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Angiogenic Imbalance and Diminished Matrix Metalloproteinase-2 and -9 Underlie Regional Decreases in Uteroplacental Vascularization and Feto-placental Growth in Hypertensive Pregnancy

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Abstract

Preeclampsia is a form of hypertension-in-pregnancy (HTN-Preg) with unclear mechanism. Generalized reduction of uterine perfusion pressure (RUPP) could be an initiating event leading to uteroplacental ischemia, angiogenic imbalance, and HTN-Preg. Additional regional differences in uteroplacental blood flow could further affect the pregnancy outcome and increase the risk of preeclampsia in twin or multiple pregnancy, but the mechanisms involved are unclear. To test the hypothesis that regional differences in angiogenic balance and matrix metalloproteinases (MMPs) underlie regional uteroplacental vascularization and feto-placental development, we compared fetal and placental growth, and placental and myoendometrial vascularization in the proximal, middle and distal regions of the uterus (in relation to the iliac bifurcation) in normal pregnant (Preg) and RUPP rats. Maternal blood pressure and plasma anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt-1)/placenta growth factor (PlGF) ratio were higher, and average placenta number, placenta weight, litter size, and pup weight were less in RUPP than Preg rats. The placenta and pup number and weight were reduced, while the number and diameter of placental and adjacent myoendometrial arteries, and MMP-2 and MMP-9 levels/activity were increased, and sFlt-1/PlGF ratio was decreased in distal vs proximal uterus of Preg rats. In RUPP rats, the placenta and pup number and weight, the number and diameter of placental and myoendometrial arteries, and MMP-2 and -9 levels/activity were decreased, and sFlt-1/PlGF ratio was increased in distal vs proximal uterus. Treatment with sFlt-1 or RUPP placenta extract decreased MMP-2 and MMP-9 in distal segments of Preg uterus, and treatment with PlGF or Preg placenta extract restored MMP levels in distal segments of RUPP uterus. Thus, in addition to the general reduction in placental and fetal growth during uteroplacental ischemia, localized angiogenic imbalance and diminished MMP-2 and MMP-9 could cause further decrease in placental and myoendometrial vascularization and placental and fetal growth in distal vs proximal uterus of HTN-Preg rats. Regional differences in uteroplacental perfusion, angiogenic balance and MMPs could be a factor in the incidence of preeclampsia in multiple pregnancy.

Keywords

angiogenesis; hypertension; matrix metalloproteinases; multiple pregnancy; preeclampsia

1. Introduction

Normal pregnancy is associated with significant uteroplacental, hemodynamic and vascular changes. Uterine hypertrophy and distention provide sufficient space for the growing fetus. Placental remodeling and trophoblast invasion of spiral arteries maintain adequate blood supply to the developing fetus. Hemodynamic changes in the maternal circulation including increased heart rate, plasma volume and cardiac output, and decreased vascular resistance maintain blood supply to different tissues with little change in blood pressure (BP) [1, 2]. These pregnancy-related uteroplacental and vascular alterations involve substantial structural and functional changes in the uterus, placenta and maternal circulation [3, 4].

In 5–8% of pregnancies women develop preeclampsia [5], manifested as hypertension-in-pregnancy (HTN-Preg) and often proteinuria. If untreated, preeclampsia could progress to eclampsia with severe hypertension, cerebral edema and seizures, and may account for 15–20% of maternal deaths [6–8]. Preeclampsia is also associated with intrauterine fetal growth restriction (IUGR), accounting for 10–15% of preterm births [7, 9]. However, the mechanisms of preeclampsia are not clearly understood. Because of the difficulty to perform mechanistic studies in pregnant women, animal models of HTN-Preg have been developed [10]. Studies in late pregnant rats with reduced uterine perfusion pressure (RUPP) [11–14] and in rat models of gestational hypoxia [15] have shown some of the features of preeclampsia, and suggested that impaired spiral artery remodeling could be an initiating event leading to placental ischemia/hypoxia in the setting of HTN-Preg.

Placental ischemia/hypoxia could trigger the release of various bioactive factors in the maternal circulation [16–21], and changes in the angiogenic vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) could play a role in preeclampsia [22–24]. *VEGF* gene expresses a family of proteins including VEGF-A, -B, -C, -D and PlGF [25]. VEGF-A, VEGF-B and PlGF bind to tyrosine kinase receptor Flt-1 (VEGFR-1) and VEGF-A binds to VEGFR-2 (Flk-1 or KDR) to promote placental vascularization [25]. Measurements of circulating levels of VEGF in preeclampsia have not been consistent, with studies showing decreased [26], unchanged [27, 28] or even increased levels [29–31]. On the other hand, PlGF has only 1/10th the affinity for VEGFR-1 compared to VEGF, but its levels are ~40 times higher than VEGF during normal pregnancy [32]. PlGF has four alternatively spliced mRNA species (PlGF 1–4), and the circulating levels of its predominant isoform PlGF-1 is decreased in preeclampsia [29, 33]. In contrast with angiogenic factors, soluble fms-like tyrosine kinase-1 (sFlt-1, sVEGFR-1) is an anti-angiogenic factor expressed as an alternatively spliced variant of VEGFR-1 that lacks both the transmembrane and cytoplasmic domains. sFlt-1 binds VEGF and PlGF in the circulation and inhibits their action on cell surface VEGFR-1 [34]. Circulating levels of sFlt-1 are 10-fold higher in pregnant than non-pregnant women, remain almost stable during the first and second trimester, then increase after the 36th week of gestation and throughout the third trimester

[20]. The circulating sFlt-1 levels and sFlt-1/PlGF ratio are much higher in preeclamptic than normal pregnant women from the second trimester onwards [25, 29, 33, 35, 36]. These observations suggest that angiogenic imbalance could be a mechanism linking RUPP to HTN-Preg. In support, restoring the angiogenic balance by infusing recombinant VEGF or PlGF lowers BP and improves renal function in RUPP rat model of placental ischemia and HTN-Preg [22–24].

Matrix metalloproteinases (MMPs) degrade various components of the extracellular matrix (ECM) and play a role in vascular remodeling [37], as well as endometrial tissue remodeling during the estrous cycle, menstrual cycle and pregnancy [37–40]. We have previously shown upregulation of MMPs in uterus and aorta of normal pregnant (Preg) rats [41, 42]. We have also shown that MMPs are altered in uterine, placental and aortic segments of RUPP rats, and suggested a role of MMPs in growth-restrictive remodeling in HTN-Preg [13].

In contrast with the extensive vascularization and growth-permissive environment during healthy singleton pregnancy [3, 4], twin or multiple pregnancy could pose a challenge to the maternal circulation and uteroplacental remodeling. Uteroplacental and vascular remodeling may require critical physiological adjustments in twin or multiple pregnancy [43–45]. Regional differences in uteroplacental blood flow could affect the pregnancy outcome, and may be a factor in the increased risk of preeclampsia in twin or multiple pregnancy [46–49]. Also, circulating levels of angiogenic and anti-angiogenic factors are altered in twin vs singleton pregnancies [45] and in twin pregnancies with suspected preeclampsia [44, 49]. However, the extent of uteroplacental vascularization and the molecular mechanisms regulating uteroplacental remodeling in normal multiple pregnancy versus multiple pregnancy complicated with preeclampsia are unclear. Specifically, the role of angiogenic imbalance and MMPs in placental and fetal development and vascular remodeling in the different regions of the uterus during multiple pregnancy are unclear.

The present study was designed to test the hypothesis that localized angiogenic imbalance and changes in MMPs underlie regional differences in uteroplacental vascularization and placental and fetal development during multiple pregnancy, and that these regional differences are exaggerated during placental ischemia. We used normal Preg rats and RUPP rat model of placental ischemia to test whether: 1) Placental and fetal growth and uteroplacental vascularization are different in the proximal, middle and distal regions of the uterus (relative to the iliac bifurcation) in Preg rats, and the differences are exaggerated in the corresponding regions in RUPP rats. 2) The regional differences in uteroplacental vascularization and placental and fetal growth reflect regional changes in MMP-2 and MMP-9 expression/activity. 3) The regional differences in uteroplacental remodeling and MMPs reflect regional changes in angiogenic and anti-angiogenic factors and their effect on MMPs.

2. Materials and Methods

2.1. Animals

Timed-pregnant (day 11) Sprague-Dawley rats (12 weeks of age, Charles River Laboratories, Wilmington, MA) were housed in the animal facility and maintained on *ad*

libitum standard rat chow and tap water in 12 hour light-dark cycle. There are two main sources of blood supply to the uteroplacental circulation in the rat; the uterine arteries which originate from the iliac arteries, and the uterine branches of the ovarian arteries, which originate from the abdominal aorta on the right uterine horn and the left renal artery on the left uterine horn [50, 51]. On gestational day 14, some of the rats underwent surgical procedure to reduce uteroplacental perfusion pressure (RUPP) by banding the lower abdominal aorta above the iliac bifurcation and the main uterine branches of the ovarian arteries as previously described [17, 24]. Briefly, the abdominal aorta near the iliac bifurcation was carefully dissected free of surrounding tissue and perivascular fat and separated from the vena cava. A blunt plastic rod (OD, 0.3 mm) was placed parallel to the aorta, and a 4–0 silk braided ligature was knotted twice around both the aorta and the adjacent plastic rod. Once taut, the rod was carefully removed from the knotted ligature, thus creating a constrictive band (ID, 0.3 mm) and reducing blood flow through the aorta. This procedure has been shown to reduce uterine perfusion pressure in the gravid rat by ~40% [52]. Since some compensation of blood flow to the placenta could occur through an adaptive increase in ovarian blood flow [53], a blunt plastic rod (OD, 0.1 mm) was used to place a ligature band (ID, 0.1 mm) on the main uterine branches of both the right and left ovarian arteries, specifically avoiding the main ovarian artery, such that the blood supply to the ovary would not be compromised. Normal pregnant (Preg) rats were sham operated. RUPP rats in which the banding procedure resulted in total reabsorption of the pups were not included in the data analyses. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the guidelines of American Physiological Society and the Institutional Animal Care and Use Committee at the Brigham and Women Hospital.

2.2. Blood Pressure (BP)

On gestational day 19, rats were anaesthetized with isoflurane, a PE-50 catheter was inserted in the carotid artery, and exteriorized at the back of the neck. The rats were allowed to recover from anesthesia for at least 1 h. The carotid arterial catheter was connected to a pressure transducer attached to an amplifier and pressure recorder (Living System Instrumentation, Burlington, VT), and BP in conscious rats was recorded [54].

2.3. Plasma sFlt-1 and PlGF

After measuring BP, blood samples were collected via the arterial catheter into sterile heparin tubes (Tyco Healthcare, Mansfield, MA). Plasma was separated by blood centrifugation at 2000×g for 10 min, and stored at –80°C. Plasma sFlt-1 levels were measured using rat sFlt-1 ELISA microplate kit (MBS2602003, MyBiosource) according to the manufacturer instructions, with 0.05 ng/ml sFlt-1 sensitivity, intra-assay and inter-assay precision, and coefficient of variability (CV) <12 %. Plasma PlGF levels were measured using rat PlGF ELISA microplate kit (MBS703282, MyBiosource) according to the manufacturer instructions, with <0.78 pg/ml PlGF sensitivity, intra-assay and inter-assay precision, and coefficient of variability (CV) <10 %. Absorbance was measured at 450 nm on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) as previously described [24].

2.4. Placental, Uterine and Fetal parameters

After measuring BP, rats were euthanized by inhalation of CO₂, the abdominal cavity was opened, and the two uterine horns (right and left) were exposed. For each uterine horn, three uterine regions were assigned as proximal, middle and distal (relative to the iliac bifurcation), such that the proximal region is the closest to the iliac bifurcation, the distal region is farthest from the iliac bifurcation, and the middle region is in between. The total number of pups and placentae as well as the number of pups and placentae in the proximal, middle, and distal regions of the uterus were recorded. Given that in the control Preg rat each uterine region contained 2 to 3 pups and placentae, each region was further subdivided into sections and assigned a number, such that sections 1 to 2 represent the proximal region, sections 3, 4 and 5 represent the middle region, and sections 6, 7 and 8 represent the distal region (Fig. 1). It could be argued that because RUPP rats generally have a lower number of pups/placentae, this may cause an overlap in the region classification in as much as what is proximal region in the controls may include middle region in RUPP and what is middle region in the controls may include distal region in RUPP. However, this should not affect our region classification as the uterine regions and subsections were assigned relative to the iliac bifurcation, and regardless of the presence or absence of pups/placentae. Also, images of the uterus were acquired using digital camera and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Images were magnified and the placental arteries feeding the placenta, and the adjacent myoendometrial arteries feeding the myometrial region between two placentae were defined, and their number and external diameter (in μm) were recorded. The pregnant uterus was then cut open, the placentae and pups were separated, gently blotted between filter papers, and the individual pup and placenta weight were recorded.

The placenta and uterus were cut into 5 mm wide segments to facilitate tissue homogenization and protein extraction. Placental segments were obtained from the entire placenta, and uterine segments containing the decidua were used. Placental and uterine segments from proximal, middle and distal regions of the uterus of Preg and RUPP rats were homogenized, the tissue homogenate was centrifuged at $10,000 \times g$ for 10 min at 4°C, the supernatant was collected, and the placental and uterine extracts were prepared for measurement of sFlt-1 and PlGF as described above, or used for Western blot or gelatin zymography experiments. Some placental and uterine segments from the distal region of the Preg uterus were incubated in the presence of the anti-angiogenic factor sFlt-1 (0.1 $\mu\text{g/ml}$, R&D Systems, Minneapolis, MN) [13] or placental extract from the distal placentae of RUPP rats for 48 h in tissue culture medium. For comparison, some placental and uterine segments from the distal region of the RUPP uterus were incubated in the presence of the angiogenic factor PlGF (0.1 $\mu\text{g/ml}$, MyBioSource, San Diego, CA) or placental extract from the distal placentae of Preg rats for 48 h in tissue culture medium. We used placental extract because our hypothesis and other studies suggest that the placenta is the main culprit in preeclampsia and a major source of sFlt-1 and PlGF [23, 24, 34, 49]. Tissues were treated with the same amount of placental extract protein (10 $\mu\text{g/ml}$), and maintained in organ culture medium. The culture medium was changed and fresh sFlt-1 or PlGF or placental extract was added every 24 h. We selected PlGF over the other angiogenic factor VEGF because most studies show decreased PlGF levels in preeclampsia [29, 33, 35], while

measurements of VEGF have not been consistent, with studies showing decreased [26], no change [27, 28] or even increased levels [29–31]. Also, PlGF is specific for VEGFR-1 and its soluble form sFlt-1, while VEGF also binds to VEGFR-2, causing increased vascular permeability and edema [55]. We avoided using higher concentrations of PlGF as excess PlGF may induce microvascular abnormalities [56]. Following the respective treatment, the tissues were stored at -80°C for Western blot and gelatin zymography analysis.

2.4. Western blots

Placental and uterine strips were homogenized in a homogenization buffer containing 20 mM 3-[N-morpholino] propane sulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol, 1.2 mM EDTA, 0.02% BSA, 5.5 μM leupeptin, 5.5 μM pepstatin, 2.15 μM aprotinin and 20 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, using a 2-ml tight-fitting homogenizer (Kontes Glass Co., Vineland, NJ). The tissue homogenate was centrifuged at $10,000 \times g$ for 10 min and the supernatant was collected. If the supernatant contained floating debris, centrifugation was repeated to obtain a clear supernatant. Protein concentration in the supernatant was determined using a protein assay kit (Bio-Rad, Hercules, CA). Protein extracts (20 μg) were combined with an equal volume of $2\times$ Laemmli loading buffer, boiled for 5 min, and size fractionated by electrophoresis on 8% SDS-polyacrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane by electroblotting. Membranes were incubated in 5% nonfat dry milk in phosphate buffered saline (PBS)-Tween for 1 h and then with polyclonal rabbit anti-MMP-2 (sc-10736, 1:1000), or anti-MMP-9 antibody (sc-10737, 1:1000) (Santa Cruz Biotechnology, Dallas, TX) for 24 h at 4°C . Negative control experiments were performed with the omission of primary antibody, and exhibited no detectable immunoreactive bands. In other more stringent control experiments, the specimens were treated with heat-inactivated MMP-2 or MMP-9 antibody (Repeated 10 times heating at 75°C for 30 sec and cooling at 4°C for 1 min) [57], and no immunoreactive bands could be detected using the heat-inactivated antibody. Membranes were washed 3 times for 15 min each in PBS-Tween then incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 2 h. Membranes were washed another 5 times 15 min each in PBS-Tween, and the immunoreactive bands were detected using enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The blots were subsequently re-probed for β -actin (1:5000). The reactive bands were analyzed by optical densitometry and ImageJ software. The densitometry values represented the pixel intensity normalized to β -actin to correct for loading [13, 41, 42].

2.6. Gelatin zymography

Placental and uterine tissue homogenates (without dithiothreitol) were subjected to electrophoresis on 8% SDS polyacrylamide gel containing 0.1% gelatin (Sigma, St. Louis, MO). The gel was then incubated in a zymogram renaturing buffer containing 2.5% Triton X-100 (Sigma) with gentle agitation for 30 min at room temperature. The gel was then equilibrated in a zymogram developing buffer (pH 6.7) containing 50 mM Tris-base, 0.2 M NaCl, 5 mM CaCl_2 , 0.02% Brij35 (Fisher Scientific, Pittsburgh, PA), and 1 μM ZnCl_2 (Sigma) for 30 min at room temperature, then incubated in the zymogram developing buffer at 37°C for 16 h. The gel was stained with 0.5% coomassie blue R-250 (Sigma) for 30 min, then destained with an appropriate coomassie R-250 destaining solution (methanol: acetic

acid: water = 50:10:40). Areas corresponding to MMP-2 and MMP-9 gelatinolytic activity appeared as clear bands against a blue background, while actin appeared as a dark blue band at 43 kDa against a light blue background. In order to control for the inter-gel variation, similar amount of loading protein (1 μ g) from different groups was used in different gels. Also, the clear MMP bands were analyzed by optical densitometry and ImageJ software, and the integrated protease activity density was measured as pixel intensity $\times$ mm² normalized to actin intensity to correct for loading and inter-gel variation as previously described [13, 41, 42].

2.7. Solution and Drugs

Krebs solution was used for tissue dissection and contained in mM 120 NaCl, 5.9 KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 11.5 dextrose, 2.5 CaCl₂, 1.2 MgCl₂, and bubbled with 95% O₂ and 5% CO₂, at pH 7.4. The tissue culture medium used to pretreat the tissues with PIGF, sFlt-1 or placental extract from Preg or RUPP rats was composed of Minimum Essential Medium supplemented with penicillin, streptomycin, and amphotericin B (Invitrogen, Grand Island, NY). PBS was used in Western blot experiments and contained in mM: 137 NaCl, 2.7 KCl, 8 Na₂HPO₄, 2 KH₂PO₄, at pH 7.4. All other chemicals were of reagent grade or better.

2.8. Statistical Analysis

Data from Preg and RUPP rats were analyzed and presented as means $\pm$ SEM with “n” representing the number of rats per group (Preg n=13, RUPP n=10). Data were analyzed and plotted using Prism (v.5.01; GraphPad Software, San Diego, CA). Data were first analyzed using ANOVA with multiple classification criteria [rat group (Preg vs. RUPP) or region of the uterus (distal vs. proximal and middle)]. When a statistical difference was observed, the data were further analyzed using Bonferroni's *post-hoc* test for multiple comparisons. Student's unpaired *t*-test was used for comparison of two means. Also, for comparison of groups with 2–3 pups/uterine region, Student's unpaired *t*-test was used. Differences were considered statistically significant if *P* < 0.05.

3. Results

3.1. Average Maternal and Fetal Parameters

Maternal measurements on gestational day 19 showed that BP was significantly higher in RUPP than Preg rats (Table 1). After animal euthanasia and excising the uterus, the uterine weight (without placentae or pups) was significantly lower in RUPP than Preg rats (Table 1). The total number of placentae per uterus varied between 9 to a maximum of 16 in Preg rats and between 5 to a maximum of 12 in RUPP rats (Table 1) (Fig. 1). The total number of placentae per uterus was significantly reduced in RUPP compared to Preg rats (Table 1). Also, the average placenta weight was significantly less in RUPP compared to Preg rats (Table 1). Fetal measurements showed that both the number of pups per uterus (litter size) and the average pup weight were significantly reduced in RUPP vs Preg rats (Table 1). Calculation of placental efficiency as individual pup weight/placenta weight [58, 59] indicated that it was significantly reduced in RUPP compared with Preg rats (Table 1).

3.2. Regional Differences in Number and Size of Placentae and Pups

Further analysis of the proximal, middle and distal regions of the uterus, in relation to distance from the iliac artery bifurcation, indicated that there were regional differences in placental and fetal development in Preg rats (Fig. 1A), and these regional differences were more pronounced in RUPP rats (Fig. 1B). The frequency and weight of placenta (Fig. 1C and 1E) and the frequency and weight of pups (Fig. 1C and 1G) in Preg rats were decreased in distal compared to proximal regions of the uterus.

In general, the frequency of the placenta or pup was less in RUPP vs Preg rats (Fig. 1C and 1D). The RUPP uterus also showed further reductions in the frequency of placenta or pup in distal vs. proximal and middle regions (Fig. 1D). In effect, the RUPP uterus showed complete absence of placenta or pup in the far distal region (Fig. 1D). The individual placenta weight in the proximal, middle and distal regions of the uterus of RUPP rats was generally less than that in the corresponding regions of Preg rats (Fig. 1E and 1F). Also, the individual placenta weight was less in distal vs proximal and middle region of the RUPP uterus (Fig. 1F). In general, the individual pup weight in the proximal, middle and distal regions of the uterus of RUPP rats was less than that in the corresponding regions of Preg rats (Fig. 1G and 1H). Also, the individual pup weight was significantly less in distal vs proximal and middle regions of RUPP uterus (Fig. 1H).

3.3. Regional Differences in Placental and Myoendometrial Vessels

Regional differences in uteroplacental vascularization were observed in the Preg (Fig. 2A) and RUPP uterus (Fig. 2B). Interestingly, the number and external diameter of placental arteries were greater in the distal compared to proximal region of the Preg uterus (Fig. 2C and 2E). Also, the number and external diameter of myoendometrial arteries were greater in the distal compared to proximal regions of the Preg uterus (Fig. 2G and 2I).

Analysis of the RUPP vessels showed a trend for an increase in the number and diameter of placental and myoendometrial arteries in the middle region and part of the distal region compared to the proximal region of the RUPP uterus (Fig. 2D, 2F, 2H, 2J). However, this trend ceased in the far distal region, where the number and diameter of placental and myoendometrial arteries were significantly reduced compared to the proximal and middle regions of the RUPP uterus (Fig. 2D, 2F, 2H, 2J). Additionally, the number and diameter of placental and myoendometrial arteries in the proximal, middle and distal regions of the RUPP uterus (Fig. 2D, 2F, 2H, 2J) were generally less than those in the corresponding regions of the Preg uterus (Fig. 2C, 2E, 2G, 2I).

3.4. Regional Differences in Gelatinase Activity of Uteroplacental MMPs

Gelatin zymography analysis of placenta and uterine tissue homogenates from Preg and RUPP rats revealed proteolytic bands corresponding to pro-MMP-9, MMP-9, pro-MMP-2, and MMP-2 (Fig. 3A and 3B). The gelatinolytic activity corresponding to pro-MMP-9, MMP-9, pro-MMP-2, and MMP-2 was increased in placental (Fig. 3C, 3E, 3G and 3I) and uterine segments (Fig. 3D, 3F, 3H and 3J) from distal vs. proximal regions of the Preg uterus. In contrast, the gelatinase activity corresponding to pro-MMP-9, MMP-9, pro-MMP-2, and MMP-2 was reduced in placenta (Fig. 3C, 3E, 3G, 3I) and uterine segments

(Fig. 3D, 3F, 3H, 3J) from distal vs. proximal region of the RUPP uterus. The zymography data also showed a consistent reduction of gelatinase activity of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 in the placental and uterine segments in the distal region of the uterus of RUPP vs. Preg rats (Fig. 3).

We also measured the protein levels of uteroplacental MMPs in Preg and RUPP rats. Western blots revealed immunoreactive bands corresponding to pro-MMP-9 (92 kDa), MMP-9 (82 kDa), pro-MMP-2 (72 kDa) and MMP-2 (63 kDa) in placental and uterine tissue homogenates of proximal, middle and distal regions of the Preg and RUPP uterus (Fig. 4A and Fig. 4B). The amount of pro-MMP-9, MMP-9, pro-MMP-2, and MMP-2 in the placenta (Fig. 4C, 4E, 4G, 4I), and the amount of MMP-9 and MMP-2 in the uterus (Fig. 4F, 4J) were increased in distal vs. proximal regions of the Preg uterus. In contrast, the amount of MMP-9 and MMP-2 in placenta and uterine segments was decreased in the distal vs. proximal regions of the RUPP uterus (Fig. 4). Western blots also showed a consistent reduction in the amount of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 in the placenta and of MMP-9 and MMP-2 in uterine segments from the distal region of RUPP vs. Preg uterus (Fig. 4).

3.5. Regional differences in uteroplacental sFlt-1, PlGF, and sFlt-1/PlGF Ratio

Plasma measurements showed increased sFlt-1 levels, decreased PlGF levels and increased sFlt-1/PlGF ratio (Table 1). Placental and uterine extracts were used to measure sFlt-1, PlGF, and sFlt-1/PlGF ratio in different regions of the uterus of Preg and RUPP rats (Fig. 5). The levels of sFlt-1 were increased in the placenta and uterine segments from the distal compared to proximal and middle regions of the Preg uterus. In general, sFlt-1 levels were higher in the placenta and uterine segments from the proximal, middle and distal regions of the RUPP uterus compared with the corresponding regions of the Preg uterus. The levels of sFlt-1 were also increased in the placenta and uterine segments from the distal compared with proximal and middle regions of the RUPP uterus (Fig. 5A, 5B). In comparison, the levels of PlGF were reduced in the placenta and uterine segments from the distal compared with proximal regions of the Preg uterus. PlGF levels were generally less in the placenta and uterine segments from the proximal, middle and distal regions of the RUPP uterus compared with the corresponding regions of the Preg uterus. The levels of PlGF were also reduced in the placenta and uterine segments from the distal compared with proximal regions of the RUPP uterus (Fig. 5C, 5D). The calculated sFlt-1/PlGF ratio was increased in the placenta and uterine segments from the distal compared with proximal regions of the Preg uterus. The sFlt-1/PlGF ratio was generally greater in the placenta and uterine segments from the proximal, middle and distal regions of the RUPP uterus compared to the corresponding regions of the Preg uterus. The sFlt-1/PlGF ratio was also increased in the placenta and uterine segments from the distal compared with proximal regions of the RUPP uterus (Fig. 5E, 5F).

3.6. Effects of sFlt-1, PlGF and Placental Extracts on Activity/Levels of MMPs

To test the effects of growth modulators, placental and uterine segments from the distal regions of the Preg uterus were treated with PlGF, sFlt-1 or RUPP placental extract for 48 h in organ culture. In comparison, placental and uterine segments from the distal regions of the

RUPP uterus were treated with sFlt-1, PIGF or Preg placental extract for 48 h in organ culture.

Gelatin zymography showed that the gelatinase activity of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 in the placenta or uterus of Preg rats treated with PIGF was not significantly different from that in non-treated placenta or uterus of Preg rats (Fig. 6). Pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 activity was significantly reduced in placenta and uterus isolated from Preg rats and treated with sFlt-1 or RUPP placenta extract compared to non-treated tissues of Preg rats (Fig. 6). In comparison, the gelatinase activity of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 from placenta or uterine segments isolated from RUPP rats and treated with sFlt-1 was not significantly different from that in non-treated tissues of RUPP rats (Fig. 6). On the other hand, the amount of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 was significantly increased in placenta and uterine segments isolated from RUPP rats and treated with PIGF or Preg placenta extract when compared to non-treated tissues of RUPP rats (Fig. 6).

Western blots analysis showed that the levels of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 in the placenta or uterine segments isolated from Preg rats and treated with PIGF were not significantly different from non-treated tissues of Preg rats (Fig. 7). The levels of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 were significantly reduced in placenta and uterus isolated from Preg rats and treated with sFlt-1 or RUPP placenta extract when compared to non-treated tissues of Preg rats (Fig. 7). In general, the levels of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 were reduced in placenta and uterine segments of RUPP vs Preg uterus. The levels of pro-MMP-9 and MMP-9 were not significantly different and the levels of pro-MMP-2 and MMP-2 were further reduced in placenta and uterine segments isolated from RUPP rats and treated with sFlt-1 compared with non-treated tissues of RUPP rats (Fig. 7). On the other hand, the levels of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 were significantly increased in placenta and uterine segments isolated from RUPP rats and treated with PIGF or Preg placenta extract when compared to non-treated tissues of RUPP rats (Fig. 7).

4. Discussion

The main findings are: **1)** Placental and fetal growth were decreased in the distal vs proximal and middle regions of Preg uterus, and the regional decreases were more pronounced in the distal region of RUPP uterus. **2)** Uteroplacental vascularization was generally reduced in the proximal, middle and distal regions of RUPP uterus compared to the corresponding regions of Preg uterus. **3)** Uteroplacental vascularization in the distal vs proximal and middle regions was increased in Preg uterus, but decreased in RUPP uterus. **4)** The levels/activity of uteroplacental MMP-2 and MMP-9 in the distal vs proximal and middle regions were increased in Preg uterus, but decreased in RUPP uterus. **5)** sFlt-1/PIGF ratio in distal vs proximal and middle regions was decreased in Preg uterus, but increased in RUPP uterus. **6)** Treatment with anti-angiogenic sFlt-1 or RUPP placental extract decreased MMP-2 and -9 in placenta and uterus of Preg rats, while treatment with angiogenic PIGF or Preg placental extract increased MMP-2 and MMP-9 in placenta and uterus of RUPP rats.

4.1. Increased BP and reduced placental and fetal development during RUPP

Inadequate trophoblast invasion of spiral arteries and the ensuing uteroplacental ischemia/hypoxia are important events in the pathogenesis of preeclampsia [7, 15, 60]. The risk of preeclampsia increases in twin and multiple pregnancy [44, 45, 49], where relative increases in placental ischemia and inadequate uteroplacental vascularization [46] could further affect placental and fetal development. Studies in RUPP and hypoxic pregnant rats have supported placental ischemia/hypoxia as an initiating mechanism of HTN-Preg and IUGR [10, 15, 17]. Consistent with previous reports [24, 54, 61, 62], the present study showed that BP was increased and the average number and individual weight of placentae and pups were reduced in RUPP model of placental ischemia compared to Preg rats. Changes in placental efficiency occur as a result of alterations in the weight of the fetus, placenta or both [58, 59]. In addition to natural variations in placental efficiency, the fetal to placental weight ratio can be altered experimentally by manipulation of uterine blood flow [59]. We found that placental efficiency was significantly reduced in RUPP compared with control Preg rats, further suggesting reduced placental development relative to the fetal size and nutrient demand in the RUPP model of induced placental ischemia. It is important to further examine how multiple pregnancy particularly during placental ischemia could affect the uteroplacental circulation and the placental and fetal growth in different regions of the uterus, and to determine the potential molecular mechanisms involved in the regional differences in uteroplacental remodeling.

4.2. Regional differences in placental and fetal development in RUPP vs Preg rats

The rat uterus is composed of two horns, and during normal pregnancy each horn carries between 4 to 8 placentae and pups. The vast majority of blood flow to each uterine horn is supplied by the uterine artery, which originates from the iliac artery [63, 64]. Because of the finite uterine blood supply, the blood flow to each placenta and pup could be inversely proportionate to their number, such that the more the multiple pregnancy, the less the blood flow to each placenta and pup. Likewise, the blood flow to each placenta and pup could be inversely proportionate to the distance from the origin of the main uterine artery [63, 64], such that the farther the distance from the iliac bifurcation the less the blood flow to each placenta and pup. The present study showed that the frequency and individual weight of placentae and pups were reduced in distal compared to proximal and middle regions (relative to the iliac bifurcation) of the Preg uterus. Thus, in the presence of finite uterine blood flow, multiple pregnancy could cause insufficient blood supply to the individual placenta and fetus, particularly in the regions distal from the main uterine arterial supply. If this is the case, then these regional reductions in placental and fetal development are expected to be exaggerated during placental ischemia induced by RUPP.

In the RUPP rat model of HTN-Preg, restricting blood flow to the uterine artery reduces uteroplacental perfusion pressure in the gravid uterus by ~40% [52], making it a good model to study the effects of uteroplacental ischemia on placental and fetal growth in multiple pregnancy. The present study suggest that regional differences in placental and fetal growth are exaggerated during uteroplacental ischemia because: 1) The placental and fetal number and weight were generally reduced in the proximal, middle and distal regions of the RUPP uterus compared to the same regions in the Preg uterus. 2) The placenta number and weight

were markedly reduced in the distal vs proximal and middle regions of the RUPP uterus. 3) No placentae or pups were observed in the far distal regions of the right or left horn of the RUPP uterus.

4.3. Regional differences in placental and myoendometrial vascularization

Because of the marked uteroplacental vascularization during pregnancy [3, 4, 43, 53], we tested whether the regional changes in placental and fetal growth reflect changes in the vascularization of the placenta, which provides nutrients to the developing fetus, as well as the uterus, which undergoes expansive remodeling to accommodate the growing fetus [3, 4]. The Preg uterus showed adequate number and diameter of placental and myoendometrial arteries in the proximal and middle regions, that coincided with adequate placental and fetal growth in these regions. Contrary to the reduced placenta and fetal growth in the distal region of the Preg uterus, the number and diameter of placental and myoendometrial arteries were increased. This can be explained by possible compensatory mechanisms to increase vascularization and maintain adequate blood supply in an attempt to rescue the underdeveloped placenta and fetus in the distal regions of the Preg uterus. This is supported by reports that during normal pregnancy the entire placental and uterine vasculature enlarges in order to accommodate the necessary increase in blood flow to the developing placenta and fetus [53, 65]. Also, gestational growth of uteroplacental arteries is not expected to be uniform, as some arteries are recruited to supply the placenta, while other arteries are recruited to accommodate the growth and expansion of the myoendometrium [66]. This could also be related to placental regulation of the arterial-venous exchange, a mechanism by which the placenta might exert some control over its own blood supply. Nevertheless, despite the compensatory vascularization mechanisms, reduction in placental and fetal development are still observed in distal regions of the multiple Preg uterus.

In comparison with the Preg uterus, there was a trend for compensatory increase in uteroplacental vascularization in the middle region and parts of the distal region compared to the proximal region of the RUPP uterus. However, these trends of increased vascularization ceased in the far distal region of the RUPP uterus, and the number and diameter of placental and myoendometrial arteries were significantly reduced in the far distal compared to the proximal region of the RUPP uterus. This marked decrease in uteroplacental vascularization coincided with the severe placental and fetal growth restriction in the distal region of the RUPP uterus. Additionally, the number and diameter of placental and myoendometrial arteries were generally less in the proximal, middle and distal regions of the RUPP uterus compared to the corresponding regions in the Preg uterus. Thus while compensatory mechanisms may improve uteroplacental vascularization and rescue the placenta and fetus in the middle regions of the RUPP uterus, these physiological adjustments are generally impaired and fail to rescue the placenta and fetus in the far distal regions of the RUPP uterus.

4.4. Role of MMPs in regional differences in uteroplacental growth and vascularization

MMPs play a major role in uterine tissue remodeling during the menstrual and estrous cycle, embryo implantation, and through the course of pregnancy [37, 41, 42]. We examined the role of MMP-2 and MMP-9 in the regional differences in placental and fetal development

and uteroplacental vascularization. Gelatin zymography and Western blots revealed that MMP-2 and MMP-9 were abundant in the placental and uterine segments from the proximal, middle and distal regions of the Preg uterus. These observations are consistent with previous reports that increased MMP-2 and MMP-9 plays a role in the uteroplacental and vascular remodeling during healthy pregnancy [13, 41, 42, 67–69]. Also, MMP-2 and MMP-9 were increased in the distal compared to proximal regions of the Preg uterus, and paralleled the observed compensation in uteroplacental vascularization in the distal regions of the Preg uterus. These findings suggest a role of MMP-2 and MMP-9 in promoting uteroplacental remodeling to maintain adequate vascularization and rescue the underdeveloped placenta and fetus in the distal regions of the Peg uterus during multiple pregnancy.

The role of MMPs in uteroplacental remodeling appeared to be reduced during placental ischemia, as the levels/activity of MMP-2 and MMP-9 were reduced in placental and uterine segments from the proximal, middle and distal regions of the RUPP uterus compared with the corresponding regions in the Preg uterus. These observations are consistent with previous reports of decreased MMP levels in umbilical cord artery and microvascular endothelial cells of preeclamptic women [70, 71], and reduced levels/activity of MMP-2 and MMP-9 in the uterus, placenta and aorta of RUPP vs Preg rats [13, 41]. The data are also in accordance with reports that pharmacological inhibition of MMPs is associated with decreased placenta weight and cause IUGR in both normal Preg and HTN-Preg rats, and reduce trophoblast invasion and placental perfusion in HTN-Preg rats [72–74]. Importantly, in contrast with the increase in MMPs in the distal region of the Preg uterus, MMP-2 and MMP-9 were markedly decreased in the distal region of the RUPP uterus. The downregulation of MMPs paralleled the lack of compensation in uteroplacental vascularization and the severe placental and fetal growth restriction in the far distal region of the RUPP uterus. These data suggest that downregulation of MMPs could contribute to the aggravated growth-restrictive uteroplacental and vascular remodeling during multiple pregnancy complicated with placental ischemia.

4.5. Role of angiogenic imbalance

We examined the possible upstream factors that could drive the changes in MMPs and in turn the uteroplacental remodeling during placental ischemia and HTN-Preg. Preeclampsia remits after delivery of the placenta, implicating the placenta as a central culprit in the disorder. Studies have suggested that the ischemic placenta could trigger the release of bioactive factors in the maternal circulation, causing some of the features of preeclampsia including HTN-Preg and IUGR [21, 61, 75, 76]. Changes in anti-angiogenic/angiogenic balance have been suggested as a potential factor in HTN-Preg and preeclampsia [17, 21, 27, 34, 35]. Angiogenic imbalance could play a role in the observed regional changes in placental and fetal growth, uteroplacental vascularization and MMPs in the Preg and RUPP uterus because: **1)** The levels of sFlt-1 and the sFlt-1/PlGF ratio increased in the plasma of RUPP vs Preg rats. The observed increases in plasma sFlt-1 levels and sFlt-1/PlGF ratio in RUPP rats are consistent with reports that circulating sFlt-1 and the sFlt-1/PlGF ratio are elevated in preeclamptic women [29, 33, 35, 36, 77], and in RUPP and other animal models of HTN-Preg [17, 24, 78–82]. The sFlt-1/PlGF ratio can be modulated by not only sFlt-1, but also PlGF. The present observation that plasma levels of PlGF are decreased in RUPP vs

Preg rats is consistent with reports that PIGF levels are decreased in preeclamptic women [29, 33, 36] and in RUPP and Dahl salt-sensitive rat models of HTN-Preg [17, 24, 80, 82]. **2)** Because plasma angiogenic factors represent the global release from different maternal tissues, we measured the angiogenic factors in the different regions of the uterus and found that sFlt-1 was increased, PIGF was decreased and sFlt-1/PIGF was increased in the placenta and uterus of proximal, middle and distal regions of the RUPP uterus compared to the corresponding regions in the Preg uterus. The placental and uterine angiogenic imbalance paralleled the reduced placental and fetal growth, uteroplacental vascularization, and MMP levels/activity in the proximal, middle and distal regions of the RUPP vs Preg uterus. These findings are consistent with reports that sFlt-1 levels are greater in villous explants from preeclamptic than normal Preg women [83], and that sFlt-1 increases while PIGF decreases in twin vs singleton pregnancies [45] and in twin pregnancies with suspected preeclampsia [44, 49]. **3)** The levels of sFlt-1 were further increased, PIGF was further decreased, and sFlt-1/PIGF ratio was further increased in the distal vs proximal and middle regions of the RUPP uterus. These observations paralleled the severe reduction in fetal and placental growth, uteroplacental vascularization and MMPs in distal vs proximal regions of the RUPP uterus. **4)** Treatment with the anti-angiogenic factor sFlt-1 or RUPP placental extract, which we found to contain high sFlt-1 and low PIGF levels, decreased MMP-2 and MMP-9 levels/activity in placenta and uterus of Preg rats. The inhibitory effects of sFlt-1 on MMP-2 and -9 are consistent with reports that treatment with sFlt-1 reduces the gelatinolytic activity of MMP-2 and MMP-9 in an experimental model of abdominal aortic aneurysm [84]. The data also agree with reports that exposure of endothelial cells to preeclamptic plasma decreases angiogenesis, and removal of sFlt-1 or treatment with a sFlt-1 antibody reverses the anti-angiogenic effects of sFlt-1 and restores angiogenesis and endothelial cell function [83]. Future studies in genetically-altered animals should test whether downregulation of sFlt-1 would improve not only the general circulating levels, but also the localized uterine expression of MMP-2 and MMP-9, and in turn uteroplacental vascularization and fetoplacental growth. **5)** Treatment with the angiogenic factor PIGF or Preg placental extract, which we found to contain high PIGF and low sFlt-1 levels, increased MMP-2 and MMP-9 levels/activity in placenta and uterus of RUPP rats. The present observations with PIGF are consistent with our previous report that the angiogenic factor VEGF prevents the decreases in MMP-2 and MMP-9 in uterine, placental and aortic segments of RUPP rats [13], and other reports that PIGF promotes endothelial cell growth, vasculogenesis, and placental development [20].

The regional changes in MMPs induced by anti-angiogenic and angiogenic factors could underlie the observed regional differences in placental growth and uteroplacental vascularization. Angiogenic factors such as PIGF and VEGF may act in an autocrine or paracrine fashion to accelerate angiogenesis, and MMPs may mediate these effects by virtue of their proteolytic activity and degradation of different protein components of ECM. MMPs could also promote angiogenesis by detaching pericytes from the vessels undergoing angiogenesis, releasing ECM-bound angiogenic growth factors, exposing cryptic pro-angiogenic integrin binding sites in ECM, generating pro-migratory ECM component fragments, and cleaving endothelial cell-cell adhesions [85]. Thus downregulation of MMPs by anti-angiogenic sFlt-1 particularly in uterine regions distal from the origin of uterine

artery could cause decreases in angiogenesis, trophoblast invasion of spiral arteries, uteroplacental vascularization and feto-placental growth during HTN-Preg. On the other hand, upregulation of MMPs by PIGF would promote angiogenesis, trophoblast invasion of spiral arteries, uteroplacental vascularization and feto-placental growth during normal pregnancy, and could be a promising target in the management of HTN-Preg and preeclampsia.

4.6. Other observations/considerations

Other observation/considerations include: **1)** Reduction in placental and fetal development in the distal uterine regions was associated with vascular compensation and upregulation of MMPs in the Preg uterus, but not the RUPP uterus. The angiogenic balance is tightly controlled by feedback mechanisms such that modest increases in the sFlt-1/PIGF ratio in the distal uterine region could trigger a feed-back compensatory increase in other angiogenic factors such as VEGF to maintain angiogenesis and rescue the underdeveloped placenta and fetus. These compensatory mechanisms may not be available during placental ischemia, as the marked increases in sFlt-1 and sFlt-1/PIGF ratio could reach supraphysiological levels and neutralize any VEGF available for compensation. Of note, VEGF may stimulate sFlt-1 production in the placenta through an action on VEGFR-2 [86]. Also, VEGF levels are controlled at the maternal-fetal interface, partly through feedback modulation of sFlt-1, in order to prevent damage to the placenta or fetus by excess VEGF [86], and dysregulation of this feedback mechanism may prevent compensatory MMP upregulation and uteroplacental vascularization and lead to further restriction of placental and fetal growth. This is supported by reports that serum levels of sFlt-1 and the sFlt-1/PIGF ratio are markedly increased while PIGF levels are markedly decreased in twin pregnancies with preeclampsia compared with twin pregnancies with an uneventful pregnancy outcome [44, 45]. **2)** Other anti-angiogenic factors may be released and affect regional MMP levels and uteroplacental vascularization in HTN-Preg. For instance, sFlt-1 e15a, a splice variant of sFlt-1 that is abundantly expressed by syncytiotrophoblasts and released by the placenta, has been shown to bind VEGF and decrease endothelial cell migration, invasion, and tube formation, and its serum levels show 10-fold increase in preeclamptic vs normal pregnant women [87]. Also, soluble endoglin (sEng) is an anti-angiogenic factor that binds TGF- β 1 and inhibits its signaling effects [25]. The levels of sEng are higher in preeclampsia and the hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome compared with normal pregnant women [21, 88], and the serum and placenta levels of sEng are increased while serum TGF- β levels are decreased in RUPP rats [61]. When co-administered with sFlt-1 in pregnant rats, sEng induces vascular permeability, proteinuria, IUGR, severe HTN-Preg and HELLP syndrome-like features [89]. The expression of sEng is increased in placental explants exposed to hypoxia [90], and sEng impairs tube formation in human umbilical vein endothelial cells (HUVECs) [88]. The role of sEng in the regional differences in feto-placental development, uteroplacental vascularization and MMP expression/activity in HTN-Preg should be examined. **3)** The present study was performed in the RUPP rat model of placental ischemia and HTN-Preg, and whether regional differences in placental and fetal development, uteroplacental vascularization and MMPs occur in other models of HTN-Preg such as the Dahl salt-sensitive HTN-Preg rat and the catechol-O-methyltransferase deficient mice should be examined [80, 91]. **4)** The present study measured fetoplacental development and

uteroplacental vascularization at specific time point on day 19 of pregnancy. Time course studies at earlier gestational days would help determine whether the loss of vascular development precedes, and therefore potentially causes, the loss of the pup, or if there is simply no vascularization since there is no demand from a developing pup, and these studies should represent an important area for future investigations. 5) MMPs are a large family of at least 28 proteolytic enzymes [37, 92]. While we examined MMP-2 and MMP-9, other MMPs have been detected in the uterus and placenta, and the changes in these MMPs in HTN-Preg need to be examined. For instance, trophoblast- and vascular smooth muscle-derived MMP-12 may mediate proteolysis and uterine spiral artery remodeling during pregnancy [93]. Also, MMP activity could be influenced by other MMP activators and inhibitors. In effect, some MMPs may cleave other pro-MMPs, and membrane-type 1-MMP is a key activator of pro-MMP-2 [37, 92, 94]. Also, tissue inhibitors of metalloproteinases (TIMPs) are endogenous modulators of MMPs [92, 94, 95], and the regional changes in uteroplacental TIMPs in HTN-Preg need to be examined.

4.7. Perspective

The present study showed that during multiple pregnancy modest angiogenic imbalance in distal regions of the uterus is associated with compensatory upregulation of MMPs and increased uteroplacental vascularization in order to maintain blood supply to the growing fetus. In addition to the general angiogenic imbalance and reduction in placental and fetal growth and uteroplacental vascularization observed during placental ischemia, there is marked angiogenic imbalance and lack of compensatory mechanisms to upregulate MMPs, resulting in decreased uteroplacental vascularization and restricted fetoplacental growth in the distal regions of the RUPP uterus. The regional differences in angiogenic balance, MMP levels/activity could affect blood flow and placental perfusion in HTN-Preg and could be a factor in the increased incidence of preeclampsia in multiple pregnancy.

While the present study raises the possibility of some similarities in the molecular mechanisms regulating fetoplacental growth and uteroplacental vascularization during multiple pregnancies among mammals, the present results in rats should be carefully interpreted and extrapolated to other species particularly in relation to human multiple pregnancy. Currently, delivery of the baby and placenta are the only effective measures to manage preeclampsia. Preterm birth is another undesirable outcome of preeclampsia. One approach to manage preeclampsia is to remove sFlt-1 from the circulation of preeclamptic patients. Some studies have shown that extracorporeal removal of circulating sFlt-1 in preeclampsia patients decreases sFlt-1/PlGF ratio and improves symptoms and prolongs pregnancy [96]. However, extracorporeal immunoadsorption therapy to reduce the levels of sFlt-1 in preeclampsia women may require advanced apheresis equipment and highly trained clinical staff that may only be feasible in large medical centers. Although PlGF may have potentially limiting side-effects, localized administration of PlGF in the vicinity of the fetoplacental interface using direct injection of PlGF or incorporation of PlGF into implantable scaffolds or matrices could counteract the downregulation of MMPs induced by placental ischemia and sFlt-1, and restore the activity and remodeling capacity of growth-permissive MMPs, thus maintaining fetal growth and prolonging gestation, and thereby provide future approaches for management of preeclampsia particularly in multiple pregnancies. Regional

changes in MMPs may also represent novel markers for detection, and potential localized targets for management of HTN-Preg and preeclampsia in twin and multiple pregnancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

BP	blood pressure
ECM	extracellular matrix
HELLP	hemolysis, elevated liver enzymes, and low platelets
HIF	hypoxia-inducible factor
HUVECs	human umbilical vein endothelial cells
HTN-Preg	hypertension-in-pregnancy
IUGR	intrauterine growth restriction
MMP	matrix metalloproteinase
PIGF	placental growth factor
Preg	normal pregnant
RUPP	reduction of uteroplacental perfusion pressure
sEng	soluble endoglin
sFlt-1	soluble fms-like tyrosine kinase-1
VEGF	vascular endothelial growth factor

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Fig. 1.

Frequency and weight of placentae and pups in proximal (Prox), middle and distal regions of uterus of Preg and RUPP rats. The uterus of Preg (A) and RUPP rats (B) was exposed. For each uterine horn, three uterine regions were assigned as proximal, middle and distal (relative to the iliac bifurcation), such that the proximal region is the closest to the iliac bifurcation, the distal region is farthest from the iliac bifurcation, and the middle region is in between. Given that in the control Preg rat, each uterine region contained 2 to 3 pups and placentae, each region was further subdivided into sections and assigned a number, such that sections 1 to 2 represent the proximal region, sections 3, 4 and 5 represent the middle region, and sections 6, 7 and 8 represent the distal region. The placentae and pups were separated, and the frequency (likelihood of presence of placenta and pup in specific uterine region such that 1 = Detected pup and placenta, and 0 = No detected pup or placenta) (C and D) and weight of placenta (E and F) and pups (G and H) in the proximal, middle and distal regions of the uterus were recorded. Bar graphs represent means $\pm$ SEM in Preg (n=13) and RUPP (n=10) groups.

* Significantly different ($P<0.05$) vs proximal region (1) of the uterus of Preg or RUPP rats.

Significantly different ($P<0.05$) in RUPP vs Preg rats.

Fig. 2.

Number and diameter of placental and adjacent myoendometrial arteries in proximal (Prox), middle and distal regions of uterus of Preg and RUPP rats. The uterus of Preg (A) and RUPP rats (B) was exposed. For each uterine horn, three uterine regions were assigned as proximal, middle and distal (relative to the iliac bifurcation), such that the proximal region is the closest to the iliac bifurcation, the distal region is farthest from the iliac bifurcation, and the middle region is in between. Given that, in the control Preg rat, each uterine region contained 2 to 3 pups and placentae, each region was further subdivided into sections and assigned a number, such that sections 1 to 2 represent the proximal region, sections 3, 4 and 5 represent the middle region, and sections 6, 7 and 8 represent the distal region. Images of uterus were magnified in order to examine the placental and uterine vessels of Preg (A) and RUPP rats (B). The number (C and D) and external diameter (E and F) of placental arteries, as well as the number (G and H) and external diameter (I and J) of myoendometrial arteries

in proximal, middle and distal regions of uterus of Preg and RUPP rats were recorded. Bar graphs represent means $\pm$ SEM in Preg (n = 13) or RUPP (n=10) rats.

* Significantly different (P<0.05) vs proximal region (1) of uterus in Preg or RUPP rats.

Significantly different (P<0.05) in RUPP vs Preg rats.

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Fig. 3.

Gelatinase activity of placental and uterine MMP-9 and MMP-2 in proximal (Prox), middle and distal regions of uterus of Preg and RUPP rats. Equal protein amount (1 µg) of tissue homogenates from the placenta (A) and uterus (B) of proximal, middle and distal regions of the uterus of Preg and RUPP uterus were prepared for gelatin zymography analysis. The densitometry values of the proteolytic bands corresponding to pro-MMP-9 (C and D), MMP-9 (E and F), pro-MMP-2 (G and H) and MMP-2 (I and J) were presented as pixel intensity × mm² and normalized to actin to correct for loading. Bar graphs represent means ± SEM in Preg (n = 13) or RUPP (n=10) rats.

* Significantly different (P<0.05) vs Prox region of the uterus in Preg or RUPP rats.

Significantly different ($P < 0.05$) in RUPP vs Preg rats.

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Fig. 5.

Levels of anti-angiogenic sFlt-1 and pro-angiogenic PlGF in placenta and uterus in proximal (Prox), middle and distal regions of uterus of Preg and RUPP rats. Tissue homogenates of the placenta (A, C and E) and uterus (B, D and F) from Prox, middle and distal regions of the uterus of Preg and RUPP rats were prepared centrifuged twice and the supernatant was used for measurements of sFlt-1 (A and B) and PlGF (C and D) using ELISA, and the sFlt-1/PlGF ratio was calculated (E and F). Bar graphs represent means $\pm$ SEM in Preg (n = 6) or RUPP (n=6) rats.

* Significantly different (P<0.05) vs Prox region of the uterus in Preg or RUPP rats.

Significantly different (P<0.05) in RUPP vs Preg rats.

Fig. 6.

Effect of sFlt-1 and PIGF and placental extracts on gelatinase activity of placental and uterine MMP-2 and -9 in distal regions of uterus of Preg and RUPP rats. Placenta (A) or uterus (B) from distal region of uterus of Preg or RUPP rats were treated with PIGF (0.1 µg/ml) or sFlt-1 (0.1 µg/ml) or placental extract from RUPP or Preg rats for 48 h in organ culture. Tissue homogenates were prepared for gelatin zymography analysis (A and B). The densitometry values of the proteolytic bands corresponding to proMMP-9 (C and D), MMP-9 (E and F), proMMP-2 (G and H) and MMP-2 (I and J) were presented as pixel intensity × mm² and normalized to actin to correct for loading. Bar graphs represent means ± SEM, n=6/group.

* Significantly different (P<0.05) vs control Preg group.

Significantly different (P<0.05) vs control RUPP group.

Fig. 7.

Effect of sFlt-1 and PIGF and placental extracts on placental and uterine levels of MMP-2 and -9 in distal regions of uterus of Preg and RUPP rats. Placenta (A) or uterus (B) from distal region of uterus of Preg or RUPP rats were treated with PIGF (0.1 μ g/ml) or sFlt-1 (0.1 μ g/ml) or placental extract from RUPP or Preg rats for 48 h in organ culture. Tissue homogenates were prepared for Western blot analysis using antibodies to MMP-9 (1:1000) and MMP-2 (1:1000) (A and B). Immunoreactive bands corresponding to pro-MMP-9 (C and D), MMP-9 (E and F), pro-MMP-2 (G and H) and MMP-2 (I and J) were analyzed by optical densitometry and normalized to β -actin to correct for loading. Bar graphs represent means $\pm$ SEM, n=6/group.

* Significantly different ($P<0.05$) vs control Preg group.

Significantly different ($P<0.05$) vs control RUPP group.

Table 1

Average maternal and fetal parameters in Preg and RUPP rats.

Parameter	Preg	RUPP
Blood pressure (mmHg)	103.8 ± 0.8	129.0 ± 2.7 *
Whole uterus weight without placentae or pups (g)	4.5 ± 0.2	3.3 ± 0.3 *
Total number of placentae per rat	12.08 ± 0.54 (9–16)	9.5 ± 1.17 (5–12) *
Average placenta weight (mg)	439 ± 10	397 ± 6 *
Litter size (number of pups)	12.08 ± 0.54 (9–16)	9.5 ± 1.17 (5–12) *
Average pup weight (g)	2.03 ± 0.04	1.12 ± 0.06 *
Placental efficiency (Pup weight/Placenta weight) (g/g)	4.63 ± 0.42	3.09 ± 0.60 *
Plasma sFlt-1 (pg/ml)	979.2 ± 43.6	1435.0 ± 167.3 *
Plasma PIGF (pg/ml)	2.57 ± 0.30	1.33 ± 0.13 *
Plasma sFlt-1/PIGF ratio	469.3 ± 62.0	1304.0 ± 178.1 *

Data are expressed as means±SEM in Preg (n = 13) and RUPP rats (n=10). Numbers in parenthesis indicate range.

* Significantly different (P<0.05) in RUPP vs Preg rats.