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A Deficiency of Placental IL-10 in Preeclampsia

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Accommodation of the fetoplacental unit in human pregnancy requires maternal immune tolerance to this "semiallograft". Local antiplacental immunity is modified by synthesis of uncommon histocompatibility Ags (e.g., HLA-G), growth factors, and cytokines by the placenta. Placental interleukins have been identified in reproductive tissues, but their roles in adaptive maternal immunity and determining term pregnancy outcomes have not been fully clarified. This study examined the distribution of IL-10 and TNF-α staining in term placentas. Women with proteinuric hypertension (PE, $n = 10$) were compared with an age-matched group with normal pregnancy (NP, $n = 14$) and gestational hypertension (GH, $n = 6$). Using immunohistochemistry of parrafin-fixed tissues, trophoblast cells were identified by cytokeratin 7 and cytokeratin 18 staining. The cytokine binding of villous trophoblast cells was scored depending on the extent of circumferential cytoplasm staining (<25%; intermediate or >75%). The cytokine positive decidual cells were scored as a percentage of total extravillous trophoblast cells. There was a reduction in villous IL-10 immunostaining compared with normal term placenta (PE, 10.2 ± 1.1, mean ± SEM; NP, 14.07 ± 1.16 Mann-Whitney *U* test; $p = 0.02$). In these patients, there was an increase in TNF-α immunostaining. Sparse endovascular extravillous trophoblast cells demonstrated nuclear IL-10 staining in 30% of patients with preeclampsia. Serum IL-10 was diminished in women with preeclampsia compared with normal pregnancy. In conclusion, villous trophoblast demonstrated diminished immunostaining of IL-10 in preeclampsia. This abnormality may be associated with heightened maternal antifetal immunity and therefore inadequate placental development in preeclampsia. ***The Journal of Immunology,*** **1999, 163: 3491–3495.**

The acceptance of the fetoplacental unit by the maternal uterine surface requires an element of immunological tolerance. The presence of immune cells in the decidual tissue of the uterus presents a potential barrier, both physical and immunological, to the development of the placenta (1). Some characteristics of the fetoplacental unit encourage endometrial and myometrial invasion (2) and others modify the immunological barrier (e.g. HLA-G) (3). These characteristics of placental function may be abnormal at the formative stages of placental development, and thus the presumptive uterine barriers may be abnormal in preeclampsia, a medical complication of human pregnancy related to shallow placental development (4).

The role of the placenta as an immune modifier has been identified by classical and nonclassical MHC production (5, 6). Other [1]cytokines and growth factors have been identified as functional proteins in the placenta, but their roles in normal placental development and therefore in pathological placental disease have not been determined (7, 8). IL-10 has been shown in normal placental cells (trophoblast cells) to suppress the mixed lymphocyte responses in vitro (9). Maternal bone marrow-derived cells in the uterine wall include NK-like cells and T cells (10) that may be modified by placental IL-10 production. Modification of the local maternal antifetal immune response has been shown to be important in patients with recurrent spontaneous abortion (11), but the longer term consequences of an abnormal local immune reaction have not been determined. Preeclampsia is a disease of later pregnancy characterized by increased maternal blood pressure and proteinuria associated with end-organ damage in the mother and neonatal prematurity if severe (12).

This study aimed to investigate the quantity and distribution of an immunosuppressor cytokine IL-10 and pro-inflammatory cytokine TNF-α in placentas at term. The hypothesis tested was that a decrease in immunosuppressor cytokine IL-10 and an increase in TNF-α in preeclampsia are evidence of a heightened Th1 response in this disease. Immunostaining of the placental villi and sloughed decidua in normal pregnancy was compared with preeclampsia and gestational hypertension. The serum concentration of IL-10 was also measured in preeclampsia compared with normal pregnancy to establish whether circulating levels of IL-10 were reflective of placental production.

Materials and Methods

Patient selection

Consecutive patients with preeclampsia, gestational hypertension, or a normal pregnancy outcome were included from the King George V Hospital (Sydney, Australia). There were 10 patients with preeclampsia. Age-matched normal controls were selected ($n = 14$). Patients with gestational hypertension were included to define the effect of hypertension alone in the absence of other evidence of maternal endothelial dysfunction on placental immune staining ($n = 6$). The clinical parameters of the patient population are shown in Table I. Gestational hypertension was defined as an increase in blood pressure after 20 wk gestation not associated with proteinuria, thrombocytopenia, increased creatinine concentration, or elevated liver function tests. Preeclampsia was defined as an increase in blood pressure associated with proteinuria occurring after 20 wk gestation. There was a significant difference in fetal weight and gestational age at delivery.

Immunolocalization of IL-10 and TNF-α

Placental villous surface biopsies were collect fresh at the time of delivery. A short fixation time in formalin was undertaken and then tissue was set in paraffin. Serial 4-μm sections of tissue were prepared and stained. Haematoxylin and eosin were used to determine the villous and sloughed decidual architecture. Rabbit anti-human cytokeratin 7 diluted 1/50 in 1% BSA in PBS and cytokeratin 18 diluted 1/25 (Dako, Carpinteria, CA) were used to localize villous and extravillous trophoblast cells, and as positive controls

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[2] Abbreviation used in this paper: C.I., confidence interval.

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Table I. *Clinical parameters of patients with normal pregnancy, gestational hypertension, and preeclampsia included for immunostaining of the placenta (mean ± SEM)*

	Preeclampsia	Normal Pregnancy	Gestational Hypertension	*p* Value
Maternal Age (yr)	32.8 ± 1.4	30.6 ± 1.5	33.2 ± 1.0	0.528
Gestational age at delivery (wk)	36.2 ± 1.1	39.9 ± 0.4	36.8 ± 1.7	0.005
Range (wk)	29–40	37–42	34–41	
Fetal Weight (g)	2523 ± 280	3560 ± 100	3160 ± 161	0.004
Blood pressure (mm Hg)				
Maximum systolic	170 ± 8.8	108 ± 2.5	155 ± 7.2	
Range	240–140		170–140	
Maximum diastolic	100 ± 5.3	65 ± 1.8	100 ± 4.1	NS
Range	140–80		110–90	
Proteinuria (mg/24 h)	3065 ± 1026	Nil		
Range (mg/24 h)	790–8400		10–130	NS

for standard streptavidin (1/200; Dako), HRP staining against a biotinylated secondary Ab (1/500, Polylink; Dako). Tissues were hydrated after xylene preparation in decreasing alcohol concentration. The tissue was then quenched with 0.3% H_2O_2 in methanol. General Ab binding was controlled with 10% swine serum in sterile PBS incubated for 30 min at room temperature (22°C). The goat anti-human polyclonal anti-human IL-10 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) at 40 μg/ml or TNF-α at 10 μg/ml were incubated at room temperature for 60 min. Goat serum at an equivalent concentration was used as a negative control.

Quantification of staining

The number of extravillous decidual cells that were positive and negatively stained was expressed as a ratio. The extent of immunostaining in the villi was determined in 10 high power fields (×200 magnification). The total number of villi were counted and the extent of circumferential cytoplasmic staining of the cytotrophoblast and syncytiotrophoblast in a given villus was graded as 3, 75–100%; 2, 25–75%; or 1, <25%. The total staining score was divided by the number of whole villi per high power fields in 10 fields. These scores (between 1 and 3) were added for each high power field, and a score between 10 and 30 was gained for each sample. The scorer was blinded to the clinical diagnosis of the tissues at the time of assessment, and tissues were independently assessed by two observers.

Serum IL-10

Serum was collected at the time of diagnosis of preeclampsia ($n = 8$) and compared with women with a normal pregnancy outcome ($n = 9$). The demographics of the two groups showed that there was no difference in maternal ages (35 vs 31 yr, NS), gestational age at delivery (35 vs 39 wk, NS), parity rate (70% primipara in each group), and mode of delivery (cesarean section rate 50 vs 40%), but fetal weight was significantly reduced in the preeclamptic group (2534 vs 3368 g; $p = 0.03$). Serum IL-10 was determined by ELISA (Genzyme, Cambridge, MA). Patients with preeclampsia had marked hypertension (170/105 mmHg) with proteinuria (>++), which completely resolved postpartum. The intraassay variability was 12%.

Statistical analysis

Clinical differences (parity, age at gestation and blood pressure) were determined by ANOVA. The difference in rates of end-organ complications were determined by χ^2 analysis. The differences in scoring of immunostaining was determined by Kruskal-Wallis ANOVA and significance reached if $p < 0.05$. The effect of gestation on IL-10 staining was determined by Spearman's Rank coefficient. The difference in IL-10 concentration between preeclampsia and normal pregnancy was determined by Mann-Whitney *U* test. The correlation between TNF-α and IL-10 staining was determined by regression analysis.

Results

The preeclamptic patients had significantly raised blood pressure ranging from 240–140 mmHg systolic to 140–80 mmHg diastolic. The range of proteinuria was 790-8480 mg/24 h in those who had a 24-h determination (60%) or 2+ or greater on dipstick. The increase in blood pressure in the gestational hypertension group (170 mmHg/105 mmHg) was not associated with proteinuria.

There was a statistically significant decrease in villi staining for IL-10 in placentas from preeclampsia compared with normal pregnancy and gestational hypertension ($p = 0.006$; Fig. 1). The rate of extravillous trophoblast cell staining in preeclampsia compared with normal pregnancy was not significantly different (Fig. 2). There was a marginally significant correlation between villi staining and decidual positive cells for IL-10 ($p = 0.05$, $r^2 = 0.5$). There was no significant effect of gestational age at delivery on staining in any area of placental tissue. Figs. 3–6 demonstrate the extent of staining in positive and negative controls and IL-10 staining in preeclampsia and normal pregnancy.

In endovascular trophoblast in the placentas of three patients with preeclampsia demonstrated nuclear staining with IL-10 (Fig. 7). This was not seen in other trophoblast cells associated with terminal villi or stromally located trophoblast. This staining was seen in cytokeratin-positive cells in this location. There was no nuclear staining seen with equal concentrations of goat immune serum. Figs. 8 and 9 demonstrate TNF-α staining in patients with preeclampsia and normal pregnancy. There was a tendency to increased decidual cell TNF-α staining in preeclampsia ($p = 0.09$) compared with normal pregnancy. There was no difference in staining in patients with gestational hypertension and in villous

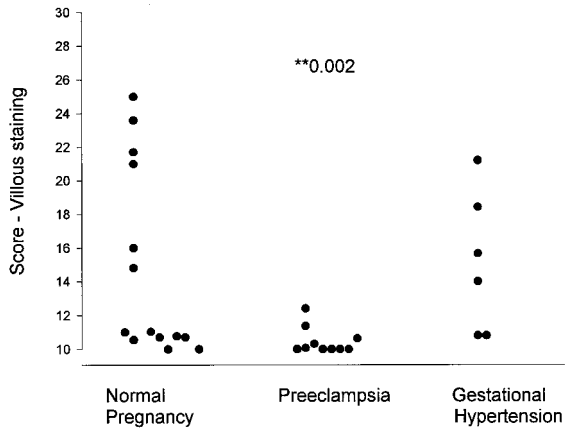

FIGURE 1. The extent of villous IL-10 Ab staining was statistically reduced in placentas from women with a preeclamptic outcome compared with normal pregnancy. Gestational hypertension did not differ significantly from patients with preeclampsia (Kruskal-Wallis ANOVA, $p < 0.006$).

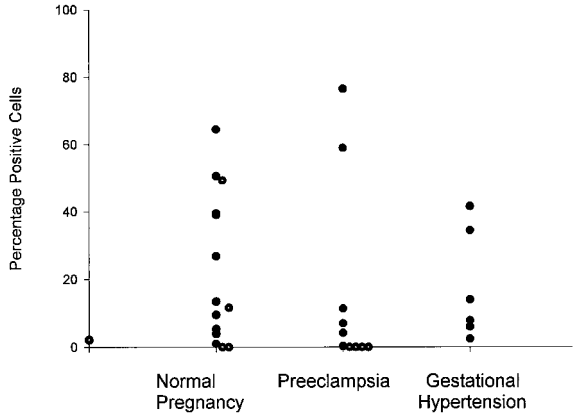

FIGURE 2. Distribution of staining for IL-10 in decidual trophoblast elements. There was no significant difference between the three groups examined, preeclampsia, normal pregnancy, and gestational hypertension, with moderate IL-10 staining in cytokeratin positive cells in all tissues.

staining in any group. The mean percentage of stained cells for TNF-α in preeclampsia was 30.7% (confidence interval (C.I.)[2], 0.4–61.0%) and 17.07% (C.I., 4.65–29.49%). The percentage of positive cells for IL-10 was 15.84 (C.I., 4.16–35.84%) in preeclampsia and 20.95 (C.I., 8.75–33.16%) in normal pregnancy.

The patients with preeclampsia showed a significantly lower circulating IL-10 concentration compared with normal pregnancy ($p < 0.004$, Mann-Whitney *U* test) (Fig. 10).

Discussion

This is the first study to demonstrate changes in IL-10 immunolocalization in term placentas from women with preeclampsia compared with those with a normal pregnancy outcome. There was a general decrease in cytoplasmic trophoblast villi IL-10 content in preeclampsia compared with placentas from normal pregnancy. There was a decrease in IL-10 in trophoblast cells located in decidual tissue, but a few samples demonstrated nuclear staining of IL-10 in sparse endovascular trophoblast cells. There was a weak

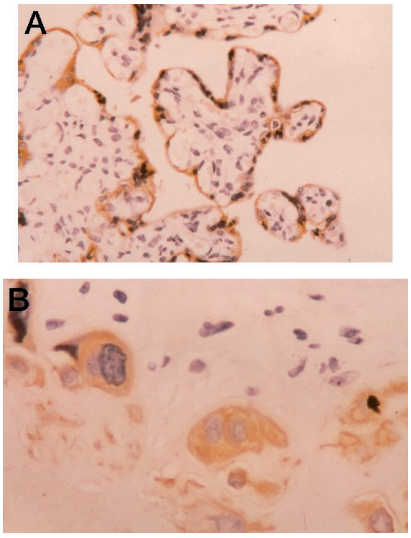

FIGURE 3. *A,* Normal pregnancy villi demonstrating cytoplasmic cytokeratin staining evenly distributed throughout the placenta. *B,* Cytokeratin 7 and 18 Ab binding of extravillous trophoblast cells in sloughed maternal decidua derived from term placentas.

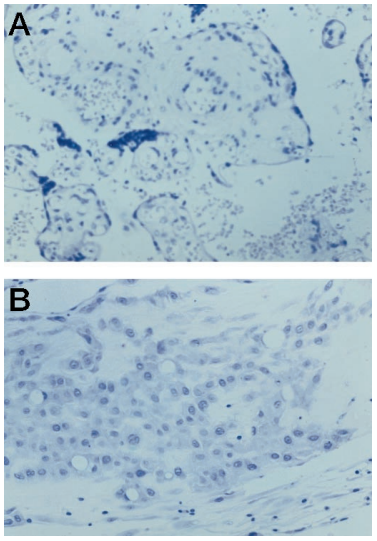

FIGURE 4. Goat immune serum shows no trophoblast or other cellular binding in paraffin-fixed placental tissues at term. Placentas with negative goat serum binding were used as controls for term villi (*A*) (×200) and extravillous trophoblast (*B*) (×100)

correlation between the decrease in IL-10 seen in preeclampsia and an increase in TNF-α in decidual trophoblast cells.

IL-10 has been identified as an important cytokine in pregnancy. IL-10 may be involved in the maintenance of pregnancy by corpus luteum maturation and progesterone production (13). Ovarian corpus luteum cell growth was stimulated by exogenous IL-10 and also in the presence of Th2 type lymphocytes derived from early pregnancy. In a well-known mouse cross that is prone to spontaneous abortion a deficiency of IL-10 has been demonstrated to alter the net fetal number and outcome (14). Longitudinal studies in mice demonstrate a sequential change in the cytokine profile including IL-10 in peripheral blood and release from spleen elements as pregnancy advances (15, 16). IL-10 inhibition in the second half of pregnancy in mice causes fetal growth retardation (17). Progesterone has been shown to increase Th2-type responses in T cells (18). Taken together, these data suggest that early pregnancy is associated with an increase in circulating Th2 cytokine IL-10

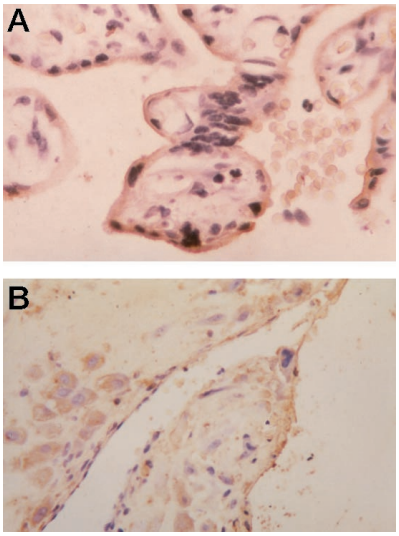

FIGURE 5. Normal pregnancy placental villi demonstrating IL-10 (40 μg/ml) staining in the villi (*A*) and extravillous trophoblast (*B*) (×200).

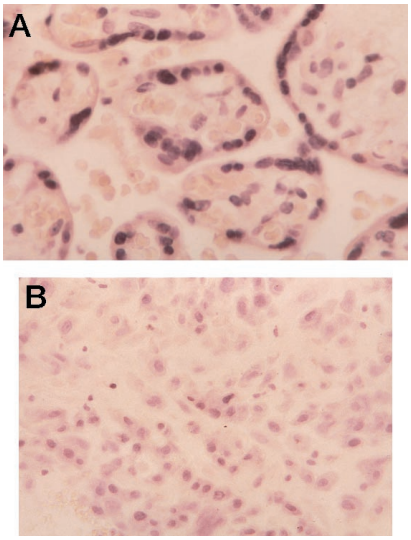

FIGURE 6. Preeclampsia villous trophoblast demonstrating that IL-10 (*A*; ×200) is significantly reduced compared with normal pregnancy (*B*; ×200). The difference in extravillous trophoblast staining with IL-10 Ab (*B*) was not statistically different to normal pregnancy .

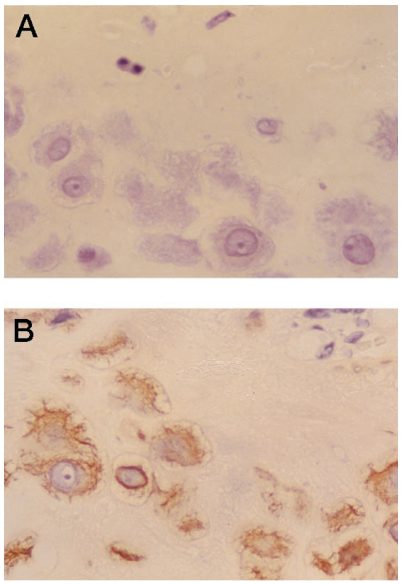

FIGURE 8. *A*, Absence on TNF-α in the decidual tissue of placenta in normal pregnancy. *B*, Serial section of normal placenta showing positive cells with cytokeratin staining (×200).

and that fetal and placental growth and development are depend on adequate IL-10 production.

A degree of maternal tolerance to fetal presenting cells has been identified in pregnancy (19), but the relationship of this tolerance to cytokine production has not been defined. In human pregnancy there is a shift of cytokine production from Th1-type inflammatory cytokines to Th2 type cytokines with a predominance of IL-10 and IL-4 over IL-2 and TNF-α in stimulated PBMC (20). This balance is altered in preeclamptic mononuclear cells with the alternative result, a relative decrease in IL-10 compared with the pro-inflammatory cytokine production (20).

There is a significant population of NK cells and other inflammatory cells at the uteroplacental interface whose response may be altered by the cytokine environment and other immune modifiers (21). Consistent with the finding that there is decreased Th2 cytokine IL-10 in preeclampsia, there is evidence from other studies of an increase in inflammatory markers, notably TNF-α, in preeclampsia at the level of message in the placenta (22) and in the maternal circulation (23). The level of IL-10 production relative to inflammatory cytokines IFN-γ and IL-2 in other tissues has been shown to determine T cells proliferation and macrophage activation (24). Therefore, the reduction in IL-10 production seen in preeclamptic fetally derived tissues may support a pro-inflammatory cytokine response in the mother. The finding of an increase in TNF-α relative to the IL-10 production in this study is consistent with other published findings.

The link between abnormalities in cytokines expression and a widespread endothelial disease such as preeclampsia is a feasible

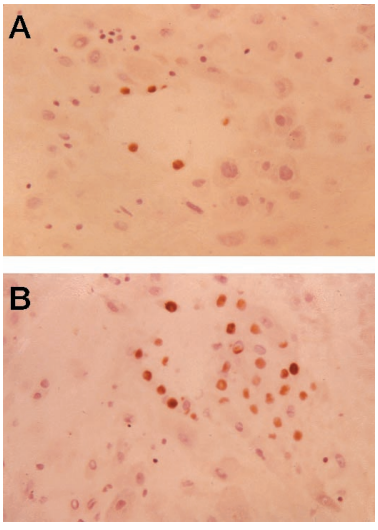

FIGURE 7. Preeclamptic samples (*A* and *B*) demonstrating nuclear staining of endovascular extravillous trophoblast. These samples show a lack of staining to IL-10 in stromally placed decidual trophoblast cells and around villi. These changes were not seen in tissue obtained from women with a normal pregnancy outcome.

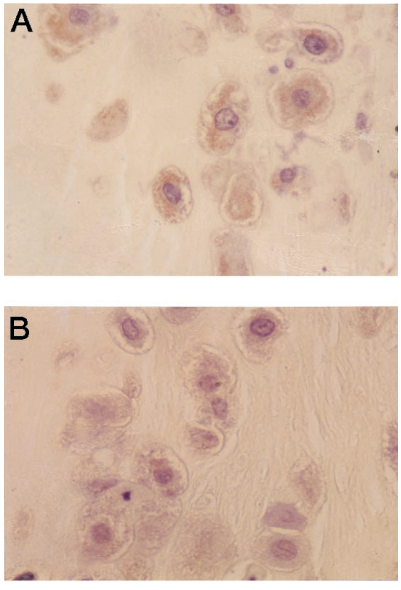

FIGURE 9. Serial sections of placenta in preeclampsia shows an increase in TNF-α in preeclampsia (*A*) in extravillous trophoblast. Adjacent sections show these cells as negatively staining wth goat serum (*B*). There was a weak correlation between the presence of TNF-α in preeclampsia and a deficiency of tissue IL-10 in these patients (×200).

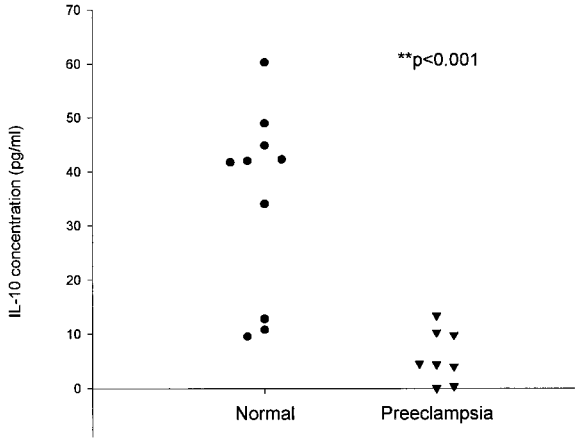

FIGURE 10. Serum IL-10 concentration in women with preeclampsia compared with a normal pregnancy outcome. There was a significant decrease in IL-10 serum concentration in women with preeclampsia compared with normal ($p = 0.004$).

one. TNF-α, a proinflammatory cytokines, has been shown to decrease endothelium-dependent vasodilation (25), one of the clinical hallmarks of the vasoconstriction in preeclampsia. E-selectin and other endothelial cell adhesion molecules are up-regulated by TNF-α (26). Therefore a shift toward a proinflammatory cytokine response may lead to endothelial activation.

Although villi showed less staining for IL-10 in preeclampsia, there were elements of maternal sloughed arteries containing IL-10 nuclear staining. Several cytokines and growth factors have been shown to localize to the cell nucleus via mechanisms including specific NF binding (IL-6-NF) and directly (basic fibroblast growth factor) (27). Nuclear IL-10 may be explained by an association with a transcription factor (28). IL-10 is anti-inflammatory by means of its binding with IκB and therefore preventing the release of nuclear factors related to up-regulating the production of inflammatory cytokines. Factors relating to transcription factor release and binding may be involved in the alteration of cellular IL-10 binding in vascular trophoblast in preeclampsia.

This study demonstrated that there is a significant alteration in the IL-10 staining of the trophoblast in preeclampsia compared with normal pregnancy and in a similar groups of patients, a decrease in circulating IL-10 in maternal serum. Recent studies have examined the IL-10 effect in preeclampsia and have found no difference in results in serum alone (29, 30). The effect of gestational age at collection of the sample and assay sensitivity may explain the difference in these studies. This study included groups of patients at comparable gestational ages with a correlation between placental IL-10 production and circulating maternal levels of cytokine. Thus a decrease in placental IL-10 is present in preeclampsia. The ratio of IL-10 to other cytokines in preeclampsia may be critical in determining the extent of placental acceptance by the uterine tissues and thus in determining the extent of placental development and invasion. These processes are critical to the eventual pregnancy outcomes in terms of maternal well-being and fetal growth.

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