 30 min, washed and toned in 0.2% gold chloride for 2 min. After washing, specimens were placed in 3% sodium thiosulfate for 2 min, and then stained with HE. PTAH stainings were performed as follows. Deparaffinized tissues were immersed in 4% CrO₃ for 1 h. After washing, the sections were incubated in 1% NaHSO₃ for 1 min and washed. Successively, they were placed in 1% KMnO₄ for 5 min. This was followed by 3 min incubation in 2% oxalic acid and further stained in hematoxylin with tungstophosphoric acid and KMnO₄.

Film *In Situ* Zymography

Frozen sections were cut and mounted on gelatin film coated with 7% gelatin solution (Fuji Film, Tokyo, Japan), and incubated at 37°C for 8 h. Then the films were treated with Amido Black 10B. *In situ* proteolytic activity was detected as unstained areas.¹⁴ The experiments were repeated three times.

Apoptosis Assay

Apoptosis was evaluated using TUNEL assay. Specimens were undertaken with *in situ* apoptosis detection kit (Takara, Ohtsu, Japan) using the instructions provided by the manufacturer. Apoptotic cells were detected as FITC positive. The experiments were repeated at least three times.

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) system kit for sVEGFR-1 was purchased from R&D. The assay was performed according to the manufacturer's instructions. Four maternal plasma samples in WT and PAH group at E19 and two plasma samples in each group at E10 were examined. The absorption at 450 nm was measured using a microtiter plate spectrophotometer.

Gelatin Zymography

Each homogenized uteroplacental sample was diluted in equal amount of distilled water and electrophoresed at 4°C in 10% sodium dodecyl sulfate-polyacrylamide crosslinked gels (SDS-PAGE), containing 0.1% gelatin (Invitrogen). Following electrophoresis, the gel was washed with 2.5% Triton X-100 followed by incubation in Tris-HCl, 0.5 mM CaCl₂,

10^{-6} M ZnCl_2 , pH 8.0, at 37°C for 16 h. Coomassie brilliant blue staining was then carried. The experiments were repeated at least three times.

Statistical Analysis

Values were expressed as mean $\pm$ s.e. Student's *t*-test and one-way ANOVA with *post hoc* test were used for statistical evaluation. Statistical significance was assumed when $P < 0.05$ was obtained.

RESULTS

Placental Infarction was Observed in PAH

We had previously reported that PAH pregnant mice exhibited systemic disorders including hypertension, proteinuria and cardiac hypertrophy and that the fetus from PAH mice resulted in severe IUGR and high rates of mortality.^{9,10} To understand the pathophysiological events seen in PAH placenta and the intrauterine milieu of fetus during pregnancy, we compared the placentas between WT and PAH mice (Figure 1). The decidua-side surface of WT placenta at E19 was smooth and reddish, whereas that of PAH placenta was rough and whitish with multiple infarcts (Figure 1a). A mouse placenta is known to be divided into three layers by the constituent cells of each layer, that is, (D) decidua basalis composed mainly of maternal vasculature and invading trophoblasts, (J) junctional zone that contains spongiotrophoblasts and maternal vasculature which is separated from decidua by giant trophoblasts layer and (L) labyrinth that contains fetal-derived microvessels and specialized trophoblast subtypes that form maternal blood canals.¹⁵ Time course analysis of the placenta showed that at E13 these three layers were clearly detected in PAH as well as in WT placenta (Supplementary Figure 1E and F). At E16, microscopic foci of fibrinoid degeneration were noted in decidua and junctional zone in PAH placenta (Supplementary Figure 2). At E19, it was shown in PAH placenta marked fibrinoid degeneration and decidual vasculopathy, that is, atherosclerosis (Figure 1b and c, arrows and asterisks). PTAH stainings confirmed the fibrinoid necrosis of vascular walls in the spiral arteries of PAH placenta (Figure 1c, inset). TUNEL stainings in uteroplacental side at E19 demonstrated that the apoptotic cells were clustered in decidua and were also significantly detected in junctional zone in PAH (Figure 1d).

Terminal Fetal Microvessels of PAH Were Devoid of Pericyte Coverage and Basement Membrane Support

Since fetal blood contains nucleated red blood cells, fetal vasculature can be distinguished from maternal blood canal. To detect ECs-supporting basement membrane, PAM stainings were carried out. It was revealed that at E13 only larger fetal vessels in labyrinth were lined by PAM-positive basement membrane both in WT and PAH placentas (Figure 2a, left, arrows). At E16, in addition to large vessels, some of smaller fetal vessels were stained for PAM in WT placenta (Figure 2a, middle, arrows), whereas PAM-lining small

vessels were scarcely observed in PAH placenta (Figure 2a, middle, arrowheads). At E19, striking difference was demonstrated between WT and PAH labyrinth (Figure 2a, right). PAM-positive basement membrane traced every terminal vessel in WT labyrinth, demonstrating accomplished maturation of vascular networks. On the other hand, in PAH labyrinth, PAM linings disrupted, branched or formed irregular webs.

We next confirmed that fetoplacental vasculature of WT mice was stained for endothelial marker CD31 and that these ECs tubules were gradually covered by α -SMA-positive pericytes at later stages (Supplementary Figure 3, WT). At E13 and E16, the numbers of CD31-positive vessels in PAH labyrinth were not significantly different from those in WT (Supplementary Figure 3, PAH). However, at E19 there was marked difference that CD31-positive microvessels were finely distributed in WT labyrinth, whereas they were rough and irregular in number and shape in PAH labyrinth (Figure 2b, upper panels and Figure 2c, upper panel). Furthermore, α -SMA-positive vessels in PAH labyrinth were far less in number than those in WT (Figure 2b, lower panels and Figure 2c, lower panel). Fluorescence double stainings revealed that CD31-positive terminal microvessels in WT labyrinth were accompanied by α -SMA-positive pericytes. On the other hand, the majority of CD31-positive microvessels in PAH labyrinth were poorly covered by α -SMA-positive pericytes (Figure 2b, insets).

Irregular Interaction Between Maternal Blood Sinuses and Fetal Microvessels in PAH Labyrinth

Maternal blood spaces in the labyrinth are known to be composed of specialized trophoblast subtypes which are detected by CK (Figure 3).^{15,16} By using serial sections, we confirmed that CK-positive trophoblasts form maternal blood pathways showed distinctive distribution pattern from CD31-positive fetal-derived blood vessels in WT labyrinth (data not shown). At E13, maternal blood canals lined by CK-positive trophoblasts were rough in both WT and PAH labyrinth (Figure 3a, left). At E16, blood canals in WT became differentiated into more complicated sinuses of regular width, whereas the blood pathways lined by CK-positive trophoblasts in PAH placenta looked somewhat varied in size and shape (Figure 3a, middle). At E19, further differentiation of blood canals was observed in WT labyrinth. CK-positive specialized trophoblasts finely traced each terminal lumen, forming loop lines (Figure 3a, right). To the contrary, blood sinuses in PAH labyrinth were rough and irregular, and notched lumens extended branches of various sizes. These results suggested that distorted maternal blood sinuses in PAH labyrinth are unable to interact with fetal-derived microvessels appropriately at the circulatory terminus. Thus, we did double stainings against fetal microvessels and maternal blood sinuses. In WT labyrinth, at E19 it was shown that CD31-positive fetal ECs were closely encircled by CK-positive trophoblasts (Figure 3b, upper panels). On the

Figure 1 Histology of PAH placenta (E19). **(a)** Macroscopic appearances of WT and PAH placenta. Decidual sides are shown. Infarct areas are observed widely in PAH as whitish color changes. **(b)** Microscopic appearances of WT and PAH placenta. Three layers of decidua basalis (D), junctional zone (J) and labyrinth (L) are indicated. Diffuse fibrin deposition between D and J are shown (arrows). **(c)** Higher magnification of uteroplacental sites. Note the atherosclerosis, i.e. mural fibrinoid necrosis (arrows) and diffuse fibrin deposition (asterisk) in PAH. Insets: PTAH stainings of a spiral artery. Fibrinoid necrosis is highlighted as strong blue stainings in PAH. **(d)** TUNEL stainings of uteroplacental sites in E19 placenta. Positive cells are labeled by FITC (green).

Figure 2 Abnormal development of fetal-derived microvessels in PAH labyrinth. (a) PAM stainings of labyrinth. Fetal-derived vessels that contain nucleated red blood cells are indicated by arrows. PAM-positive basement membrane formation in fetal small vessels is detectable from E16 and accomplished at E19 in WT placenta, whereas it is poorly performed in PAH placenta. (b) Immunohistochemical stainings for CD31 and α -SMA of labyrinth at E19. Insets represent a double stainings for CD31 (green) and α -SMA (red). (c) The results of CD31-positive microvessel density (MVD) (upper) and α -SMA-positive MVD (lower). The data was obtained from every two center areas of five different samples in each group.

other hand, in PAH labyrinth many fetal ECs tubules were distantly located from blood sinuses (Figure 3b, lower panels).

Changes in Neovascularization-Related Gene Expression in PAH Labyrinth and Maternal Plasma Level of sVEGFR-1

We looked for the molecules that are involved in pathological neovascularization of PAH labyrinth, and the expression levels of angiogenesis/vasculogenesis-related genes were screened in E13, E16 and E19 labyrinth by RT-PCR ($n = 3$ per group) (Figure 4). As a result, nine molecules showed distinctive gene expression patterns between WT and PAH placentas (Figure 4a). In the regulation of neovascularization,

angiopoietin-1 (Ang-1) and Ang-2, and their specific receptor tyrosine kinase with Ig and EGF homology domain-1 (Tie-1) and Tie-2 are thought to play a critical role for ECs-pericytes interaction.^{17,18} It is known that Ang-2 antagonizes the activity of Ang-1 in ECs-pericytes interaction, and the balance between Ang-1 and Ang-2 is very important for vascular development.¹⁹ In PAH labyrinth, Ang-1 was downregulated at E16, and Ang-2 was upregulated at E13. In addition, Tie-1 and Tie-2 were markedly downregulated at E13 and E16 (Figure 4a, left), indicating that the Ang/Tie system was deranged in PAH labyrinth. Claudin-5, which belongs to tight junction molecules and contributes to cell-cell adhesion in ECs, was also downregulated in PAH labyrinth at E13 and E16 (Figure 4a, left). Recently, putative

Figure 3 Morphological abnormality of fetomaternal vascular network in PAH labyrinth. (a) Differentiation of maternal blood sinus in WT (upper) and PAH labyrinth (lower) is demonstrated by CK stainings. At E19, abnormal differentiation of PAH sinus becomes apparent as irregularly notched lumens (lower, right). (b) Double stainings of ECs (green) and trophoblasts (red) at E19 labyrinth. Encircled ECs tubules by trophoblasts are indicated by dotted lines.

receptor protein related to the angiotensin receptor AT1 (APJ) that belongs to a G protein-coupled receptor and its endogenous peptide ligand apelin were identified.^{20,21} Apelin and APJ are thought to be a new member for promoting the development of vasculature^{20,21} and regulating the BP as vasodilator.^{22,23} In this study, APJ and apelin expressions were clearly detected in WT labyrinth at E13 and E16, whereas the levels of those in PAH labyrinth were

concurrently suppressed (Figure 4a, right). In addition, in PAH labyrinth, the expression of eNOS, the main regulator of vasodilation, was also downregulated at E13, and strong vasoconstrictor ET-1 was upregulated from E16 to E19 (Figure 4a, right).

We next performed quantitative RT-PCR to compare precise gene expression levels including time-dependent expression patterns of these molecules between WT and PAH

Figure 4 Changes in the gene expression of key molecules in vascular development and functions, and maternal plasma level of sVEGFR-1. **(a)** Total RNA was prepared from the labyrinth of E13, E16 and E19, and RT-PCR was performed. Remarkable differences in the expression levels between PAH and WT labyrinths are marked by dotted rectangles. Representative results obtained from three independent samples of each gestational period are presented. **(b)** Absolute expression levels of each molecule against GAPDH are shown. White columns indicate the expression levels in WT and black columns indicate in PAH. Significant differences in the expression levels between PAH and WT labyrinths are marked by dotted rectangles. $^{**}P < 0.01$. **(c)** ELISA of sVEGFR-1. Maternal plasma sVEGFR-1 levels at E19 are significantly higher in PAH than those in WT ($n = 4$ per group at E19 and $n = 2$ per group at E10). $^{**}P < 0.01$.

labyrinth. Consistently with the results of RT-PCR analysis, the expression levels of these molecules were distinctively different at certain stages of placental vascular development (Figure 4b). In WT labyrinth, Ang-2, Tie-1, claudin-5, APJ, apelin and eNOS showed the highest expression at E13, and the peaks of Ang-1, Tie-2 and ET-1 were at E16. On the other hand, in PAH labyrinth the expression levels of Tie-1, Tie-2, claudin-5, APJ and eNOS were severely downregulated and that of Ang-2 was markedly upregulated within 24 h after the onset of hypertension. At E16, the expression levels of Ang-1, Tie-1, Tie-2, claudin-5, APJ and apelin were shown to be significantly suppressed in PAH labyrinth.

We hypothesized that angiogenic milieu of maternal circulation, in addition to the fetoplacental site, might be disturbed in PAH. Recent studies on human pregnancy-induced hypertension (PIH) demonstrated that sVEGFR-1 was significantly increased in the blood of PIH patients.²⁴ Therefore, we examined the circulatory levels of sVEGFR-1 in PAH females. Maternal plasma sVEGFR-1 levels in WT and PAH at E10 were 0.43 ± 0.07 and 0.38 ± 0.001 ng/ml, respectively (NS). At E19, plasma sVEGFR-1 levels in WT and PAH were 5.01 ± 0.26 and 6.93 ± 0.06 ng/ml, respectively, showing significant elevation in PAH females ($P < 0.01$) (Figure 4c). This result indicates that systemic maternal ECs dysfunction that represents the pathophysiology of human preeclampsia might also be the case in this model.

Suppression of Proteolytic Activities at Uteroplacental Site in PAH Placenta

Since invasive trophoblasts and trophoblast stem cells are known to regenerate and actively produce extracellular matrix for the normal vascular development in placenta,^{25,26} we hypothesized that tissue remodeling at the uteroplacental site might be disturbed in PAH mice. To investigate the proteolytic activities of uteroplacental site directly, we performed the film *in situ* zymography (FIZ) analysis in E19 placenta. Strong proteolytic activities were demonstrated in WT placenta (Figure 5a, lower left, bright area), and the active sites were found to coincide with uteroplacental site by comparison using serial section of HE stainings (Figure 5a, upper left, dotted circle). On the contrary, the proteolytic activity of PAH placenta was almost undetectable (Figure 5a, lower right, dark area). To explore the pathogenesis in proteolytic activity of PAH placenta, uteroplacental site, that is, decidua/junctional zone, was carefully separated from labyrinth, and the expression patterns of MMPs and tissue inhibitor of MMPs (TIMPs) were investigated. In RT-PCR

analysis ($n = 3$ per group), the expression of MMP-2 in uteroplacental site was strikingly downregulated in PAH at E16 (Figure 5b). On the other hand, the expression of its endogenous inhibitor TIMP-2 in uteroplacental site did not show significant difference between PAH and WT at E16. In addition, MMP-9 was also downregulated at E16, and its endogenous inhibitor TIMP-1 was suppressed in uteroplacental site of PAH (Supplementary Figure 4). By using quantitative RT-PCR, we confirmed that the expression of MMP-2 mRNA in uteroplacental site at E16 was severely suppressed in PAH (Figure 5c). The expression level of TIMP-2 in uteroplacental site was not different between PAH and WT at E16, and it was slightly higher in PAH than in WT at E19. These results demonstrated that tissue-remodeling enzymes at uteroplacental site increased exponentially around E16 in normal pregnancy, whereas their expression was severely suppressed in PAH.

We next evaluated the active form of MMP-2 in uteroplacental site by gelatin zymography analysis. Active form of MMP-2 was clearly detected in the uteroplacental site of WT (Figure 5d, left lane, arrow), whereas it was scarcely observed in PAH (Figure 5d, right lane). Similar result was obtained from the uteroplacental samples at E16, although active form of MMP-2 was faintly detected in PAH placenta (Supplementary Figure 4). Then, we confirmed by immunohistochemistry that the trophoblasts of these layers were positive for MMP-2 (data not shown). It has been noted that trophoblast stem cells undergo epithelial–endothelial transformation to become invasive, and that these cell types synthesize extracellular matrix.^{25,27,28} Therefore, we investigated progenitor cell population. By immunohistochemical stainings, decidua layer at E19 was shown to be diffusely positive for spinocerebellar ataxia type 1 (Sca-1) in WT placenta, indicating that this site contains abundant progenitor cells (Figure 5e, left, upper panel). Sca-1-positive cells in the decidua layer of PAH mice were far less in number than those of WT (Figure 5e, right, upper panel). Taken together with MMP-2 downregulation, these findings suggested that tissue-remodeling activities of uteroplacental site were suppressed in PAH. In addition to uteroplacental site, Sca-1 was detected in some of microvessels in the labyrinth of WT placenta (Figure 5e, left, lower panel). These Sca-1-positive microvessels of WT were round tubule in section whereas those of PAH were irregularly distorted (Figure 5e, right, lower panel). It was supported from our findings that the fetoplacental vascular development in the labyrinth was affected by maternal complication.

Figure 5 Suppression of tissue-remodeling activities in the uteroplacental site of PAH placenta. **(a)** FIZ at E19 placenta. Uteroplacental sites are encircled by dotted lines. In WT placenta, strong proteolysis is demonstrated in the area coincided with uteroplacental site. On the other hand, in PAH placenta, no significant proteolysis is detected. **(b)** RT-PCR analysis of the uteroplacental site of the placenta in each period. MMP-2 mRNA at E16 in PAH is scarcely detected (dotted rectangle). Representative results obtained from three independent samples of each gestational day are presented. **(c)** Quantitative RT-PCR analysis of the uteroplacental site of the placenta. Marked downregulation of MMP-2 mRNA at E16 (dotted rectangle) and upregulation of TIMP-2 mRNA at E19 in PAH are demonstrated. $^{**}P < 0.01$, $^{*}P < 0.05$. **(d)** Gelatin zymography analysis in uteroplacental site at E19. The expression of pro-enzymatic form of MMP-2 (72 kDa) and active form of MMP-2 (arrow) is stronger in WT than in PAH. **(e)** Immunohistochemical stainings of Sca-1 in uteroplacental sites (upper) and labyrinths (lower) at E19. Intensive stainings for Sca-1 are detected in decidua basalis of WT whereas the stainings are weak and sparse in PAH.

Comparison of Fetal Organs Between WT and PAH at E19

It is known that the presence of nucleated red blood cells (RBCs) in later stage of pregnancy reflects severe hypoxia and

anemia of the fetus.²⁹ We therefore counted the number of nucleated RBCs in the labyrinth at E19. The number of nucleated RBCs in E19 PAH placenta was significantly higher

than that in WT placenta (Figure 6a and b). Furthermore, PAH placenta contained nucleated RBCs islands in several sections (Figure 6a, inset). It was also shown by whole mount HE stainings that most of thoracic and visceral organs in PAH fetus were notably smaller in size (Figure 6c, upper panel). We compared the fetal organs, such as brain, lung, kidney and heart, macroscopically between WT and PAH. These organs in PAH fetus at E19 looked extremely whitish, and the sizes were smaller than those in WT except for the heart ventricle, which was dilated (Figure 6c, lower panels). These results indicated that the fetuses in PAH pregnancy suffered from severe hypoxia. This pathology was probably due to the defect of maternal–fetal circulatory exchanges induced by impaired fetoplacental vascular maturation and uteroplacental tissue remodeling in the later stage of pregnancy.

DISCUSSION

The roles of RAS in the placenta of human PIH remain to be controversial. Vascular AT-1 was downregulated in the placentas of PIH and IUGR cases.³⁰ The levels of active renin and angiotensin-II in the placentas of PIH did not differ from those in normal pregnancies³¹ and the plasma concentration of angiotensin-II was decreased in PIH.³² These studies suggest that RAS may not contribute directly to the pathogenesis of PIH. On the other hand, it is widely accepted as a classical knowledge that the systemic vascular sensitivity to angiotensin-II is elevated in PIH.³³ Furthermore, it was revealed the presence of AT-1-bradykinin B2 heterodimer³⁴ and agonistic autoimmune antibody against AT-1³⁵ in PIH. These studies suggest that AT-1-mediated signalings in PIH are activated. Therefore, pathological feature of PAH pregnancy should be carefully considered from both side, that is, aberrant AT-1-mediated signalings that are frequently observed in PIH and excessive angiotensin-II that does not reflect the pathogenesis of PIH in most cases. We previously investigated transgenic hRN^{+/+} female mated with transgenic hANG^{+/+} male, and hANG^{+/+} mAT-1a^{-/-} female mated with hRN^{+/+} male.^{9–11} In the former study, local level of angiotensin in the placenta was as high as that in PAH pregnancy, but neither significant histological changes in the placenta nor maternal hypertension occurred.^{9,11} In the latter study, maternal symptoms and fetal growth were markedly improved.¹⁰ Therefore, both ‘maternal hypertension’ and ‘AT-1a-mediated signalings in maternal vasculature and in decidua’ may concordantly play critical roles in PAH pregnancy. Moreover, we found that sVEGFR-1, a key molecule in PIH,²⁴ was elevated in the plasma of PAH female (Figure 4c), which supports our hypothesis that PAH may become a useful tool for investigating pathophysiology of human preeclampsia.

Time course analysis of fetoplacental vasculature showed that fetal side microvessels develop and differentiate actively during the second half of pregnancy, and that they are gradually stabilized by basement membrane and pericytes.

The present study demonstrated that the maternal hypertension, which started in the second half of pregnancy, suppressed the development and maturation of fetoplacental vasculature. CD31-positive fetal microvessels at term were abnormal in size and number, and poorly supported by pericytes and basement membrane in PAH placenta. These morphological abnormalities became overt at term. However, the expression of several key molecules required for ECs tubule formation and stabilization such as Ang-1/Tie-2 system was hampered within a few days after BP elevation (E13–E16). These results suggested that the fate of pathological neovascularization might be determined in the early phase after the onset of hypertension and that the morphological abnormality became overt during later stage.

In physiological neovascularization, cellular and structural maturation of vasculature is critical to establish stable blood flow. Ang and Tie are representative molecules that control crosstalk between ECs and pericytes.^{18,19} Ang-1 is known to be produced by pericytes and to collaborate with its receptor Tie-2 expressed in ECs. On the other hand, Ang-2 is stored in ECs and competitively inhibits the function of Ang-1. It has previously been reported that Ang-1, Ang-2 and Tie-2 were expressed in normal and preeclamptic human placenta in the first and third trimesters, and that Tie-2 interacted with Ang-1 in human umbilical vein ECs and cultured-trophoblast cell line.^{36,37} Time course analysis in our current study revealed that the expression levels of Ang/Tie were much higher at E13 and E16 than at term, suggesting the important roles of Ang/Tie signaling in the maturation of developing vasculature at midterm of gestation. We further demonstrated that in PAH placenta the expression level of Ang-2 was upregulated at E13, following marked suppression of Ang-1/Tie-2 at E16, and the microvessel densities were decreased in PAH placenta at E19. These results strongly suggest that downregulation of Ang-1/Tie-2 between E13 and E16 decelerated microvessel maturation, which was manifested morphologically as fragile vasculature at term. Although Tie-1 is an orphan receptor whose function remains unknown, Tie-1 is thought to associate physically with Tie-2 and to contribute to angiogenesis.³⁸ In the present study, the differential expression pattern of Tie-1 between WT and PAH placenta at E13 and E16 was correlated with that of Tie-2. Although it is expected that Tie-1 plays an important role, probably in concert with Tie-2, in the pathological neovascularization of preeclampsia, the detail of the physiological interaction between Tie-1 and Tie-2 is a subject for future study.

Other important findings in this study include that tissue remodeling at uteroplacental junction is suppressed from middle stage after BP elevation (E16) in PAH mice. It has been generally accepted that impaired pseudovasculogenesis and shallow invasion are determined at an early stage of gestation.³⁹ However, the constituents of uteroplacental junction keep remodeling throughout pregnancy by produ-

Figure 6 IUGR in PAH mice. **(a)** Significant numbers of nucleated RBCs are still detectable in PAH labyrinth at E19 (right). In some parts, nucleated RBCs are accumulated as an island (inset). **(b)** Mean number of nucleated RBCs in sagittal section, counted from ten specimens of each group is shown. **(c)** Comparison of sagittal body sections and the brains, lungs, kidneys and hearts between WT fetus (left) and PAH fetus (right) at E19. **(d)** Schema of pathological events in the placental labyrinth of PAH induced by RAS-mediated maternal hypertension. The downregulation of Ang/Tie system disrupts the interactions and crosstalk between ECs and pericytes. In addition, the downregulation of claudin-5, Apelin/APJ and eNOS on mid-pregnancy and the upregulation of ET-1 on later phase in PAH placenta enhance BP elevation and suppress the normal vascular maturation in placenta. These induce the poorly developed fetal vasculature, which cannot interact with maternal blood sinus appropriately, and lead to insufficient supply of oxygen and nutrients, resulting in the preeclamptic complications including maternal pathology and fetal IUGR.

cing extracellular matrix, and it has been poorly understood whether and how maternal hypertension affects the remodeling activities of uteroplacental junction. In this point, it should be noted that striking downregulation of MMP-2 occurred at E16, and the suppression of proteolytic activities *in situ* became obvious at E19 in PAH uteroplacental site. Re-expression of MMP-2 mRNA at E19 in PAH uteroplacental site may be due to extensive apoptosis and leukocytic accumulation in response (Figure 1d). We detected MMP-2-positive leukocytes by immunohistochemistry in PAH uteroplacental site at E19 (data not shown). However, the expression of TIMP-2 mRNA was higher in PAH than WT (Figure 5c). MMP-2 and TIMP-2 proteins are known to form complexes and their balance is important to determine tissue proteolytic activity.⁴⁰ Taken together, it is strongly suggested that the tissue remodeling of uteroplacental area is hampered by maternal hypertension at later stage of gestation, and that hypertension-induced local milieu leads to weakened placental anchorage to uterine wall. Further study is required whether human gestational hypertension unrelated to initial failure of pseudovasculogenesis would result in shallow placental-myometrial interaction during the second half of pregnancy.

APJ is a recently identified G protein-coupled receptor and expressing mainly in the cardiovascular system. We previously revealed by using APJ knockout mice that APJ exerted hypotensive effect and antagonistic role against angiotensin-II in BP regulation.⁴¹ In this study, it was demonstrated that the expression of apelin/APJ in WT placenta was the highest at E13 and decreased as gestation proceeded, whereas in PAH placenta, apelin/APJ were downregulated strikingly at E13. In PAH mice, excess of angiotensin-II is produced in latter half of pregnancy by the interaction between fetoplacental-derived hRN and maternal hANG,⁹ and it is recently reported that angiotensin-II-infused rats showed decrease of cardiac apelin mRNA.⁴² Taken together, our results support the possibility that the expression of apelin/APJ may be suppressed by angiotensin-II system in this model. Several studies have substantiated that apelin/APJ signaling causes vasodilation and hypotension via the activation of NO system.^{23,41,43} On the other hand, angiotensin-II was reported to increase total eNOS protein in ovine fetoplacental artery-derived ECs.⁴⁴ Therefore, concurrent downregulation of eNOS mRNA with apelin/APJ mRNAs in PAH placenta under excessive angiotensin-II needs further investigation. Claudin-5 belongs to tight junction molecules and contributes to cell-cell adhesion in ECs.⁴⁵ Interestingly, in this study, claudin-5 showed similar suppression pattern as apelin/APJ in PAH placenta. We have recently reported that apelin/APJ signaling enhanced the focal adhesion formation by using 293T cell line stably expressing APJ.⁴⁶ Although it remains unknown possible interaction between apelin/APJ system and claudin-5, the apelin/APJ signaling and claudin-5 might contribute in concert to the pathological placental neovascularization.

The present study provided important information about pathogenesis of abnormal development of placental vascular network in later periods of pregnancy in PAH mice. We demonstrated that maternal hypertension suppressed fetoplacental vascular maturation and uteroplacental tissue remodeling. We also showed that RAS-mediated hypertension caused sVEGFR-1 elevation in maternal blood. These factors probably induced deterioration of maternal-fetal circulatory exchanges and led to severe fetal hypoxia and IUGR. We summarize the events of pathogenesis of maternal and fetal complications in PAH mice in Figure 6d. Some of the key molecules in PAH, such as Ang-1, Tie-2, eNOS and ET-1, in addition to sVEGFR-1, are known to participate in the pathophysiology of human preeclampsia.^{37,47,48} Currently, very limited information is available about the changes in time-dependent expression of these molecules in human PIH. Therefore, these molecules might be noted as sensitive markers for early diagnosis of this disease. Further investigation is necessary to understand the pathological cascade of abnormal development of placental vasculature that must be critical for the collapse of circulatory interaction between mother and fetus.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGEMENT

We thank to the members of Fukamizu laboratory, Drs M Nishiyama, T Takenouchi, Y Nakatani, M Takiguchi and H Usui for discussion, and Mrs T Matsui and N Yamamura for their excellent technical assistance. This work was supported by Grant-in-Aid for Scientific Research (S) (AF), Grant-in-Aid for Exploratory Research (AF), and Grant-in-Aid for Scientific Research (C) (MF) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

1. National high blood pressure education program working group on high blood pressure in pregnancy report of the national high blood pressure education program working group on high blood pressure in pregnancy. *Am J Obstet Gynecol* 2000;183:S1-S22.
2. Parham P. NK cells and trophoblasts: partners in pregnancy. *J Exp Med* 2004;200:951-955.
3. Hiby SE, Walker JJ, O'Shaughnessy KM, *et al*. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 2004;200:957-965.
4. Hu Y, Dutz JP, MacCalman CD, *et al*. Decidual NK cells alter *in vitro* first trimester extravillous cytotrophoblast migration: a role for IFN-gamma. *J Immunol* 2006;177:8522-8530.
5. Kobashi G, Hata A, Shido K, *et al*. Association of a variant of the angiotensinogen gene with pure type of hypertension in pregnancy in the Japanese: implication of a racial difference and significance of an age factor. *Am J Med Genet* 1999;86:232-236.
6. van Dijk M, Mulders J, Poutsma A, *et al*. Maternal segregation of the Dutch preeclampsia locus at 10q22 with a new member of the winged helix gene family. *Nat Genet* 2005;37:514-519.
7. Johnson MP, Fitzpatrick E, Dyer TD, *et al*. Identification of two novel quantitative trait loci for pre-eclampsia susceptibility on chromosomes 5q and 13q using a variance components-based linkage approach. *Mol Hum Reprod* 2007;13:61-67.
8. Reynolds LP, Borowicz PP, Vonnahme KA, *et al*. Animal models of placental angiogenesis. *Placenta* 2005;26:689-708.
9. Takimoto E, Ishida J, Sugiyama F, *et al*. Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen. *Science* 1996;274:995-998.

10. Saito T, Ishida J, Takimoto-Ohnishi E, *et al.* An essential role for angiotensin II type 1a receptor in pregnancy-associated hypertension with intrauterine growth retardation. *FASEB J* 2004;18:388–390.
11. Takimoto-Ohnishi E, Saito T, Ishida J, *et al.* Differential roles of renin and angiotensinogen in the fetomaternal interface in the development of complications of pregnancy. *Mol Endocrinol* 2005;19:1361–1372.
12. Kasai A, Shintani N, Oda M, *et al.* Apelin is a novel angiogenic factor in retinal endothelial cells. *Biochem Biophys Res Commun* 2004;325:395–400.
13. Boven LA, Middel J, Breij EC, *et al.* Interactions between HIV-infected monocyte-derived macrophages and human brain microvascular endothelial cells result in increased expression of CC chemokines. *J Neurovirol* 2000;6:382–389.
14. Furuya M, Ishikura H, Nemori R, *et al.* Clarification of the active gelatinolytic sites in human ovarian neoplasms using *in situ* zymography. *Hum Pathol* 2001;32:163–168.
15. Adamson SL, Lu Y, Whiteley KJ, *et al.* Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol* 2002;250:358–373.
16. Johansen M, Redman CWG, Wilkins T, *et al.* Trophoblast deportation in human pregnancy—its relevance for pre-eclampsia. *Placenta* 1999;20:531–539.
17. Davis S, Aldrich TH, Jones PF, *et al.* Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 1996;87:1161–1169.
18. Suri C, Jones PF, Patan S, *et al.* Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 1996;87:1171–1180.
19. Imhof BA, Aurrand-Lions M. Angiogenesis and inflammation face off. *Nat Med* 2006;12:171–172.
20. O'Dowd BF, Heiber M, Chan A, *et al.* A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 1993;136:355–360.
21. Tatemoto K, Hosoya M, Habata Y, *et al.* Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998;251:471–476.
22. Kleinz MJ, Davenport AP. Immunocytochemical localization of the endogenous vasoactive peptide apelin to human vascular and endocardial endothelial cells. *Regul Pept* 2004;118:119–125.
23. Tatemoto K, Takayama K, Zou MX, *et al.* The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept* 2001;99:87–92.
24. Maynard SE, Min JY, Merchan J, *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003;111:649–658.
25. Kraus FT, Redline RW, Gersell DJ, *et al.* Placental Pathology. AFIP: Washington, DC, 2004, pp 1–22.
26. Teesalu T, Masson R, Basset P, *et al.* Expression of matrix metalloproteinases during murine chorioallantoic placenta maturation. *Dev Dyn* 1999;214:248–258.
27. Rossant J, Cross JC. Placental development: lessons from mouse mutants. *Nat Rev Genet* 2001;2:538–548.
28. Zhou Y, McMaster M, Woo K, *et al.* Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol* 2002;160:1405–1423.
29. Lewis SH, Benirschke K. Placenta. In: Sternberg SS (ed). *Placenta In Histology for Pathologist*, 2nd edn. Lippincott-Raven: New York, 1997, pp 961–996.
30. Knock GA, Sullivan MH, McCarthy A, *et al.* Angiotensin II (AT1) vascular binding sites in human placentae from normal-term, preeclamptic and growth retarded pregnancies. *J Pharmacol Exp Ther* 1994;271:1007–1015.
31. Kalenga MK, Thomas K, de Gasparo M, *et al.* Determination of renin, angiotensin converting enzyme and angiotensin II levels in human placenta, chorion and amnion from women with pregnancy induced hypertension. *Clin Endocrinol (Oxf)* 1996;44:429–433.
32. Hanssens M, Keirse MJ, Spitz B, *et al.* Angiotensin II levels in hypertensive and normotensive pregnancies. *Br J Obstet Gynaecol* 1991;98:155–161.
33. Gant NF, Daley GL, Chand S, *et al.* A study of angiotensin II pressor response throughout primigravid pregnancy. *J Clin Invest* 1973;52:2682–2689.
34. AbdAlla S, Lother H, el Massiery A, *et al.* Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. *Nat Med* 2001;7:1003–1009.
35. Wallukat G, Homuth V, Fischer T, *et al.* Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. *J Clin Invest* 1999;103:945–952.
36. Dunk C, Shams M, Nijjar S, *et al.* Angiopietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development. *Am J Pathol* 2000;156:2185–2199.
37. Zhang EG, Smith SK, Baker PN, *et al.* The regulation and localization of angiopoietin-1, -2, and their receptor Tie2 in normal and pathologic human placentae. *Mol Med* 2001;7:624–635.
38. Tsiamis AC, Morris PN, Marron MB, *et al.* Vascular endothelial growth factor modulates the Tie-2: Tie-1 receptor complex. *Microvasc Res* 2002;63:149–15827.
39. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 2005;308:1592–1594.
40. Fernandez-Catalan C, Bode W, Huber R, *et al.* Crystal structure of the complex formed by the membrane type 1-matrix metalloproteinase with the tissue inhibitor of metalloproteinases-2, the soluble progelatinase A receptor. *EMBO J* 1998;17:5238–5248.
41. Ishida J, Hashimoto T, Hashimoto Y, *et al.* Regulatory roles for APJ, a seven-transmembrane receptor related to AT1, in blood pressure *in vivo*. *J Biol Chem* 2004;279:26274–26279.
42. Iwanaga Y, Kihara Y, Takenaka H, *et al.* Down-regulation of cardiac apelin system in hypertrophied and failing hearts: Possible role of angiotensin II-angiotensin type 1 receptor system. *J Mol Cell Cardiol* 2006;41:798–806.
43. Zhong JC, Huang DY, Liu GF, *et al.* Effects of all-trans retinoic acid on orphan receptor APJ signaling in spontaneously hypertensive rats. *Cardiovasc Res* 2005;65:743–750.
44. Zheng J, Bird IM, Chen DB, *et al.* Angiotensin II regulation of ovine fetoplacental artery endothelial functions: interactions with nitric oxide. *J Physiol* 2005;565:59–69.
45. Morita K, Sasaki H, Furuse M, *et al.* Endothelial claudin: claudin-5/TMVCf constitutes tight junction strands in endothelial cells. *J Cell Biol* 1999;147:185–194.
46. Hashimoto Y, Ishida J, Yamamoto R, *et al.* protein-coupled APJ receptor signaling induces focal adhesion formation and cell motility. *Int J Mol Med* 2005;16:787–792.
47. Kharfi A, Giguère Y, Sapin V, *et al.* Trophoblastic remodeling in normal and preeclamptic pregnancies: implication of cytokines. *Clin Biochem* 2003;36:323–331.
48. Granger JP, Alexander BT, Bennett WA, *et al.* Pathophysiology of pregnancy-induced hypertension. *Am J Hypertens* 2001;14:1785–1855.