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Multiparity increases trophoblast invasion and vascular endothelial growth factor expression at the maternal–fetal interface in mice☆

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ABSTRACT

To analyze immunomodulating effects related to parity status, we studied trophoblast invasion grade, placental expression and systemic concentration of VEGF and its receptor Flt-1 in normal fertile (CBA/J × BALB/c) mice and abortion-prone (CBA/J × DBA/2) H-2^d × H-2^k mice. BALB/c or DBA/2 mated CBA/J females were, respectively, divided into the following groups: primiparous young (3.0 ± 0.5 months old); primiparous old (8.5 ± 0.5 months old) and multiparous old (8.5 ± 0.5 months old, with 4 pregnancies). Immunohistochemical analysis of term placentae from both multiparous groups revealed various layers of invasive trophoblast tissue, identified as cytokeratin+/vimentin− cells, in contrast to the single layer detected in the placentae of primiparous animals, indicating that multiparity increases trophoblast invasion regardless of the success of the pregnancy outcome. Invasive trophoblast tissue from primiparous CBA/J × DBA/2 placentae showed diminished VEGF expression in comparison with the normal fertile group, while both multiparous groups demonstrated high expression of VEGF in the invasive trophoblast tissue. Placental expression of Flt-1 was similar in all groups. However, the primiparous CBA/J × BALB/c group showed the highest plasma concentration of sFlt-1 at term, while both multiparous groups demonstrated low circulating levels. No differences in circulating VEGF levels were observed among the groups. These results demonstrate an increase in trophoblast invasion tissue and expression of VEGF in the maternal–fetal interface in multiparous mice compared to primiparous mice. Moreover, the placenta appears to be able to regulate the circulating levels of VEGF by releasing sFlt-1.

1. Introduction

Beneficial effects of multiparity status have been previously reported (Beer et al., 1975; Chavez et al., 1987; Trupin et al., 1996; Robillard et al., 1999). Our group has been investigating immunomodulating effects related to the parity status (Lagadari et al., 2004; Litwin et al., 2005). We observed that placentae from multiparous females

Abbreviations: D, decidua, ITT: invasive trophoblast tissue; L, labyrinthine trophoblast; S, spongiotrophoblast; NP, not pregnant; PY, primiparous young (3.0 ± 0.5 months old); PO, primiparous old (8.5 ± 0.5 months old); MO, multiparous old (8.5 ± 0.5 months old with 4 pregnancies); VEGF, vascular endothelial growth factor; VIM, vimentin; CK, cytokeratin; IHC, immunohistochemistry; H&E, hematoxylin and eosin.

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showed an increased number of macrophages compared to placentae from primiparous mice. The macrophages were located in a band between the spongiotrophoblast and decidua (Lagadari et al., 2004). Syngeneic placentae also showed various layers of cells next to the maternal side of the decidua that were cytokeratin (CK) positive and vimentin (VIM) negative demonstrating their trophoblast origin, and for this reason we considered them as invasive trophoblast cells. These cells also expressed high levels of G-CSF and M-CSF (Litwin et al., 2005) which are known to regulate macrophage functions (Hidaka et al., 2002). The existence of invasive trophoblast tissue in the maternal organism has been reported in the mouse (Redline and Lu, 1989; Adamson et al., 2002). However, our work showed that parity status influences the extent to which this tissue develops.

One critical factor synthesized by macrophages is vascular endothelial growth factor (VEGF), whose role in angiogenesis has been extensively investigated (Ferrara and Davis-Smyth, 1997; Neufeld et al., 1999). Impairment of placental VEGF secretion can induce endothelial cell dysfunction and pregnancy loss in mice; inactivation of VEGF in mice resulted in embryonic lethality (Ferrara et al., 1996). The cytotrophoblast and syncytiotrophoblast of women with missed abortion have been found to be negative for VEGF (Vuorela et al., 2000). Recently, homozygosity for the VEGF 1154A gene has been proposed as a susceptibility factor in miscarriage (Coulam and Jeyendran, 2008). As well as its angiogenic properties, VEGF is involved in the plasminogen activation pathway and can degrade components of the extracellular matrix and activate growth factors and metalloproteases required for invasion (Pepper et al., 1991; Unemori et al., 1992; Mandriota et al., 1995; Anteby et al., 2004).

In the murine placenta, VEGF transcripts are detected in the labyrinthine trophoblast and trophoblast giant cells (Breier et al., 1995; Chakraborty et al., 1995; Dumont et al., 1995). The biological activity of VEGF results from interaction with its receptors, VEGF-R1 (Flt-1), VEGF-R2 (KDR/flk-1) and VEGF-R3 (Flt-4), all of which are expressed predominantly in endothelial cells. Flt-1 is expressed in spongiotrophoblast cells and monocytes, while Flk-1 is expressed in the labyrinthine trophoblast (Breier et al., 1995; Dumont et al., 1995; Hirashima et al., 2003). Furthermore, Flt-1 also can be secreted in a soluble form (sFlt-1), which acts to inhibit VEGF activity (Kendall and Thomas, 1993). It has been suggested that an excess of sFlt-1 may have a pathogenic role in pre-eclampsia by preventing the interaction of VEGF with VEGF receptors on the endothelial cell surface, thereby inducing endothelial dysfunction (Levine et al., 2004). In addition, exogenous sFlt-1 administered to pregnant rats induces hypertension, proteinuria and glomerular endotheliosis (Maynard et al., 2003). VEGF receptors also are essential for pregnancy in mice (Fong et al., 1995), and their altered expression in human decidua has been reported in recurrent miscarriages (Vuorela et al., 2000).

Considering our previous studies and the properties of VEGF in the maintenance of a successful pregnancy, the aim of the present report was to investigate the influence of multiparity status on trophoblast invasion grade and to analyze the placental expression and systemic concentration of VEGF and its receptor Flt-1 in normal fertile and in abortion-prone allogeneic mouse crossbreedings.

2. Materials and methods

2.1. Mouse crossbreedings

Two-month-old mice (CBA/J females; DBA/2 and BALB/c males) were purchased from CNEA (Buenos Aires, Argentina). Female mice were divided into three groups: primiparous young (PY): 3.0 ± 0.5 months old, primiparous old (PO) and multiparous old (MO): 8.5 ± 0.5 months old ($n = 10$ for each group).

CBA/J female mice were used as non-pregnant virgin controls or were mated with male BALB/c (CBA/J × BALB/c, normal fertile H-2^d × H-2^k crossbreeding) or DBA/2 (CBA/J × DBA/2, abortion-prone H-2^d × H-2^k crossbreeding) until the first pregnancy (PY, PO) or the fourth pregnancy (MO). PO animals were maintained until they were 7.0 ± 0.5 months old and were mated in the same combinations. The observation of a vaginal plug denoted day 0.5 of pregnancy.

All experiments were performed according to our local institutional animal care and the European Guidelines of Animal Care (Derrell Clark et al., 1996).

2.2. Biological samples

2.2.1. Plasma

Plasma from 7.5 and 18.5 ± 1.0 days of pregnancy were obtained from each group.

2.2.2. Placentae

Mice were sacrificed at 18.5 ± 1.0 days of pregnancy and placentae were removed and processed for IHC analysis (Saint Marie, 1962).

2.3. Immunohistochemical studies

Immunohistochemistry (IHC) was performed in paraffin-fixed placental sections using a standard protocol. Each placental section was stained with hematoxylin and eosin (H&E) in order to check that there was no evidence of infection or any histological alteration.

Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol. Tissue sections were incubated with a normal serum from the same species of the detection Ab used in each case, stained with the respective primary and detection Abs, developed with different substrates. Lastly, the sections were counterstained with Mayers haematoxylin and mounted using Crystal/Mount™ (Biomeda, USA).

The presence of VEGF was investigated employing a goat anti-VEGF Ab (Santa Cruz Biotechnology, USA) and a rabbit biotinylated anti-goat IgG Ab (Vector, USA). Sections were then incubated with streptavidin-peroxidase (Vector, USA) and the colour was developed using diaminobenzidine (Sigma, Germany) and hydrogen peroxide.

For the detection of Flt-1 a goat anti-mouse Flt-1 Ab was applied (R&D Systems, USA) followed by biotinylated

anti-goat Ab (as above). The expression of CK was studied with a rabbit polyclonal Ab anti-mouse CK (Sanbio BV, The Netherlands) and a biotinylated anti-rabbit IgG Ab (Vector, USA). VIM was analyzed with a monoclonal Ab anti-VIM (BD, Pharmingen, USA) and a biotinylated Ab anti-mouse IgG (DAKO, Germany). These reactions were developed using the Vectastain® ABC kit (Vector, USA), diaminobenzidine (Sigma, Germany) and hydrogen peroxide for colour development.

The histochemical labeling was assessed blindly by two independent observers, and staining intensity was scored on a scale from 0 to 4. The number of layers of invasive trophoblast tissue was counted and scored.

2.4. Measurement of VEGF and sFlt-1 levels in plasma

Plasma VEGF and sFlt-1 levels were studied employing commercial ELISA kits, following the manufacturer's instructions. VEGF levels were determined using a PeproTech ELISA kit (PeproTech, Mexico). Briefly, a purified rabbit anti-mouse VEGF Ab was used as the capture antibody. The reactions were developed by subsequent incubations with a biotinylated purified rabbit anti-mouse VEGF Ab, streptavidin–HRP and ABTS Liquid Substrate Solution (Sigma, Germany).

sFlt-1 concentrations were measured employing a R&D ELISA kit (R&D Systems, USA). The assay consisted of coating microplates with a capture goat anti-mouse Flt-1 Ab and developing the reactions by subsequent incubations with a biotinylated detection Ab, streptavidin–HRP and 3,3′,5,5′-tetramethyl-benzidine liquid substrate (MP Biomedicals, USA). The assays were stopped with 1 M H_2SO_4.

2.5. Analysis of the expression of VEGF isoforms in placenta

Placental VEGF isoforms were analyzed by SDS-PAGE and immunoblotting. Placentae were minced and homogenized with 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin (all Sigma, Germany) in 10 mM Tris pH 8.0. Placental lysates with or without prior dialysis against 8 M urea were solubilized in sample buffer containing 1% SDS, 125 mM Tris pH 6.8 in the presence or absence of 300 mM 2-ME. Protein samples (40 μg) were subjected to electrophoresis on 10% SDS-PAGE and transferred to 0.45 μm pore size nitrocellulose membranes (GE Healthcare, UK) for 1 h at 100 V and then were blocked with 0.5% TBST pH 8.0 containing 5% BSA overnight at 4 °C. Blots were incubated with a rabbit polyclonal VEGF Ab (Santa Cruz Biotech., USA) diluted 1:1000 in TBST 0.1% BSA for 1 h at room temperature or a goat Ab specific for β-actin diluted 1:1000 (Santa Cruz Biotech.) followed by treatment with anti-rabbit IgG-HRP (ZyMax, Invitrogen, USA) or anti-goat IgG-HRP (Santa Cruz Biotech.). Finally, blots were developed with ECL Advance Western Blotting Detection Kit (GE Healthcare, UK) and exposed to autoradiography films (Agfa) from 1 to 10 min.

2.6. Statistics

Results were expressed as mean ± SEM for each group. Repeated measurement analysis of variance (ANOVA) and Student–Newman–Keuls multiple comparisons' test were used to assess differences between means.

3. Results

3.1. Allogeneic placentae from multiparous females show various layers of trophoblast cells next to the decidua

As we previously observed in CBA/J syngeneic combinations, MO placentae obtained from either CBA/J × BALB/c or CBA/J × DBA/2 crossbreedings showed 5–11 layers of cells located next to the maternal side of the decidua. These cells exhibited small and flat nuclei, very different from those of decidual cells (Fig. 1A). These cells were VIM− (Fig. 1B) and CK+ (Fig. 1C) as compared to the control (Fig. 1D), thus indicating their trophoblast origin. In PY and PO placentae, they appeared restricted to a thin and single layer and this was similar in both of the crossbreedings (Fig. 1E).

3.2. Primiparous CBA/J × DBA/2 placentae show diminished expression of VEGF in the invasive trophoblast tissue, corrected by multiparity

VEGF was strongly expressed in the spongiotrophoblast and labyrinthines zone in all the primiparous and multiparous placentae (Fig. 1F) as compared to the control (Fig. 1G). In these regions, all samples showed a similar pattern and number of positive cells (Fig. 2A and B).

The differences in VEGF expression between CBA/J × BALB/c and CBA/J × DBA/2 crossbreedings were significant in the region of the uterine face of the decidua in primiparous placentae. This region appeared like a well-distinguished single layer of VEGF+ cells in the normal combination corresponding to the layer previously identified as the invasive trophoblast tissue region (Fig. 1H) in comparison to its control (Fig. 1I). In contrast, the abortion-prone mice showed a diminished expression of VEGF in this region (Fig. 1J). Each crossbreeding showed a similar pattern between the PY and PO group. Interestingly, multiparous placentae of both crossbreedings showed a similar high expression of VEGF in the invasive trophoblast tissue. A representative picture is shown in Fig. 1K, and its control is displayed in Fig. 1L. The statistical analysis of the results is represented in Fig. 2C.

3.3. Several Flt-1+ cells appear in the invasive trophoblast tissue of multiparous placentae

Flt-1 was detected neither in decidua nor in the labyrinthine by IHC. In all samples, positive cells appeared in spongiotrophoblast arranged in columns or clusters (Fig. 1M and N). No significant differences in the amount of positive cells in the spongy zone regarding the crossbreeding or parity status were detected (Fig. 2D).

Placentae from multiparous females of both crossbreedings showed several isolated Flt-1+ cells in the zone of layers of invasive trophoblast tissue (Fig. 1O and P).

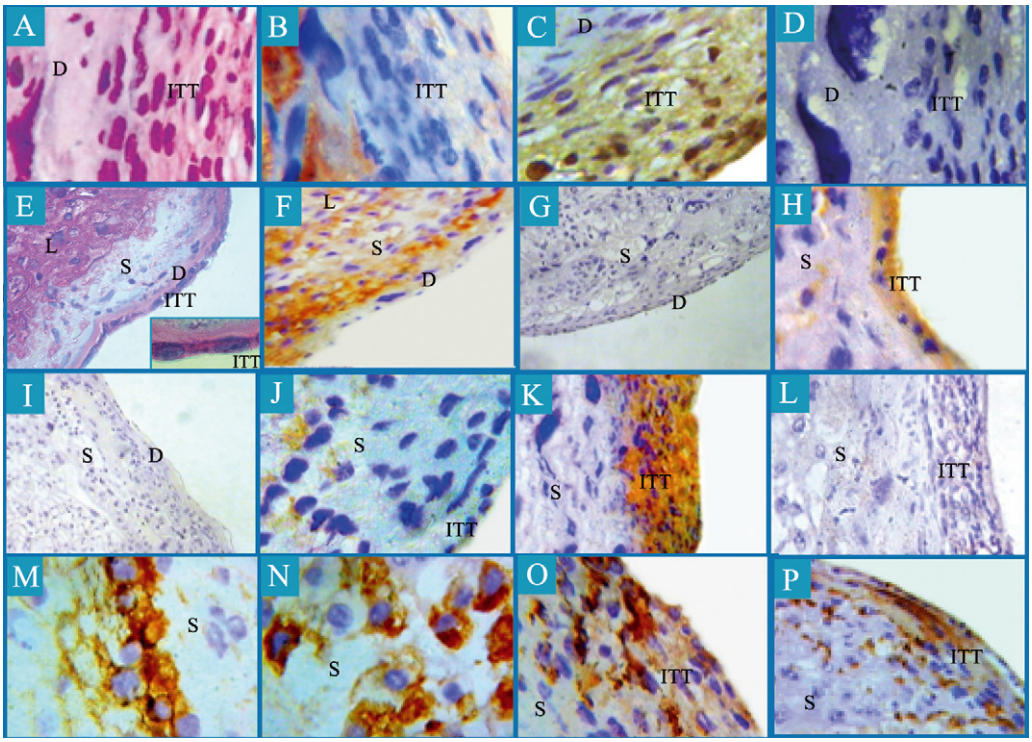

Fig. 1. Photomicrographs showing invasive trophoblast tissue in mouse allogeneic placentae. (A–D) The multilayer of invasive trophoblast cells of a representative MO placenta. (A) Invasive trophoblast tissue consisted of more than 11 layers of cells located next to the maternal side of the decidua. These cells exhibited small and flat nuclei, very different from those of decidual cells (H&E, ×1000). They proved to be vimentin negative (B) and cytokeratin positive (C), indicating their trophoblast origin. Positive cells can be recognized as brown cells (×1000). (D) Negative control (×1000). (E) H&E staining of a representative primiparous placenta (×400) with a focus on the monolayer of invasive trophoblast tissue (×1000). (F–L) VEGF expression in placentae. (F) Placental tissue from a PY female of the CBA/J × DBA/2 crossbreeding. VEGF+ cells (in brown) were detected in the spongiotrophoblast and labyrinthine zone. (G) Negative control (both ×400). (H) High VEGF expression in the invasive trophoblast tissue region of a PY CBA/J × BALB/c placenta (×1000). (I) Negative control. (J) Primiparous CBA/J × DBA/2 placentae (from both young and old females) showed a significant diminished expression of VEGF in the region of invasive trophoblast (×1000). (K) Increased amount of VEGF+ cells in the multilayer of invasive trophoblast tissue in a placenta from a MO abortion-prone combination. (L) Negative control (both ×1000). Similar images were observed in MO CBA/J × BALB/c crossbreedings. (M–P) Flt-1 expression in placentae. Flt-1+ cells (in brown) were detected in the spongiotrophoblast in all samples, regardless of crossbreeding or parity status. They appeared arranged in columns (M) or in clusters (N). Positive cells were also detected in the invasive trophoblast tissue region of MO placentae from both normal (O) and abortive (P) crossbreedings (all ×1000). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Term-pregnancy-induced upregulation of circulating sFlt-1 levels is lower in the abortion-prone crossbreeding. Multiparity diminishes and normalizes sFlt-1 concentration

Circulating levels of sFlt-1 in females at 7.5 ± 0.5 and 18.5 ± 1.0 days of pregnancy in both crossbreedings with different parity status were determined by ELISA.

Fig. 2E shows the results obtained at term pregnancy. It can be observed that gestation markedly increased sFlt-1 plasma levels. This effect was more pronounced in primiparous females belonging to the normal crossbreeding, with an increase of 11.3-fold compared to non-pregnant females (PY, CBA/J × BALB/c: 6325 ± 468 pg/ml vs non-pregnant, 559 ± 203 pg/ml, $p < 0.001$).

Interestingly, the increase in circulating sFlt-1 levels induced by term pregnancy was also detected in abortion-prone primiparous females, but to a lesser extent (5.36-fold) than in the normal ones (PY, CBA/J × DBA/2: 3003 ± 754 pg/ml vs non-pregnant, $p < 0.001$). Consequently in this group, the differences between crossbreedings were statistically significant (PY, CBA/J × BALB/c vs PY, CBA/J × DBA/2, $p < 0.001$).

Both multiparous females showed similar diminished sFlt-1 plasma concentrations (MO, CBA/J × BALB/c: 2213 ± 361 pg/ml vs MO, CBA/J × DBA/2: 1462 ± 590 pg/ml, $p > 0.5$).

In order to investigate whether the differences in the levels of circulating sFlt-1 between primiparous females could be detected earlier in pregnancy, the same ELISA assay was repeated employing plasma of day 7 pregnant mice. However, the concentration of sFlt-1 was not modified (non-pregnant, 501 ± 69 pg/ml; PY, CBA/J × BALB/c: 630 ± 185 pg/ml; PY, CBA/J × DBA/2: 613 ± 155 pg/ml, all not significant).

3.5. Free circulating VEGF levels increase in pregnancy but do not differ with crossbreeding or parity status

Circulating levels of VEGF in non-pregnant CBA/J females and in term-pregnancy females are displayed in Fig. 2F and indicate that pregnancy itself increases cir-

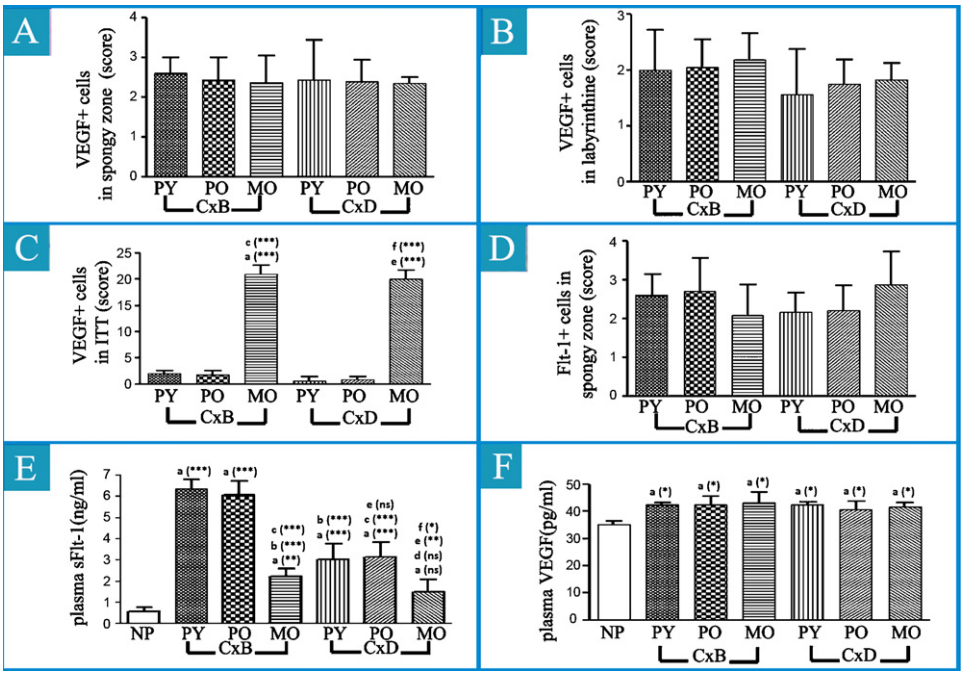

Fig. 2. (A–C) The expression of VEGF in spongiotrophoblast (A), labyrinthine (B) and invasive trophoblast tissue (C) was comparatively analyzed by IHC in the placentae obtained from the normal fertile allogeneic mouse crossbreedings CBA/J × BALB/c (C × B) and the abortion-prone combination CBA/J × DBA/2 (C × D) with different parity status (PY, PO, MO). VEGF+ cells were scored. (A, B) All groups showed a similar pattern ($p > 0.05$). (C) No statistical differences were found between CBA/J × BALB/c MO and CBA/J × DBA/2 MO. The differences between the MO groups and all other groups were significant, $p < 0.001$. (D) The expression of Flt-1 was comparatively analyzed by IHC in the spongiotrophoblast of placentae obtained from CBA/J × BALB/c and CBA/J × DBA/2 with different parity status (PY, PO, MO). VEGF+ cells were scored. There were no significant differences between groups ($p > 0.05$). (E) sFlt-1 levels were measured by ELISA in plasma from non-pregnant CBA/J female mice and term pregnant ones from the normal and abortion-prone mouse crossbreedings, divided into PY, PO and MO groups. (F) VEGF levels were measured in plasma from non-pregnant (NP) CBA/J female mice, term pregnant ones with different parity status (PY, PO, MO). The statistical analysis indicated that VEGF increased with pregnancy; however there were no differences between the crossbreedings and the parity status (non-pregnant vs all groups: $p<0.05$). The statistically significance of each comparison is expressed as: non-pregnant vs all, (a); C × B PY vs all, (b); C × B PO vs all, (c); C × B MO vs all, (d); C × B PY vs all, (e); C × B PO vs all, (f). All data is mean ± SEM. The statistical significance is expressed as: Not significant (ns) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

culating VEGF (non-pregnant, 35 ± 1.5 pg/ml vs pregnant groups, 40–44 pg/ml; $p < 0.5$). However, the concentration of free circulating VEGF remained constant, independently of the crossbreeding analyzed and the parity status of the female.

3.6. Multiparity status favours the expression of VEGF in placenta

Western blots of placental lysates probed for VEGF showed a band at 45 kDa in all mouse combinations. Additionally, MO placentae either from normal or abortive crossbreedings presented another band of higher molecular weight (>200 kDa) and a smaller fragment of 34 kDa (Fig. 3). The treatment with 2-ME did not cleave the heaviest protein.

However, when normal and abortive MO placental extracts were dialyzed against 8 M urea, the heaviest band was absent and several other bands were present between approximately 34 and 105 kDa, suggesting cleavage of the protein. Conversely, primiparous placentae of both crossbreedings showed two main bands of about 45 and 70 kDa. Moreover, western blots of MO samples showed a higher concentration of VEGF proteins than PY and PO placentae, in agreement with the immunohistochemical studies.

4. Discussion

The present work shows that multiparity increases the invasive trophoblast tissue at the maternal–fetal interface in normal and abortion-prone allogeneic mouse cross-

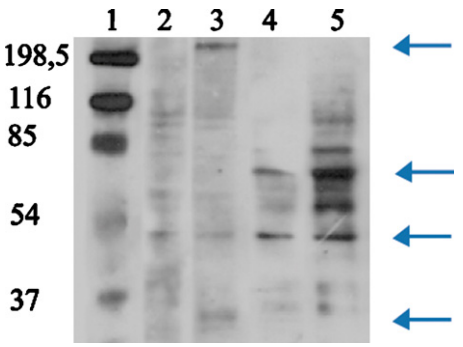

Fig. 3. Western blots of placental lysates. Placental VEGF expression was analyzed by 10% SDS-PAGE before (lanes 2 and 3) and after dialysis against 8 M urea (lanes 4 and 5). Molecular markers (MM) and placental lysates (40 μg) were loaded as follows: lane 1, MM (molecular weight in kDa); lanes 2 and 4, PY placental lysate; lanes 3 and 5, MV placental lysates. Blots were probed for VEGF. The arrows indicate main bands at 34, 45, 70, 105 and >200 kDa.

breedings to the same extent as observed previously in normal syngeneic pregnancies, indicating that this effect is independent of the differences in the major histocompatibility complex of the crossbreeding and the success of the pregnancy outcome. Interestingly, human studies have shown that first trimester endovascular trophoblast invasion is more extensive in normal parous women than in nulliparous ones (Prefumo et al., 2006).

VEGF was similarly expressed in spongiotrophoblast and labyrinthine trophoblast in all groups. The single layer of invasive trophoblast tissue present in primiparous placentae showed a lower expression of VEGF in the CBA/J × DBA/2 combination with respect to the normal fertile combination. Moreover, the multilayer of invasive trophoblast tissue of multiparous placentae showed high VEGF expression in both combinations, suggesting that besides its pro-angiogenic properties, this cytokine could participate in the invasion process per se.

We further investigated the placental expression of VEGF-R1 (Flt-1) and the circulating levels of its soluble isoform. We found that VEGF-R1 was expressed in the spongiotrophoblast in all groups and, in the case of multiparous placentae, it was represented as several isolated cells in the multilayer of invasive trophoblast tissue in both crossbreedings.

At day 7.5 of pregnancy, there was no variation in sFlt-1 levels compared to non-pregnant mice, while term-pregnant primiparous females of the normal crossbreeding markedly increased circulating sFlt-1 levels; these data are in agreement with those of He et al. (1999). We observed that the pregnancy-induced sFlt-1 increase was much lower in the primiparous CBA/J × DBA/2 combination, while multiparity reduced plasma sFlt-1 levels in both crossbreedings. On the other hand, pregnancy induced an increase in free circulating VEGF levels that did not differ with regard to the parity status and the crossbreeding.

Up to this point, three different situations of VEGF/Flt-1 concentrations in placenta and peripheral blood can be represented. In a normal first pregnancy, VEGF/Flt-1 placental expression might be optimal, where an increase in circulating sFlt-1 levels would be necessary to maintain a constant systemic VEGF level. A second situation is represented in primiparous females from the abortion-prone mating combination. Our results indicate that placental Flt-1 does not vary in respect to the first situation but invasive trophoblast tissue shows decreased expression of VEGF. Consequently, its release to circulation also would be lower, and this change would be regulated by diminishing the circulation levels of sFlt-1. Maynard et al. (2003) reported that an excess of circulating sFlt-1 associated with decreased circulating levels of VEGF contribute to the pathogenesis of pre-eclampsia. It is important to note that our mice did not show endothelial dysfunction or hypertension, facts that could be explained by the maintenance of circulating VEGF concentration. Finally, a third situation is represented in the multiparous female of any crossbreeding. We observed that the invasive trophoblast tissue proliferated and the expression of VEGF and Flt-1 are enhanced.

However, these increases are not reflected in the systemic concentrations of VEGF and Flt-1. On the contrary, we observed diminished sFlt-1 levels with respect to the normal situation while free circulating VEGF levels remained constant. These observations indicate that multiparity enhances the expression of VEGF in vivo in the layers of invasive trophoblast tissue and consequently, the release of VEGF to the circulation is attenuated. Therefore, low circulating sFlt-1 would be necessary to control systemic bioactivity of VEGF. In agreement with our results, higher levels of sFlt-1 are found in human first pregnancies with respect to those observed in second pregnancies (Wolf et al., 2005).

We further investigated VEGF proteins present in the placental lysates by western blot. Three isoforms of mouse VEGF have been reported (Shima et al., 1996). $VEGF_{120}$ is a free soluble protein, $VEGF_{164}$ is also secreted; although, a significant fraction remains bound to the cell surface and the extracellular matrix and $VEGF_{188}$ is almost completely sequestered in the extracellular matrix (Park et al., 1993). VEGF proteins become active as free diffusible forms or after cleavage of the longer isoforms thus exerting its potent mitogen effects on vascular endothelium, inducing the expression of collagenase, plasminogen activator and plasminogen activator inhibitor 1 and the extravasation of plasma proteins. Plasminogen activation results in the generation of plasmin, which is able to cleave extracellular matrix-bound VEGF releasing a bioactive proteolytic fragment of approximately 34 kDa (Ferrara and Davis-Smyth, 1997).

Besides the presence of a band of approximately 45 kDa (corresponding to the reported MW for dimeric VEGF protein) present in all groups, our western blots showed the presence of bands at 34 and >200 kDa in MO placentae, which might correspond to the isoform bound to extracellular matrix proteins. This heavy protein complex was stable after treatment with 2-ME and could be cleaved into several shorter molecules between 34 and 105 kDa in the presence of 8 M urea. In this condition, primiparous placentae showed lower VEGF expression (as shown in our IHC studies) and lighter bands, reinforcing the idea that a larger protein complex is present in MO placentae. Considering that only the longest isoforms of VEGF anchor the extracellular matrix and that the 34 kDa fragment would be derived from them, we suggest that the multiparity modulates the alternative splicing of VEGF in the invasive trophoblast tissue.

In conclusion, the present work reports that multiparity status increases the invasive trophoblast tissue at the maternal–fetal interface in normal and abortive allogeneic pregnancies. Multiparity also enhanced VEGF expression in this region without inducing deleterious modifications in the systemic concentration of the cytokine due to the release of sFlt-1. This fact could partially explain the beneficial effects of multiparity status on a subsequent pregnancy and provides basic knowledge on regulation of the trophoblast invasion process and successful placental development.

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