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Increased immunohistochemical expression of thrombomodulin at placental perivascular myofibroblast in severe preeclampsia (PE)

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Summary. The presence of pro-coagulant and anti-coagulant components of the placental vascular endothelium and syncytiotrophoblast are essential for homeostasis. Vascular endothelium prevents blood clot formation in vivo by involving a cell surface thrombin-binding glycoprotein, thrombomodulin (TM), that activates plasma anti-coagulant protein C. The TM levels increase during pregnancy, but the fibrinolytic capacity diminishes. Since vascular lesions with placental coagulation disorders can be associated with preeclampsia (PE), we hypothesized that TM expression in the stem villous vasculature and syncytiotrophoblast of the placenta are impaired in PE. Plasma and placental tissue samples were collected from PE (n=12) and normotensive pregnant patients (n=11). Patient's gestational age was 35.7±1.2 (normotensive) and 30.6±1.5 weeks (PE). Blood samples were drawn 30 min before delivery. Serum PAI-1 and PAI-2 antigens were determined by enzyme-linked immunoabsorbent assay (ELISA). A monoclonal antibody specific for TM was used for immunohistochemical tissue staining (ABC) and the staining was quantified by semi quantitative scores. Results show no intensity differences at the apical syncytiotrophoblast between the two groups. However, in preeclamptic placenta, TM expression diminished in the endothelium of the stem villi arteries and increased in the perivascular and stromal myofibroblats in cases of severe PE. TM changes were associated with an increased PAI-1/PAI-2 ratio.

It is suggested that in severe PE, the decreased placental blood flow may be due to structural and functional impairment of the endothelium of the stem villi vessels and the surrounding perivascular and stromal myofibroblast, by increasing TM expression which may modulate fetal blow flow in the villous tree.

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Introduction

In the placenta, the stem villi of the villous trees are thought to control the fetal blood flow to the materno/fetal exchange area located in the peripheral villi (Demir et al., 1997). Since the placenta lacks autonomic innervations, blood flow must be regulated by humoral mechanisms and autocrine/paracrine factors produced in the placental vasculature (Myatt, 1992; Sebire and Talbert, 2001). A second perivascular contractile system (PVCS), in addition to the fetal blood vessels system, is present in the chorionic plate and the stem villi of human placenta (Graf et al., 1997). The PVCS contains a signal transduction system, allowing communication between the smooth muscle cells of this sheath and the surrounding extracellular matrix (Graf et al., 1994).

Preeclampsia (PE), is a systemic pregnancy disorder of unknown pathogenesis, although it is widely accepted to originate in the placenta (Redman, 1991). The pivotal role of placenta in PE is suggested by the occurrence of this syndrome in pregnancies where the fetus is absent, such as in the hydatidiform mole (Scott, 1958; Chun et al., 1964). In preeclamptic pregnancies, the reduction of utero-placental perfusion pressure and the ensuing placental ischemia/hypoxia, during late pregnancy may be caused by inadequate cytotrophoblast invasion of the uterine spiral arteries in the first trimester of pregnancy (Zhou et al., 1993; Di Federico et al., 1999; Many et al., 2000). Placental ischemia/hypoxia may trigger the release of placental factors that initiate a cascade of cellular and molecular events leading to endothelial and vascular smooth muscle cell dysfunction (Bosco, 2005), thereby increasing vascular resistance and arterial pressure (Khalil and Granger, 2002). Additionally, PE is characterized by maternal hypercoagulable state and

intravascular coagulation, microthromboses in several organs and impairment of utero-placental circulation (Kanfer et al., 1996; Estelles et al., 1998).

The thromboresistance of the placental endothelium is maintained as long as natural anticoagulant pathways are functionally present in the endothelial plasma membranes (Labarrere and Faulk, 1992a). The main anticoagulant pathway in the placenta is mediated by thrombomodulin (TM), an endothelial cell-surface glycoprotein (Dittman and Majerus, 1990). The down regulation of TM is associated with fibrin deposition in the vessels, resulting either from a failure in anticoagulation or fibrinolysis (Fernandez et al., 2003). These thrombi impair blood flow through the placental microcirculation and are associated with ischemic changes (Labarrere and Faulk, 1992a,b). High affinity binding of thrombin to TM results in a conformational change and an altered substrate specificity of the thrombin molecule that activates protein C. Activated protein C degrades factor Va and VIIIa proteolytically, thereby inhibiting further thrombin generation and maintaining thromboresistance in the intervillous space. Furthermore, when thrombin is bound to TM, it is not available for interaction with its substrates, particularly fibrinogen (Esmon, 2000).

PE patients with extensive placental infarction exhibit a higher plasma plasminogen activator inhibitor-1 (PAI-1) value than PE patients without extensive placental infarction. In contrast, PAI-2 levels were lower in PE patients with infarction than in patients without infarction (Estelles et al., 1991). In PE lower PAI-2 levels were shown to be associated with placental insufficiency while higher PAI-1 levels associated with endothelial dysfunction (Roes et al., 2002).

In the present study, we investigated the immunohistochemical localization of TM in the stem villi of placentas from women with severe PE and with normotensive pregnancies, its association with maternal plasma PAI-1 and PAI-2 levels and fibrin deposits.

Materials and methods

All procedures were performed using protocols approved by the local Ethics Committee of the Clinical Hospital of the University of Chile. All women gave informed consent to participate in this study. Gestational age was determined by an ultrasound examination during the 22nd-25th week of pregnancy. Color Doppler of the uterine artery was obtained in three consecutive waveform readings and the mean pulsatility index of the two vessels was calculated (Parra et al., 2003).

The study was performed during a 15 month period from January 2002 to March 2003 at the University of Chile Clinical Hospital. The inclusion criteria in the PE group required participants to be healthy prior to pregnancy with no history of hypertension, diabetes, or renal dysfunction. The inclusion criteria in the control group were, 24-42 weeks of gestation and normotensive pregnancy. The exclusion criteria in the control group were major fetal abnormalities, placental tumor, intrauterine infection, not twins, obstetrics pathology other than spontaneous preterm delivery and maternal disease.

The preeclamptic patients were enrolled between weeks 22nd-25th routinely. The gestational age was 35.7±1.2 and 30.6±1.5 weeks for normotensive and PE patients, respectively. Pregnant women with absolute blood pressure of ≥ 160/110 mm Hg and proteinuria >5gr/24 hours were considered severe preeclamptic patients based on the classification of the American College of Obstetrics and Gynecology. Both hypertension and proteinuria were required to regress after delivery. None of the individuals in the normotensive group suffered from any medical complication during pregnancy.

Plasma and placenta samples (fetal side) were collected from preeclamptic (n=12) and normotensive patients (n=11). Blood samples, obtained 30 min prior to delivery were drawn directly into vacuum tubes with anticoagulant (one volume of 0.1 M citrate for nine volumes of blood, pH 7.4), mixed thoroughly, and centrifuged at 2,800 g for 15 minutes at room temperature. Serum samples were collected and stored at -70°C until the assay was performed. Serum PAI-1 and PAI-2 antigens were determined by using an enzyme–linked immunoabsorbent assay (ELISA).

Fetal placental villous tissue was collected 2 cm off the umbilical cord immediately after delivery from normotensive and preeclamptic subjects. The tissue was fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3),embedded in paraffin wax and 5 mm sections were made. Sections were stained for fibrin deposits using two different methods, PAP (Eosin Y; Light Green SF, Yellowish; Phosphotungstic) and Sirius-Red (Bosco et al., 1989). For the histomorphometric study, sections of stem villi with and without fibrinoid deposits, were recorded using an intraocular grid with 100 square fields of one mm^2 each according to Kanfer et al. (1996). Stem villi were examined in five consecutive fields of each sample of fetal placental parenchyma, excluding chorial and basal plates. The ratio of stem villi with fibrinoid deposits to the total area of stem villi was calculated, and the results are expressed as the percentage of positive (stained) villi. Standard immunoperoxidase techniques were used to show TM distribution in the tissue sections. Mouse anti-human TM monoclonal antibody, diluted 1:50 (v/v) (M0617 DAKO) was applied to the sections for 20 min at 37°C. In negative controls for TM, monoclonal antibody was omitted. Immunostaining was performed using a horseradish peroxidase-labelled streptavidin biotin kit (DAKO) according to manufacturer's directions using diaminobenzidine as the chromogen. The sections were counterstained with Mayer's haematoxylin (DAKO) and mounted with Entellan (Merck). All sections were processed simultaneously to allow direct comparison between the groups and examined by light microscopy (Zeiss Axioplan 2). The intensity of the immunohistochemical

Table 1. Clinical characteristics of preeclamptic and control groups.

	ALL CONTROL GROUP (pretern babies)	PE GROUP
Maternal age (year)	32.7 ± 1.5	27.8 ± 2.1 *
Primiparity (%)	9.1	50.0
Gestational age (weeks)	35.7 ±1.2 (31.9± 0.72)	30.6 ± 1.5 *
Newborn weight (grams)	2772.2 ± 321.4 (1809± 236.9)	1370.0 ± 207.3 *
Cesarean section (%)	90.9	100
BMI	24.5 ± 2.0	26.3 ± 1.0
Zscore Newborn weight	0.39 ± 0.24 (0.23 ± 0.32)	- 1.46 ± 0.35 *

*: Mann-Whitney Rank Sum Test, p<0.05 between groups. Chi-square for ratios. Gestational age was not significantly different between preeclamptic (n=12) and preterm groups (n=5), although newborn weight percentile (zscore) was significantly lower in preeclamptic babies than preterm and term control groups (- 1.46 vs 0.23 and 0.51, respectively; p<0.05).

Table 2. Special characteristics of PE group.

CHARACTERISTICS	
Fetal growth restriction (< 10th centile) (n/N)	6/12
Proteinuria 24 hours (grs/24 h)	7.4± 1.4
Platelets	80.213± 19.812
GOT	94.7± 35.2
GPT	98.9± 34.1
LDH	874.6± 111.6
Creatinine	0.99± 0.1
HELLP syndrome (n/N)	4/12
Severe Preeclampsia (n/N) *	11/12

*: Severe PE defined as: BP>160/110, proteinuria>5 grs/24 hours, associated with HELLP syndrome or neurologic disorders. This classification is according to the American College of Obstetrics and Gynecology.

staining was measured using semi quantitative scores. Three randomly selected fields from each patient were evaluated by two different examiners who were blinded to the clinical information. The sections were scored for intensity as: absent [0], faint [1], moderate [2], or intense [3]. Some tissue sections were stained for actin using a mouse anti-human smooth muscle actin monoclonal antibody (diluted 1:50 v/v M0851 DAKO) to confirm the presence of myofibroblast and pericytes. Microwave heat-induced antigen retrieval in citrate buffer, pH 6.0, was required for optimal staining with the anti-human smooth muscle actin antibody.

For morphological studies of placenta, the results represent the percent in each category within the group. The semi quantitative scoring of immunostaining intensity for variables presented in Tables 4, 5, 6 and 7 were assessed by the Pearson chi^2 test for differences between groups. Differences were considered statistically significant at $p < 0.05$.

Results

The details of patient's pregnancy outcomes are shown in Table 1. The clinical details of the PE patient group are shown in Table 2. Gestational age was not significantly different between PE (n=12) and preterm groups (n=5). The newborn weight percentile (z score) was significantly lower in PE babies than both preterm and term control groups (-1.46 vs. 0.23 and 0.51 respectively; p< 0.05). Ten of eleven patients in the control group and all patients in PE group were nonsmokers.

TM staining was detected primarily in the syncytiotrophoblast but was also observed in the endothelia and some stromal cells. Table 3 shows the range of TM staining intensity in both groups of pregnancies. The immunostaining in stromal cells of the villous tissue, probably myofibroblast, was seen in the tissue from both groups. In the PE group, the endothelium of the large vessels in the villous vasculature was occasionally immunostained for TM and spread into the surrounding stroma (Fig. 1B). In contrast, TM staining was significantly more intense in the vascular endothelium of the normotensive control group (Fig. 1A). At the apical syncytiotrophoblast there was no apparent difference in the staining intensity between the two groups (Fig. 1A,B). The TM immunostaining intensity in the placentas was significantly greater in the vascular endothelium of the villous vasculature of the control compared with the PE group placenta (Table 4). The intensity of TM immunostainig was not significantly greater in the stroma of the control compared with the PE group placenta (Table 5). Instead, the TM immunostaining intensity was significantly greater in the stroma of the PE placentas compared with the endothelium (Table 6). The TM immunostaining intensity was significantly higher in the control term placentas (6/11) compared to severe PE placentas without IURG (6/12), (Table 7). In the absence of the primary antibody (anti TM), no TM immunostaining was observed in tissue sections from either group (Fig. 2).

No significant differences in the staining of perivillous and intravillous fibrinoid deposits were observed when PE and control placentas were compared (Table 8). Perivillous fibrinoid deposits were considered as the markers of intraplacental fibrin deposition. Figure 3A,B confirm the presence of cells such as

myofibroblast and pericytes with actin cytoskeleton.

Concerning the plasma PAI-1/PAI-2 ratio, it is important to stress that PAI-1 abundance was greater than PAI-2 in both groups, and the magnitude of its increase in the preeclamptic group exceeded that of PAI-2. The PAI-1/PAI-2 ratio was significantly ($p < 0.05$)

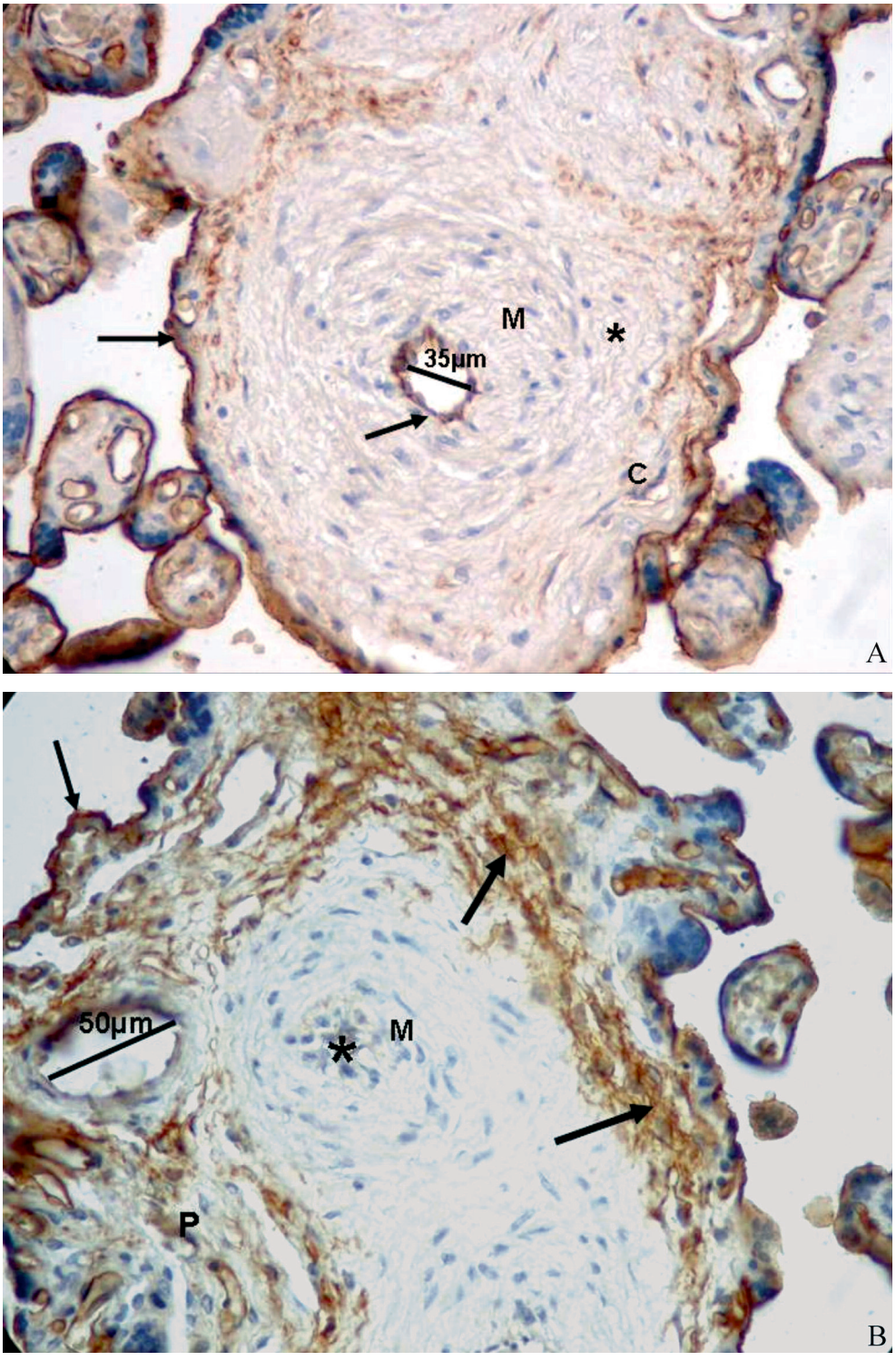


Fig. 1. A. Bright-field micrograph of human placenta from normotensive pregnancy group showing the immunohistochemical expression of TM by using a monoclonal anti-Trombomodulin antibody. Note the intense immunostaining of TM in the endothelium of stem villi artery, capillaries of the terminal villous and the apical zone of the syncytiotrophoblast (arrows). In addition, staining was negative in areas of stromal cells (*) and capillary (C) and the tunica media of the artery (M). Bar: 35 µm. **B.** Bright-field micrograph of human placenta from PE group showing the immunohistochemical expression of TM using the monoclonal anti-Trombomodulin antibody. TM immunostaining was negative in the endothelium of stem villi artery (*) and faint in the capillaries of the terminal villous, intense in the apical zone of the syncytiotrophoblast and in areas of stromal cells (arrows), possibly myofibroblasts surrounding the stem villi arteries and pericytes (P) of capillaries of the stroma and the vasa vasorum of the adventitia. Bar: 50 µm.

Table 3. Assesment of TM immunostaining in placenta.

ENDOTHELIUM		STROMAL MYOFIBROBLAST	
Control	PE	Control	PE
++	- (severe)	+	+
+ (term)	++	++	+++
+++ (term)	- (severe)	-	+++
- (term)	+	+	+
++	- (severe)	+	+++
+++	-	-	-
+++ (term)	+	-	++
+++ (term)	+ (severe)	+	+++
+++ (term)	-	-	-
+	+	+++	+
+++	+ (severe+ HELLP)	+	+++
	+ (severe+ HELLP)		+++

Three fields were scored for each slide. Intensity of staining. +, ++ or +++.

increased in the preeclamptic, as compared to normotensive subjects (Fig. 4).

Discussion

The results of this study show that in the placenta, TM is localized in the cells that are in direct contact with the vascular system and in cells of the stem villi stroma. The cells of the stroma are represented by myofibroblasts of the perivascular contractile system and pericytes of the stromal capillaries and the adventitia surrounding the arteries of the stem villi (Fig. 1). TM expression (Tables 3, 4 and Fig. 1A,B) was significantly lower in the endothelium of PE placentas compared with the controls. Notably, in the stem villi arteries of the 6/12 severe PE placentas, TM expression decreased in the endothelium and increased in the myofibroblasts. These data are in agreement with those of Labarrere and Faulk

Table 4. Immunohistochemical expression of thrombomodulin (TM) at the endothelium of the stem villi placental arteries.

GROUP	NEGATIVE	LIGHT	MODERATE	INTENSE	TOTAL
Normotensive	8.33% (n=1)	25.00% (n=2)	16.67% (n=2)	50.00% (n=6)	100.00% (n=12)
Preeclamptic	41.67% (n=5)	50.00% (n=6)	8.33% (n=1)	0.00% (n=0)	100.00% (n=12)
Total	25.00% (n=6)	37.50% (n=9)	12.50% (n=3)	25.00% (n=6)	100.00% (n=24)

The results of a total of 11 normotensive and 12 PE placentae are presented as percent of the categories within each groups. $p< 0.05$ between PE and normotensive placentae by Pearson chi^2 test.

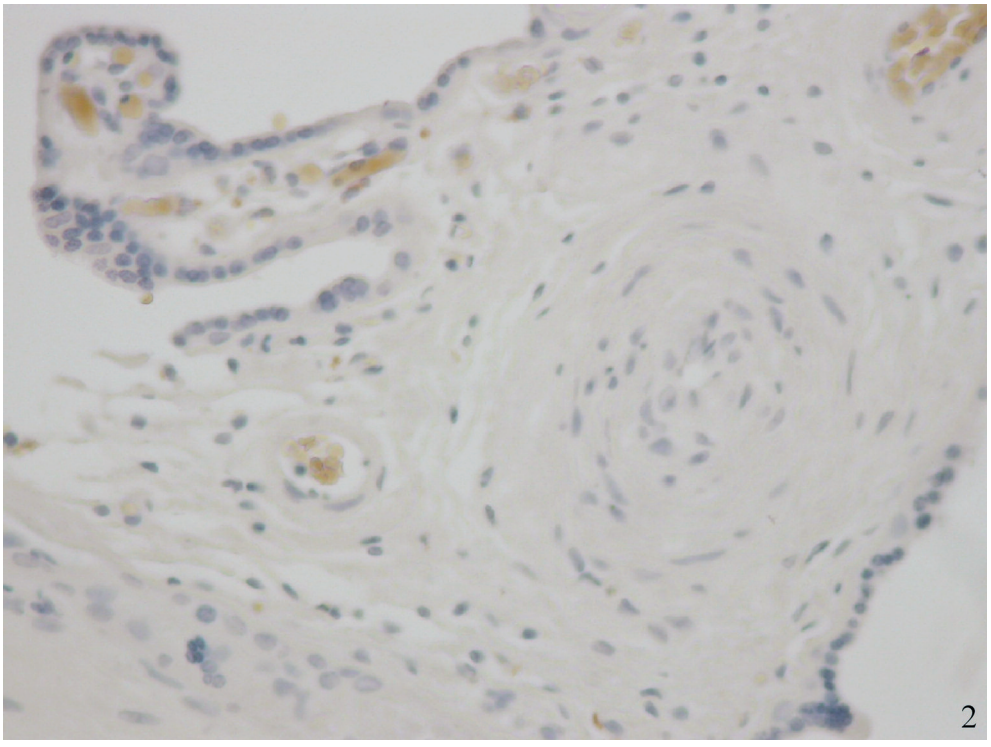
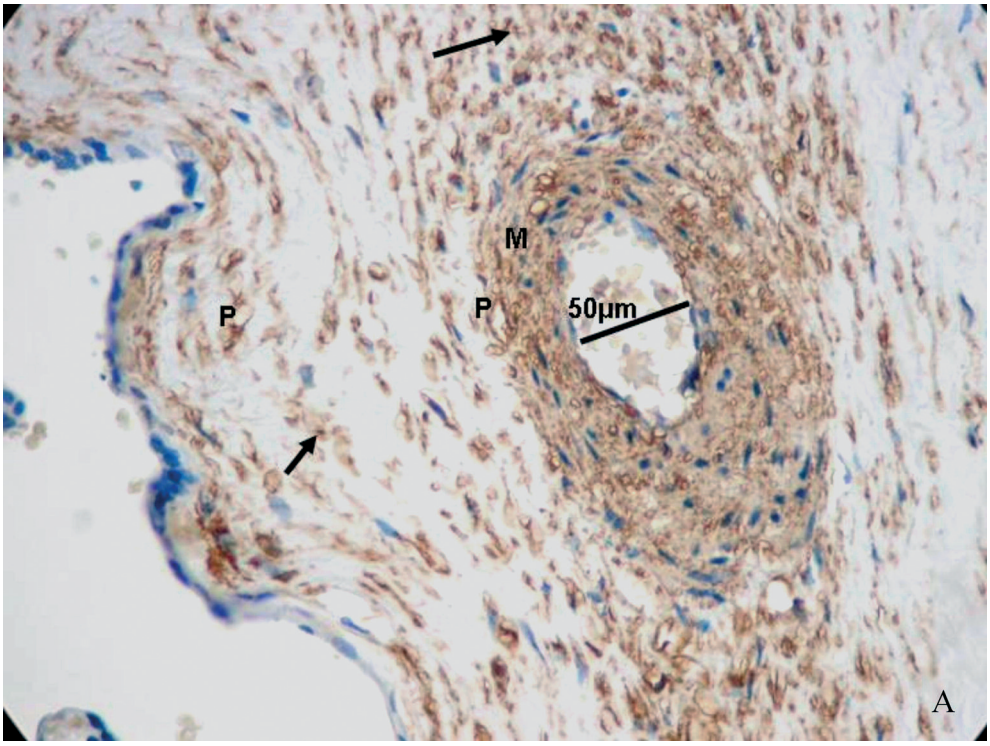


Fig. 2. Bright-field micrograph of human placenta from preeclamptic pregnancy group showing negative control TM staining. Control contained no primary antibody. x 400

(1992a,b) showing that endothelial cells were negative for TM, and positive for tissue factor and fibrin in placentas from PE and from secondary recurrent spontaneous aborts, (a placental-related disorder of pregnancy such as PE, Burton and Jauniaux, 2004). Kanfer et al. (1996) reported an excessive placental



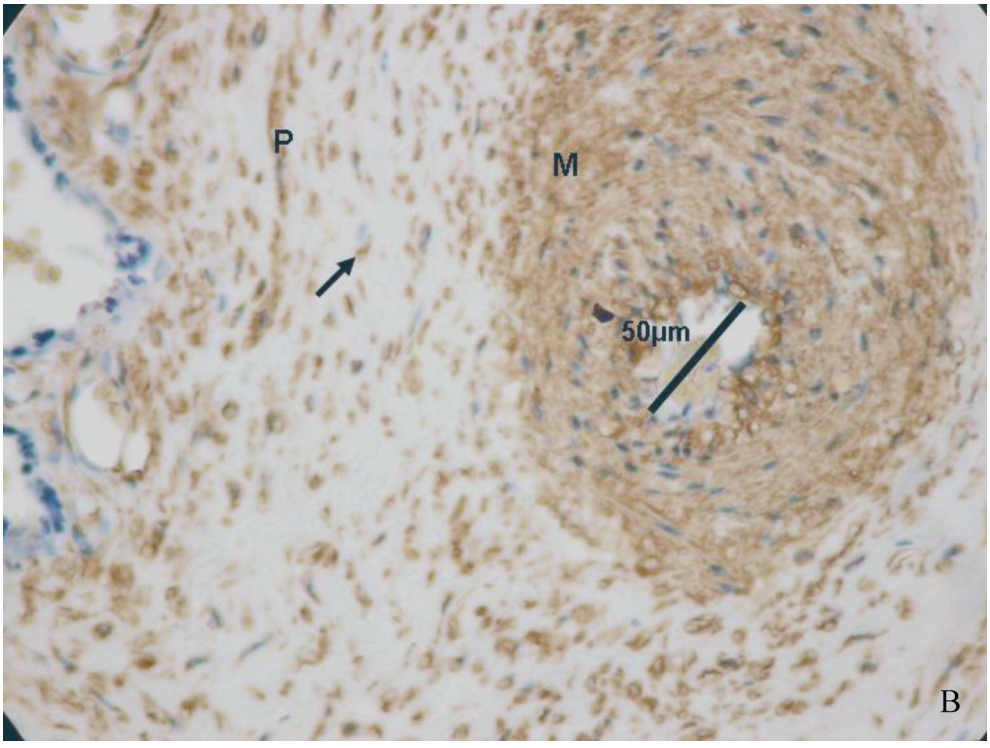


Fig. 3. A. Bright-field micrograph of human placenta from normtensive pregnancy group showing the immunohistochemical actin expression using the monoclonal anti-human smooth muscle actin antibody. Note the intense immunostaining for actin in the middle tunic (M) of a transverse section of an artery, consisting mainly of smooth muscle cells positioned circularly, and in the stromal myofibroblast (arrow) and pericytes (P) of the adventitial capillaries of the vasa vasorum. Bar: 50 µm. **B.** Bright-field micrograph of human placenta from preeclamptic pregnancy group showing the immunohistochemical actin expression using the monoclonal anti-human smooth muscle actin antibody. Note the intense immunostaining for actin in the middle tunic (M) of a semi-oblique section of an artery, consisting mainly of smooth muscle cells positioned circularly, and in the stromal myofibroblast (arrow) and pericytes (P) of the adventitial capillaries of the vasa vasorum. Bar: 50 µm.

Table 5. Immunohistochemical expression of thrombomodulin (TM) in myofibroblast and pericytes of the stem villi placenta.

GROUP	NEGATIVE	LIGHT	MODERATE	INTENSE	TOTAL
Normotensive	36.36% (n=4)	45.45% (n=5)	9.09% (n=1)	9.09% (n=1)	100.00% (n=11)
Preeclamptic	16.67% (n=2)	25.00% (n=3)	8.33% (n=1)	50.00% (n=6)	100.00% (n=12)
Total	26.09% (n=6)	34.78% (n=8)	8.70% (n=2)	30.43% (n=7)	100.00% (n=24)

The results of a total of 11 normotensive and 12 PE placentae are presented as percent of the categories within each groups. N.S. not significant between PE and normotensive placentas by Pearson chi^2 test

Table 6. Immunohistochemical expression of thrombomodulin (TM) at stem villi of Preeclamptic placentae.

TM FETAL ARTERIAL EXPRESSION	NEGATIVE	LIGHT	MODERATE	INTENSE	TOTAL
Endothelium	41.67% (n=5)	50.00% (n=6)	8.33% (n=1)	0.00% (n=0)	100.00% (n=12)
Myofibroblast and pericytes	16.67% (n=2)	25.00% (n=3)	8.33% (n=1)	50.00% (n=6)	100.00% (n=12)
Total	29.17% (n=7)	37.50% (n=9)	8.33% (n=2)	25.00% (n=6)	100.00% (n=24)

The results of TM at 12 PE placentas are presented as the percent of the categories within each group. $p<0.05$ between endothelium and myofibroblast and pericytes of PE placentae by Pearson chi^2 test.

Table 7. Immunohistochemical expression of thrombomodulin (TM) at stem villi of Control Term and Severe Preeclamptic placentas.

GROUP	NEGATIVE	LIGHT	MODERATE	SEVERE	TOTAL
Control term	50.00% (n=3)	33.33% (n=2)	16.67% (n=1)	0.00% (n=0)	100.00% (n=6)
PE severe	0.00% (n=0)	33.33% (n=2)	0.00% (n=0)	66.67% (n=4)	100.00% (n=6)
Total	25.00% (n=3)	33.33% (n=4)	8.33% (n=1)	33.33% (n=4)	100.00% (n=12)

The results of TM at 6 Control term (endothelium) and 6 PE placentas (myofibroblast and pericytes) are presented as percent of the categories within each group. $p<0.05$ between endothelium and myofibroblast and pericytes by Pearson chi^2 test.

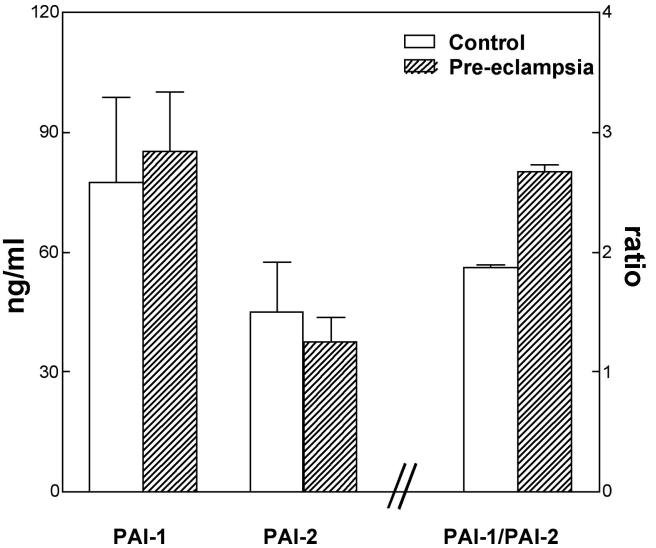


Fig. 4. Ratio of the maternal plasma PAI-1 to PAI-2 activity for normotensive pregnant women prior to delivery (n=12), and preeclampsia (n=12). The level of PAI-1 was higher than PAI-2 in both groups, and the magnitude of its increase in the preeclamptic group exceeded that of PAI-2. As a result, PAI-1/PAI-2 ratio is increased in preeclamptic compared with normotensive pregnant subjects. $P<0.05$ by Student`s t-test.

perivillous fibrin deposition in PE placentas. However, we found no significant differences in fibrin deposits of the placental stem villi between the two groups. These discrepancies (in fibrin deposition) , at least in part, could be due to the fact that our study addressed stem villi and not terminal villi. Yang et al. (1998), reported a dose-dependent reduction in cell surface TM activity in an *in vitro* HUVEC incubation with humic acid (HA) isolated from the drinking water in Taiwan. HA induces down-regulation of TM directly by increasing the cell membrane permeability, thus causing an elevation in $[Ca^{+2}]$ influx. Calcium acts as a second messenger in the activation of protein kinase C, and/or other Ca^{+2}-dependent enzymes that may play a role in down-regulation of TM expression. Cone et al., (2003) found normal endothelium TM immunoreactivity in non-inflamed but not in inflamed lung areas in a zymosan-induced rat model. They postulated that down-regulation of TM leads to a hypercoagulable endothelium, increased microvascular thrombosis, and subsequent lung injury.

The increased TM expression in myofibroblasts and pericytes representing stromal cells in the preeclamptic placentas (Fig. 1B), shows that TM is present in areas that are not in contact with the blood stream. Endothelial

TM plays a physiological role in maintaining the blood flow in the blood vessels, but its role in the tissues (except in vascular endothelial cells) is still undetermined. However, the presence of TM in non-vascular sites, such as in the anterior segment of the human eye, suggests an association of TM to cell adhesion, differentiation, and/or proliferation (Ikeda et al., 2000). Surprisingly, placentas with increased TM expression in myofibroblast showed an increased villous cytotrophoblast and decreased syncytials knots, (data not shown). It is suggested that a significantly increased TM expression in myofibroblast stromal villi in severe PE (6/12), (Table 7), may play a role in preserving the function of these cells in villous contractility and modulation of the intervillous space affecting both maternal and fetal placental circulation (Demir et al, 1997; Kacemi et al., 1999). This is further supported by a lack of IURG in these cases. In normal human and rat lungs, alveolar myofibroblast and pericytes have been observed in the stroma, and the placenta is known to have a lung function for the fetus (Kapanci et al., 1992). Bosco (1994) reported the presence of pericytes in the capillary of the stem villi by electron microscopy consistent with our observations. Although, the presence of stromal myofibroblasts were not different between PE and the normotensive groups (Fig. 3A,B), stromal TM expression increased in PE placentas and decreased in the stem villi endothelium (Table 6). Since the TM expression was significantly different between the control term placentas and severe preeclamptic placentas (Table 7), it is suggested that the increased TM expression in myofibroblasts of the villous core may counterbalance the procoagulant effect of hypoxia by vasoconstriction in the core villous tissue due to the increased thromboxane to prostacyclin ratio in PE placentas (Walsh and Wong, 1995). Myofibroblasts have also been shown to be a major cellular constituent of the villous stroma in human placenta (Feller et al., 1985). Furthermore, since the placenta lacks autonomic innervations, the blood flow must be regulated by humoral mechanisms and by autocrine/paracrine factors produced in the placenta (Myatt, 1992; Sebire and Talbert, 2001). Since the human placenta require contractile structures to generate energy for blood propulsion, and smooth muscle cells are not present in significant numbers, myofibroblast could play a role in the modulation of vascular tone of placental vessels (Feller et al., 1985). The presence of a perivascular contractile system (PVCS) in the chorionic plate and stem villi of the human placenta has been reported by Graf et al. (1997). PVCS could make communication possible between the smooth muscle cells and surrounding extracellular matrix. Graf et al. (1994) highlighted the vasodilator function of NO released by the myofibroblast and endothelial cells of the placenta and proposed that PVCS may regulate the villous turgor and the control of intervillous blood flow impedance.

The uniform expression of TM in the syncytiotrophoblast apical membrane surface observed in the PE and normotensive groups (Fig. 1A.B) indicates that this surface glycoprotein (TM) plays a major role at the feto-maternal interface related to placental architecture and function (Fazel et al., 1998). Since the maternal blood flow into the intervillous space could give rise to haemostatic problems, such as the risk of coagulation activation and fibrin deposition (Lanir et al., 2003), it is suggested that the presence of TM at apical syncytiotrophoblast surface may avoid this problem.

The antithrombotic TM activity depends on the local cell membrane status (Ishii et al., 1991, 2003), and alterations in its expression appeared to underlie the effects of hypoxia on endothelial cell. Labarrere and Faulk (1992a) showed a major role of vascular endothelium in preventing *in vivo* blood clot generation, and suggested that thromboresistance of the placental endothelium is maintained as long as natural anticoagulant pathways are functional in the endothelial plasma membranes.

A decrease in fibrinolytic activity during pregnancy is due to an increase in PAI-1 and PAI-2 activities (Estelles et al., 1991). Lower PAI-2 levels in patients with PE are associated with placental insufficiency (Nakashima et al., 1996; Roes et al., 2002). The present study showed that higher PAI-1 levels might be associated with endothelial dysfunction based on the elevated PAI-1/PAI-2 ratio in PE placentas (Fig. 4). The mechanism of endothelial TM decreases remains to be elucidated. Malek et al. (1994) explored the role of fluid shear stress (the frictional force exerted on endothelial cells by blood flow) on endothelial TM mRNA and protein expression in bovine aortic endothelial (BAE) and smooth muscle cells (BSM) in *in vitro*. They

Table 8. Percentage of placental stem villi with fibrinoid deposits.

LOCALIZATION OF FIBRINOID DEPOSITS	HEALTHY PREGNANT WOMEN (n=11)	PREECLAMPTIC WOMEN (n=12)	SIGNIFICANCE (p)
Perivillous	1.1±0.5 (0.4-2.1)	1.7±0.9 (0.5-3.1)	0.13 N.S.
Intravillous	3.7±1.5 (1.3-6.8)	4.0±1.8 (1.4-7.9)	0.60 N.S.
Total	4.9±1.7 (2.5-8.5)	5.9±2.2 (3.1-11.0)	0.34 N.S.

Results correspond to the means ± SD (range). The two groups were compared statistically, using the Mann-Whitney test. p value between preeclampsia and control group. N.S. not significant

observed a transient increase followed by a significant decrease in TM mRNA expression nine hours after the onset of blood flow in BAE but not in BSM cells. This is in agreement with our results, since we observed that TM expression was negative in the endothelial cells in PE placentas, but positive in myofibroblast and pericytes. Both myofibroblasts and pericytes contain α-actin cytoskeleton similar to BSM. Additionally, shear stress, stimulates vessel dilation, endothelial NO production, eNOS expression, (Li et al., 2003), and is a major determinant of arterial tone and vascular remodeling and atherogenesis. Jin et al. (2003) found that shear stress rapidly activates vascular endothelial growth factor receptor 2 (VEGFR-2/KDR) in a ligand-independent manner and eNOS activation in cultured endothelial cells. Helske et al. (2001) found that VEGFR-2 expression was localized exclusively to the endothelial cells of blood vessels of placental villi in either PE or normal pregnancy placentas. In this respect, the shear stress in PE placentas, could affect endothelial TM expression but not VEGF-2/KDR expression.

The procoagulant properties of the cultured vascular endothelial cells are enhanced in response to inflammatory cytokine tumor necrosis factor (TNF-α) resulting in a reduction of TM expression (Conway and Rosenberg, 1988). This is consistent with a TM decrease observed in the endothelium of the stem villi in PE placentas (Table 3). Moore et al. (1989) and Lentz et al., (1991), found that TNFα induces endothelial TM internalization and subsequent degradation, and/or inhibition of TM transcription. Increased oxidative stress in the placentas of women with PE, assessed by higher levels of malondialdehyde production (Madazli et al., 2002), suggests its stimulatory effect on cytokine TNFα. An elevation in TNFα levels in the plasma and placenta of women with PE has been reported (Conrad et al., 1998; Benyo et al., 2001). Soff et al., (1991) demonstrated that TNFα suppresses TM expression in endothelial but not in cultured rat, bovine, or human smooth muscle cells (SMC). Furthermore, following acute or chronic endothelial injury, luminal SMC with an actin cytoskeleton like myofibroblast, express TM to protect damaged blood vessels from thrombosis. Maruyama and Majerus (1985) demonstrated that in cultured HUVEC exposed to thrombin, TM expression decreased, perhaps due to internalization of thrombin-TM complex. The internalized thrombin-TM is degraded and TM reappears on the cell surface within 30 min, suggesting its recycling. When thrombin is bound to TM, it is not available to interact with its substrates platelets and fibrinogen (Grinnell and Berg, 1996; Esmon, 2000). The increased expression of TM reported here on the myofibroblast membrane would be related to the lack of differences between the fibrin deposits on the stem villi of PE versus normal pregnancies (Table 8). However, in contrast Kanfer et al. (1996) reported higher fibrinoid deposits in PE placentas compared to controls in all type of villi. In the present study fibrin deposits were measured only in stem villi.

Vascular endothelial growth factor (VEGF) is a growth factor and stimulates vasculogenesis and angiogenesis. Nomura et al. (1995), Takagi et al. (1996) and Yamagishi et al. (1999), showed that pericytes, like endothelial cells, express the VEGF gene in response to hypoxia, and become predominantly mitogenic. This promotes the growth of endothelial cells and pericytes through the synergistic actions of VEGF, endothelin-1, and platelet-derived growth factor B (PDGF-B). Endothelin-1 is significantly higher in the placental tissues from women with PE, (Singh et al., 2001), and PDGF-B released by platelets and inflammatory cells has been suggested to play a role in wound healing (Heldin, 1992). Lyall et al. (1997) reported that VEGF immunostaining was less intense in syncytiotrophoblast of term placental tissue than in stromal cells. Intensity of VEGF immunostaining in syncytiotrophoblast was significantly reduced in PE, PE plus IUGR, and IUGR, vs. normal group, but it was not different in stromal cells. It is suggested that the expression of VEGF and TM in PE syncytiotrophoblast and villi stromal cells are opposite to each other. The expression of VEGF in stromal cells of PE placentas is not affected like the expression of TM in apical syncytiotrophoblast.

Based on these results it is suggested that the endothelial damage of the stem villi arteries is characterized by a decrease in TM expression and this may be associated with an elevated PAI-1/PAI-2 ratio in the maternal plasma. Additionally, the increase of TM expression by the surrounding perivascular and stromal myofibroblast of the stem villi may be an adaptive response to modulate the fetal blood flow in the villous tree.

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References

Benyo D.F., Smarason A., Redman C.W., Sims C. and Conrad K.P. (2001). Expression of inflammatory cytokines in placentas from women with preeclampsia. J. Clin. Endocrinol. Metab. 86, 2505-2512.

Bosco C. (1994). Morphology of the capillaries in the alpha and beta zone of human term placenta: the relationship between capillary morphology and the trophoblastic layer. Med. Sci. Res. 22, 115-117.

Bosco C. (2005). Alcohol and xenobiotics in placenta damage. In : Comprehensive handbook of alcohol related pathology. Vol 2. Preedy V.R. and Watson R.R. (eds). Elsevier Ltd. London. pp 921-935.

Bosco C., Manriquez F., Molina R. and Araya J. (1989). Placentas normales de término. Vellosidades coriónicas. Matriz extracelular figurada. Tipo de colágeno y su disposición en la vecindad de los capilares fetales detectados con la técnica de picro-Sirius. Rev. Med. Chile. 117, 1102-1107.

Burton G.J. and Jauniaux E. (2004). Placental oxidative stress: from

miscarriage to preeclampsia. J. Soc. Gynecol. Invest. 11, 342-352.

Cone J.B., Ferrer T.J., Wallace B.H., Wang J. and Hauer-Jensen M. (2003). Alterations in endothelial thrombomodulin expression in zymosan-induced lung injury. J. Trauma 54, 731-736.

Conrad K.P., Miles T.M. and Benyo D.F. (1998). Circulating levels of immunoreactive cytokines in women with preeclampsia. Am. J. Reprod. Immunol. 40, 102-111.

Conway E.M. and Rosenberg R.D. (1988). Tumor necrosis factor suppresses transcription of the thrombomodulin gene in endothelial cells. Mol. Cell Biol. 8, 5588-5592.

Chun D., Braga C., Chow C. and Lok L. (1964). Clinical observation on some aspects of hydatidiform moles. J. Obstet. Gynaecol. Br. Comnwlth. 74, 180-184.

Demir R., Kosanke G., Kohnen G., Kertschanska S. and Kaufmann P. (1997). Classification of human placental stem villi : review of structural and functional aspects. Microsc. Res. Tech. 38, 29-41.

Di Federico E., Genbacev O. and Fisher S.J. (1999). Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. Am. J. Pathol. 155, 293-301.

Dittman W.A. and Majerus P.W. (1990). Structure and function of thrombomodulin: a natural anticoagulant. Blood 75, 329-336.

Esmon C.T. (2000). Regulation of blood coagulation. Biochim. Biophys. Acta 1477, 349-360.

Estelles A., Gilabert J., Espana F., Aznar J. and Galbis M. (1991). Fibrinolytic parameters in normotensive pregnancy with intrauterine fetal growth retardation and in severe preeclampsia. Am. J. Obstet. Gynecol. 165, 138-142.

Estelles A., Gilabert J., Grancha S., Yamamoto K., Thinnes T., Espana F., Aznar J. and Loskutoff D.J. (1998). Abnormal expression of type 1 plasminogen activator inhibitor and tissue factor in severe preeclampsia. Thromb. Haemost. 79, 500-508.

Fazel A., Vincenot A., Malassine A., Soncin F., Gaussem P., Alsat E. and Evain Brion D. (1998). Increase in expression and activity of thrombomodulin in term human syncytiotrophoblast microvilli. Placenta 19, 261-268.

Feller A.C., Schneider H., Schmidt D. and Parwaresch M.R. (1985). Myofibroblast as a major cellular constituent of villous stroma in human placenta. Placenta 6, 405-415.

Fernandez G.C., Te Loo M.W., van der Velden T.J., van der Heuvel L.P., Palermo M.S. and Monnens L.L. (2003). Decrease of thrombomodulin contributes to the procoagulant state of endothelium in hemolytic uremic syndrome. Pediatr. Nephrol. 18, 1066-1068.

Graf R., Langer J.U., Schonfelder G., Oney T., Hartel-Schenk S., Reutter W. and Schmidt H.H. (1994). The extravascular contractile system in the human placenta. Morphological and immunocytochemical investigations. Anat. Embryol. (Berl). 190, 541-548.

Graf R., Matejevic D., Schuppan D., Neudeck H., Shakibaci M. and Vetter K. (1997). Molecular anatomy of the perivascular sheath in human placental stem villi: the contractile apparatus and its association to the extracellular matrix. Cell Tissue Res. 290, 601-607.

Grinnell B.W. and Berg D.T. (1996). Surface thrombomodulin modulates thrombin receptor responses on vascular smooth muscle cells. Am. J. Physiol. 270, H 603-609.

Heldin C H. (1992). Structural and functional studies on platelet-derived growth factor. EMBO J. 11, 4251-4259.

Helske S., Vuorela P., Carpen O., Hornig C., Weich H. and Halmesmaki E. (2001). Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. Mol. Hum. Reprod. 7, 205-210.

Ikeda T., Ishii H., Higuchi T., Sato K., Hayashi Y., Ikeda K. and Hirabayashi Y. (2000). Localization of Thrombomodulin in the anterior segment of the human eye. Invest. Ophtalmol. Vis. Sci. 41, 3383-3390.

Ishii H., Uchiyama H. and Kazama M. (1991). Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. Thromb. Haemost. 65. 618-623.

Ishii H., Tazuka T., Ishikawa H., Takada K., Oida K. and Horie S. (2003). Oxidized phospholipids in oxidized low-density lipoprotein down-regulate thrombomodulin transcription in vascular endothelial cells through a decrease in the binding of RARß- RXRα heterodimers and Sp1 and Sp3 to their binding sequences in the TM promoter. Blood 101, 4765-4774.

Jin Z.G., Ueba H., Tanimoto T., Lungu A.O, Frame M.D. and Berk B.C. (2003). Ligand independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation on endothelial nitric oxide synthase. Circ. Res. 93, 354-363.

Kacemi A., Vervelle C., Uzan S. and Challier J.C. (1999). Immunostaining of vascular perivascular cells and stromal components in human placental villi. Cell. Mol. Biol. 45, 101-113.

Khalil R.A. and Granger J.P. (2002). Vascular mechanisms of increased arterial pressure in preeclampsia : lessons from animal models. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R29-45.

Kanfer A., Bruch J.F., Nguyen G., He C.J., Delarue F., Flahault A., Nessmann C. and Uzan S. (1996). Increased placental antifibrinolytic potential and fibrin deposits in pregnancy-induced hypertension and preeclampsia. Lab. Invest. 74, 253-258.

Kapanci Y., Ribaux C., Chaponnier C. and Gabbiani G. (1992). Cytoskeletal features of alveolar myofibroblast and pericytes normal human and rat lung. J. Histochem. Cytochem. 40, 1955-1963.

Labarrere C. A. and Faulk W.P. (1992a). Microvascular perturbations in human allografts: analogies in preeclamptic placentae. Am. J. Reprod. Immunol. 27, 109-116.

Labarrere CA. and Faulk W.P. (1992b). Fetal stem vessel endothelial changes in placentae from normal and abnormal pregnancies. Am. J. Reprod. Immunol. 27, 97-100.

Lanir N., Aharon A. and Brenner B. (2003). Haemostatic mechanisms in human placenta. Best. Pract. Res. Clin. Haematol. 16, 183-195.

Lentz S.R., Tsiang M. and Sadler J.E. (1991). Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and post- transcriptional mechanisms. Blood 77, 542-550.

Li Y., Zheng J., Bird I.M. and Magness R.R. (2003). Effects of pulsatile shear stress on nitric oxide production and endothelial cell nitric oxide synthase expression by ovine fetoplacental artery endothelial cells. Biol. Reprod. 69, 1053-1059.

Lyall F., Young., A, Boswell F., Kingdom J.C. and Greer I.A. (1997). Placental expression of vascular endothelial growth factor in placentae from pregnancies complicated by preeclampsia and intrauterine growth restriction does not support placental hypoxia at delivery. Placenta 18, 269-276.

Madazli R., Benian A., Aydin S., Uzun H. and Tolun N. (2002). The plasma and placental levels of malondialdehyde, glutathione and superoxide dismutase in pre-eclampsia. J. Obstet. Gynaecol. 22, 477-480.

Malek A.M., Jackman R., Rosenberg R.D. and Izumo S. (1994).

Endothelial expression of thrombomodulin is reversible regulated by fluid shear stress. Circ. Res. 74, 852-860.

Many A., Hubel C.A., Fisher S.J., Roberts J.M. and Zhou Y. (2000). Invasive cytotrophoblasts manifest evidence of oxidative stress in preeclampsia. Am. J. Pathol. 156, 321-331.

Maruyama I. and Majerus P.W. (1985). The turnover of thrombin-thrombomodulin complex in cultured human umbilical vein endothelial cells and A549 lung cancer cells. Endocytosis and degradation of thrombin. J. Biol. Chem. 15, 15432-15438.

Moore K.L., Esmon C.T. and Esmon N.L. (1989). Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. Blood 73, 159-185.

Myatt L. (1992). Control of vascular resistance in the human placenta. Placenta 13, 329-341.

Nakashima A., Kobayashi T. and Terao T. (1996). Fibrinolysis during normal pregnancy and severe preeclampsia : relationships between plasma levels of plasminogen activators and inhibitors. Gynecol. Obstet. Invest. 42, 95-101.

Nomura M., Yamagishi S., Harada S., Hayashi Y., Yamashima T., Yamashita J. and Yamamoto H. (1995). Posible participation of autocrine and paracrine vascular endotelial growth factors in hypoxia-induced proliferation of endotelial cells and pericytes. J. Biol. Chem. 270, 28316-28324.

Parra M., Rodrigo R., Barja P., Bosco C., Fernández V., Sepúlveda E., Aleuanlli C. and Matamala P. (2003). Oxidative stress and endothelial dysfunction is correlated with preeclampsia and fetal growth restriction. Ultasound. Obstet. Gynecol. 22, 75.

Redman C.W.C. (1991). Current topic : pre-eclampsia and the placenta. Placenta 12, 301-308.

Roes E.M., Sweep C.G., Thomas C.M., Zusterzeel P.L., Geurts-Moespot A., Peters W.H. and Steegers E.A. (2002). Levels of plasminogen activators and their inhibitors in maternal and umbilical cord plasma in severe preeclampsia. Am. J. Obstet. Gynecol. 187, 1019-1025.

Scott J.S. (1958). Pregnancy toxaemia associated with hydrops foetalis, hydatidiform mole and hydramnios. J. Obstet. Gynaecol. Br. Empire. 65, 689-701.

Sebire N.J. and Talbert D.G. (2001). The dynamic placenta : a closer look at the pathophysiology of placental hemodynamics in uteroplacental compromise. Ultrasound. Obstet. Gynecol. 18, 357-361.

Singh H. J., Rahman A., Larmie E. T. and Nila A. (2001) Endothelin-1 in feto placental tissues from normotensive pregnant women and women with pre-eclampsia. Acta Obstet. Gynecol. Scand. 80, 99-103.

Soff G.A., Jackman R.W. and Rosenberg R.D. (1991). Expression of thrombomodulin by smooth muscle cells in culture : Different effects of tumor necrosis factor and cyclic adenosine monophosphate on thrombomodulin expression by endothelial cells and smooth muscle cells in culture. Blood 77, 515-518.

Takagi H., King G.L. and Aiello L.P. (1996). Identification and characterization of vascular endothelial growth factor receptor (Flt) in bovine retinal pericytes. Diabetes 45 , 1016-1023.

Walsh S.W. and Wang Y. (1995). Trophoblast and placental villous core production of lipid peroxides, thromboxane, and prostacyclin in preeclampsia. J. Clin. Endocrinol. Metab. 80, 1888-1893.

Yamagishi S., Yonekura H., Yamamoto Y., Fujimori H., Sakurai S., Tanaka N. and Yamamoto H. (1999). Vascular endothelial growth factor acts as a pericyte mitogen under hypoxic conditions. Lab. Invest. 79, 501-509.

Yang H.L, Hseu Y.C, Lu F.J. and Tsai H.D. (1998). Humic acid reduces protein-C activating cofactor activity of thrombomodulin of human umbilical vein endothelial cells. Br. J. Haematol. 101, 16-23.

Zhou Y., Damsky C.H., Chiu K., Roberts J.M. and Fisher S.J. (1993). Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblast . J. Clin. Invest. 91, 950-960.

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