# Immunohistochemical study of the expression of interlenkin-2 (IL-2) and human leukocyte antigen G (HLA-G) in placental tissues associated with preeclampsia

妊娠中毒症胎盤における interlenkin-2 (IL-2) 及び human leukocyte antigen G (HLA-G) の発現に関する免疫組織化学的研究

原　直竜

①

# Immunohistochemical study of the expression of interleukin-2 (IL-2) and human leukocyte antigen G (HLA-G) in placental tissues associated with preeclampsia

# 妊娠中毒症胎盤における interleukin-2 (IL-2) 及び human leukocyte antigen G (HLA-G) の発現に関する免疫組織化学的研究

原　直範

# Immunohistochemical study of the expression of interleukin-2 (IL-2) and human leukocyte antigen G (HLA-G) in placental tissues associated with preeclampsia

Naonori Hara

## RESEARCH PURPOSE AND BACKGROUNDS

The fetus is a semi-allograft in that half of its histocompatibility antigens come from the father. Pregnancy seems to be an immunological paradox, in view of that the fetus can survive and develop in spite of maternal immune system for as long as 9 months. It is reasonable to speculate that some special immunological mechanisms are essential for the fetal growth and maintenance of pregnancy. Suppressed state of maternal immunity against the fetus has been considered to be the major reason for success of pregnancy, i.e. semi-allograft transplantation. Blocking antibodies to the paternal antigens of the fetus,[1,2] anti-idiotypic antibodies to the maternal T cell receptors for paternal HLA,[3] and cell-mediated immune-suppression [4] are supposed to be involved in the mechanisms of the immune-suppression. Aside from these mechanisms, the notion of immunotrophism has emerged [5] which implicates that the maternal immune cells actively recognize the fetal cells, i.e. trophoblasts, and secrete cytokines which control the growth and differentiation of trophoblasts. Cytokines such as macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are certain to augment the growth of trophoblasts.[6] These cytokines play pleiotropic roles in cytokine network in the placenta, which is considered to play a important role for maintaining pregnancy.

Another interesting issue regarding the materno-fetal immune system is human leukocyte antigen-G (HLA-G).[7] HLA protein has important roles in generation and regulation of human T cell-mediated immune responses. Recognition of surface antigens on the target cells by T cells needs the coordinated recognition of HLA antigens on the target cells. In human, all the nucleated cells express classical HLA class I antigens except trophoblasts which do not express either classical HLA class I or II.[8,9,10] However, HLA-G protein is expressed on the extravillous cytotrophoblasts which invade into the maternal decidua from anchoring villi.[11,12] HLA-G is characterized by its being almost monomorphic and its protein is expressed only in the placenta. It is also secreted as smaller-sized soluble protein.[13] The unique and restricted pattern of the expression, the lack of allelic polymorphism and the fact that placenta expresses HLA-G instead of classical HLA class I antigens have implied its specific role in the materno-fetal immune relationship during pregnancy.[14,15,16] As

for the role of HLA-G, the protection of trophoblasts from being attacked by decidual natural killer cells is postulated.[17] If the expression of HLA-G protein on extravillous trophoblasts is deranged, trophoblasts may be injured by the attack of maternal killer cells and some pregnant-complications may ensue.

Preeclampsia is a syndrome which is characterized by maternal hypertension, proteinuria and edema. Preeclampsia is detrimental for both mother and fetus. For instance, eclamptic seizure is one of severe maternal manifestations and intrauterine fetal growth retardation, fetal distress or intrauterine fetal death are fetal problems imposed by preeclampsia. Although many hypotheses on the pathogenesis of preeclampsia have been proposed, the pathogenesis of preeclampsia is still unknown. Recently, abnormally shallow invasion of extravillous cytotrophoblasts into the myometrium has been proposed as a main cause of preeclampsia.[18,19] In normal pregnancy, extravillous cytotrophoblasts invade into the decidua and further into maternal spiral arteries. By invasion of trophoblasts, spiral arteries are dilated over four-fold in diameter, which increases the amount of blood perfusion in the intervillous space.[20] The sufficient perfusion is considered to be necessary for the successful pregnancy. In preeclampsia, dilation of maternal spiral arteries does not occur because of poor invasion of extravillous cytotrophoblasts into the decidua, which induces the reduction of perfusion in intervillous space.[21] Reduced perfusion in intervillous space is thought to play a critical role in the pathogenesis of preeclampsia.[22]

On the other hand, recent emerging evidence suggests that the disruption of immune regulation during pregnancy, i.e. a cytokine network in the placenta, may be involved in the pathophysiology of complicated pregnancies such as preeclampsia. Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) levels are increased in the plasma and amniotic fluid of patients with severe preeclampsia,[23] and the serum interleukin-2 (IL-2) levels are elevated in pregnant women with preeclampsia, thus implicating the enhanced immunological status in the setting of preeclampsia.[24] IL-2 is a cytokine, most of which is secreted from the helper T cells and stimulates T cell growth and induces lymphokine-activated killer (LAK) cells. LAK cells are reported to be able to attack trophoblasts in vitro.[25] Thus, IL-2 renders lymphocytes more capable of attacking the target cells including trophoblasts. In the placenta of uncomplicated pregnancy, IL-2 is identified only in the syncytiotrophoblasts [26] but not in decidual

tissues where trophoblasts directly contact with maternal tissues including maternal immune cells.

With these backgrounds, the questions arise as to whether IL-2 is present in the decidua, which is located in close proximity to alloantigenic fetal tissue and includes plenty of maternal immune cells, and whether derangement of HLA-G expression is related to the pathophysiology of preeclampsia. To address these, I sought to determine the presence of IL-2 in the decidua and the expression of HLA-G on the trophoblasts in preeclamptic patients.

## MATERIALS AND METHODS

*Patients*

Six preeclamptic patients were recruited to this study. All of them had an elevated blood pressure more than 140/90 mmHg. None of these pregnancies were complicated by intrauterine infections, premature labor, premature rupture of the membrane (PROM), and a delivery of an infant with malformations or congenital diseases. The diagnosis of intrauterine infection was made by both clinical signs, i.e. body temperature, serum c-reactive protein (CRP) level and pathological findings of chorioamnionitis of the placenta. Obstetrical summaries of the women with preeclampsia were shown in Table 1. Fourteen uncomplicated pregnant women without preeclampsia served as controls (Table 2). All the patients were seen at the Department of Obstetrics and Gynecology, Tokyo University Hospital during the period from January 1994 to June 1995 and gave informed consent.

*Placental tissues*

The placenta was obtained soon after the vaginal delivery or the caesarean section. The blocks of placental tissues were collected from 3 or 4 cotyledons, apparently devoid of the finding of an infarction, and the tissues at the maternal surface of the blocks were mechanically cut into 2-mm cubes and embedded in Tissue-Tek II O.C.T. Compound R (Miles Laboratories, Illinois, USA). The tissues were frozen in liquid nitrogen and they were kept frozen in liquid nitrogen for 1 week to 15 months until used. The process of tissue collection and freezing was completed within 30 minutes after the vaginal delivery or the caesarean section to avoid autolysis.

The pathological examination was performed in each sample and no pathological features of chorioamnionitis were confirmed. Infarctions were observed in every preeclamptic placenta in varying degrees by macroscopic and microscopic investigation.

*Immunohistochemical staining (Labeled streptavidin biotin method)*

The placental tissues were stained by means of a labeled streptavidin biotin method. The frozen tissues were sliced into 6-$\mu$m sections by Cryostat R (Miles Laboratories, Illinois, USA). The sections were checked by microscope before immunohistochemical staining to verify whether they

included both villi and decidua. The sections were air-dried for 30 minutes to avoid artifacts and morphological changes, and fixed in the 4 ℃ acetone for 10 minutes. After being washed with cold Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.6), the sections were incubated in 0.03 % $H_2O_2$ and 0.03 % $NaN_3$ (DAKO$^R$ Peroxidase Blocking Reagent (DAKO, Carpinteria, CA, USA) ) for 10 minutes at room temperature (RT) to remove the endogenous peroxidase activity. Subsequently, in order to remove the nonspecific background staining, the sections were washed with cold TBS, incubated in 5 % rabbit serum-TBS for 10 minutes at RT. After being incubated in 0.1 % avidin-50 mM Tris-HCl with 15 mM $NaN_3$ (DAKO$^R$ Biotin Blocking System (DAKO) ) for 15 minutes at RT, the sections were washed with cold TBS and then incubated in 0.01 % biotin-50 mM Tris-HCl with 15 mM $NaN_3$ (DAKO$^R$ Biotin Blocking System (DAKO) ) for 15 minutes at RT to quench the endogenous biotin. Following these procedures, the sections were washed with cold TBS and incubated in 1 $\mu$g/ml (thousand-fold dilution) of mouse anti-human IL-2 monoclonal antibody (Genzyme, Cambridge, USA) or 0.1 $\mu$g/ml (thousand-fold dilution) of mouse anti-HLA-G monoclonal antibody (87G) [27] (kindly supplied by Dr. Daniel E. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA, USA) or 0.025 $\mu$g/ml (thousand-fold dilution) of mouse anti-human cytokeratin monoclonal antibody (CAM 5.2) (Becton Dickinson, San Jose, CA, USA), which was used to identify extravillous cytotrophoblasts in the decidua, diluted in Tris-HCl buffer containing carrier protein and 15 mM $NaN_3$ (DAKO$^R$ Antibody Diluent (DAKO) ) for 18 hours at 4 ℃. For control staining, 1 $\mu$g/ml (hundred-fold dilution) of mouse $IgG_1$ negative control (DAKO), the same IgG subclass as the anti-human IL-2 antibody, and 0.1 $\mu$g/ml (thousand-fold dilution) of mouse $IgG_{2a}$ and $IgG_{2b}$ negative control (DAKO), IgG subclass similar to the anti-HLA-G and anti-human cytokeratin antibody, were used at same concentration of the primary antibody. Control experiments were run in which the treatment of primary antibody was omitted. After being washed with cold TBS, the sections were then incubated at 1.2 $\mu$g/ml (six hundred-fold dilution) of biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulins (DAKO), diluted in Tris-HCl buffer containing carrier protein and 15 mM $NaN_3$ (DAKO$^R$ Antibody Diluent (DAKO) ), for 10 minutes at RT. They were further washed with cold TBS, followed by the incubation with peroxidase-conjugated streptavidin solution (DAKO LSAB$^R$ Kit, Peroxidase (DAKO) ) for 10 minutes at RT.

They were washed again with cold TBS and incubated with 1 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (DAKO $^{R}$ DAB Chromogen tablets (DAKO)) containing 0.02 % $H_2O_2$ for 10 minutes at RT to visualize reaction products, i.e. brown reaction end-products. Finally, the sections were soaked into excess water and lightly counterstained with Mayer's haematoxylin.

*Staining of serial sections*

To investigate the topographical relation of IL-2 positive cells and HLA-G positive cells to cytokeratin positive cells, I stained the serial sections with anti-human IL-2 antibody, anti-HLA-G antibody and anti-cytokeratin antibody.

*Statistical analysis*

The incidence of women with IL-2 positive extravillous cytotrophoblasts in normal and preeclamptic group was statistically analyzed employing direct p test (statistically significant: $p < 0.05$). Difference of the number of HLA-G positive extravillous cytotrophoblasts between normal and preeclamptic women was statistically analyzed employing Cochran-Cox test (statistically significant: $p < 0.05$).

## RESULTS

In all the sections obtained from both uncomplicated pregnant women and preeclamptic patients, cytokeratin was strongly stained by CAM 5.2 in villous cytotrophoblasts, syncytiotrophoblasts and large cytoplasm-rich decidual layered cells, a finding compatible with extravillous cytotrophoblasts (Figure 1).[28,29,30,31,32] Cytokeratin was recognized as brown color staining produced by CAM 5.2 in the cytoplasm of trophoblasts as show in Figure 1. The cytoplasm of every trophoblast was stained equally. Some of extravillous cytotrophoblasts clustered, which seemed to be the persistent cytotrophoblastic shell cells. In the serial sections, IL-2 was stained very weak as small brown granules in cytokeratin-positive decidual layered cells, if any (Figure 1, 2), and HLA-G was stained in almost all cytokeratin-positive decidual layered cells, including presumptive cytotrophoblastic shell cells in all uncomplicated pregnant women examined (Figure 3, 4, Table 4). The number of HLA-G positive cells in 1000 extravillous cytotrophoblasts was 928 $\pm$ 67 (mean $\pm$ S.D.) in seven cases examined (Table 4).

In contrast, in sections obtained from preeclamptic patients, immunoreactive IL-2 was strongly stained in cytokeratin-positive decidual layered cells in five out of six cases (Table 3). The negative case was a preeclamptic patient whose pregnancy had been uneventful until the day before delivery when she developed the signs of preeclampsia, and completely ameliorated in a few hours after the delivery. The reaction products which were recognized vividly as small brown granules were appeared to be present in the cytoplasm of the extravillous cytotrophoblasts (Figure 5, 6). In all five cases of preeclamptic patients examined, clusters of cytokeratin-positive decidual layered cells, i.e. presumptive persistent cytotrophoblastic shell cells, were devoid of the expression of HLA-G protein (Figure 7, 8). The number of HLA-G positive cells in 1000 extravillous cytotrophoblasts was 353 $\pm$ 212 (mean $\pm$ S.D.) in five cases examined (Table 3). However, interspersed extravillous cytotrophoblasts surrounding cytotrophoblastic shell cells were positive for HLA-G as was seen in the uncomplicating setting. Thus, the incidence of the women with positive staining of IL-2 in extravillous cytotrophoblasts was higher in preeclamptic group than that in normal pregnant group ($p < 0.05$, direct p test) and the number of the positive staining of HLA-G in extravillous

cytotrophoblasts was smaller in preeclamptic women than that in normal pregnant women ($p < 0.05$, Cochran-Cox test).

In control sections that were treated with $IgG_1$, IgG2a and IgG2b negative control instead of the anti-human IL-2, anti-human cytokeratin and anti-HLA-G monoclonal antibody (Figure 9, 10, 11, 12), or stained without primary-antibody staining (Figure 9, 11), no apparent staining was observed in both uncomplicated pregnant women and preeclamptic patients.

The degrees of staining intensity of each primary antibody were not altered by the length of storage periods of placental samples.

## DISCUSSION

Suppression of cell-mediated immunity of the mother against the fetus is considered to be a plausible explanation for the unique success of the semi-allogenic fetus in utero. This suppression might be ascribed to mixed lymphocyte reaction (MLR)-blocking antibodies to the paternal antigens of the fetus,[1,2] anti-idiotypic antibodies to the receptors of maternal lymphocytes,[3] and cell-mediated immune-suppression.[4] Recently, an increasing interest has been focused on the concept of immunotrophism, which emphasizes that maternal lymphocytes and macrophages actually recognize the fetal allograft and thereby secrete certain cytokines, possible regulators for growth and differentiation of trophoblasts.[5] Cytokines such as macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are shown to augment placental growth,[6] and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is supposed to regulate the growth of trophoblasts such that they grow to invade into the decidua but not penetrate through the myometrium.[33] These cytokines form a cytokine network in the placenta, which is considered to play a important role for the successful pregnancy.

Recent several lines of evidence suggest the perturbation of the exquisite cytokine network may result in the malfunction of the placenta and thus bring on pregnant complications such as preeclampsia. In the preeclamptic patients, increased serum activity of IL-2 has been reported,[24] suggesting the link between immunoactivated state and preeclampsia. The presence of IL-2 in the decidual layer tissue may point to the enhanced state of the maternal immune cells in the decidua, including lymphocytes and macrophages. It could be that IL-2 released from the extravillous cytotrophoblasts may stimulate the decidual lymphocytes and augment their cytotoxicity, which may be detrimental to trophoblasts and induce inflammation in the placenta; that, in turn, may result in the development of pregnancy-related disorders including preeclampsia. In fact, the administration of IL-2 to decidual large granular lymphocytes (LGLs), which have natural killer activity against K562 cells but not against normal human trophoblasts, enhances their natural killer activity against the normal trophoblasts in vitro.[25,34]

It is well known that the fetus can survive not only in the uterus but also in the fallopian tube and

even on the peritoneum. These ectopic pregnancies lead us to suggest that the immune regulation during pregnancy that prevents the fetus from being rejected by the maternal immune system can be workable not only in the uterus but also in the fallopian tube and in the peritoneal cavity as well. I surmised that the first signal that induces the immune regulation during pregnancy might come from the fetal cells in the placenta, that is, the trophoblasts. In 1987, a non-classical HLA class I gene, HLA-G, was cloned.[7] HLA-G protein is expressed only on the extravillous cytotrophoblasts, that are in derect contact with maternal tissues, and to a limited extent on early villous cytotrophoblasts. Although the classical HLA antigens are polymorphic, extremely little polymorphism of HLA-G has been reported except for that in African American people.[35,36,37] A soluble protein form is also expressed by translation of alternative spliced mRNA containing intron 4.[13] Extravillous cytotrophoblasts, which invade into the decidua basalis and exist in the close vicinity of the maternal immune cells, express HLA-G but not classical HLA class I antigens.[11,12] In regard to the function of HLA-G protein, the protective role for the trophoblasts is thought. Given that natural killer cell effector function is triggered by recognition of the absence of class I antigens on target cells, the expression of HLA-G, a HLA class I antigen, on the trophoblasts may avert the attack of maternal natural killer cells on the trophoblasts. In fact, HLA-G transfectants show resistance against the natural killer activity of decidual LGLs.[17] As HLA-G protein is almost monomorphic, it is unlikely that maternal cytotoxic T cells which recognize target cells that express classical HLA class I antigens attack the trophoblasts. Even though the type of fetal HLA-G is minimally different from that of maternal HLA-G in amino acids sequence and the maternal cytotoxic lymphocytes could recognize the fetal HLA-G protein, secreted soluble HLA-G protein may bind to the receptor of the T cells and the cytotoxic T cell-recognition might be blocked. Thus, this protein is assumed to be the first signal for inducing the immune regulation which prevents the fetus from being rejected during pregnancy.[16]

The noteworthy histopathological feature of the placenta associated with preeclampsia is the limitation of extravillous cytotrophoblast invasion into the myometrium [18] and the limitation of extravillous cytotrophoblast breach into maternal arterioles.[19] In normal pregnancy, extravillous cytotrophoblast invasion induces more than four-fold increase in maternal spiral arteries' diameters at fetal implantation-site [20] and, therefore, the amount of blood perfusion in the intervillous space

increases. In preeclampsia, the maternal spiral arteries fail to dilate because of the limitation of extravillous cytotrophoblast invasion into the decidua and, as a result, the amount of blood perfusion in the intervillous space does not increase eventually.[21] This reduction of placental perfusion is considered to be one of the pathologies of preeclampsia.[22]

In this study, I demonstrated that the expression of HLA-G protein in the presumptive persistent cytotrophoblastic shell cells was lacking, whereas IL-2 was present in the extravillous cytotrophoblasts in preeclampsia. These findings suggest that extravillous cytotrophoblasts may be susceptible of the attack of maternal lymphocytes. The attenuation of HLA-G expression on the extravillous cytotrophoblasts may result in the derangement of immunoregulatory mechanism necessary for the fetus from being rejected and IL-2 may be expressed by extravillous cytotrophoblasts. Enhanced expression of IL-2 may stimulate the natural killer activity of decidual lymphocytes and thereby, further deteriorate extravillous cytotrophoblasts. Detriment of extravillous cytotrophoblasts may cause the failure of extravillous cytotrophoblast invasion into the decidua and incomplete angiogenesis of maternal spiral arteries, leading to the development of preeclampsia.

SUMMARY

The disruption of the materno-fetal immune regulation could account for preeclampsia. In this study, the presence of IL-2 in the decidual tissue and HLA-G in the extravillous cytotrophoblasts was determined in 6 preeclamptic patients and 14 normal pregnant women, employing an immunohistochemical technique. The presence of IL-2 in the extravillous cytotrophoblasts in 5 out of 6 preeclamptic patients and the attenuation of HLA-G expression in the extravillous cytotrophoblasts in all five preeclamptic patients examined were observed. In contrast, little, if any, presence of IL-2 and no attenuation of HLA-G expression were observed in normal pregnant women. The presence of IL-2 and diminished expression of HLA-G in extravillous cytotrophoblasts could be at play in the pathophysiology of preeclampsia.

REFERENCES


1. Rocklin RE, Kitzmiller JL, Carpenter CB, Garovoy MR, David JR: Absence of an immunologic blocking factor from the serum of women with chronic abortions. N. Engl. J. Med. 1976; 295: 1209-1213.
2. McIntyre JA, Faulk WP: Maternal blocking factors in human pregnancy are found in plasma not serum. Lancet 1979; October 20: 821-823.
3. Suciu-Foca N, Reed E, Rohowsky C, Kung P, King DW: Anti-idiotypic antibodies to anti-HLA receptors induced by pregnancy. Proc. Natl. Acad. Sci. USA. 1983; 80: 830-834.
4. Kovithavongs T, Dossetor JB: Suppressor cells in human pregnancy. Transplant. Proc. 1978; 10: 911-913.
5. Wegmann TG, Athanassakis I, Guilbert L, Branch D, Dy M, Menu E, Chaouat G: The role of M-CSF and GM-CSF in fostering placental growth, fetal growth, and fetal survival. Transplant. Proc. 1989; 21: 566-568.
6. Athanassakis I, Bleackley RC, Paetkau V, Guilbert L, Barr PJ, Wegmann TG: The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. J. Immunol. 1987; 138: 37-44.
7. Geraghty DE, Koller BH, Orr HT: A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment. Proc. Natl. Acad. Sci. USA. 1987; 84: 9145-9149.
8. Faulk WP, Temple A: Distribution of $\beta 2$ microglobulin and HLA in chorionic villi of human placentae. Nature 1976; 262: 799-802.
9. Goodfellow PN, Barnstable CJ, Bodmer WF, Snary D, Crumpton MJ: Expression of HLA system antigens on placenta. Transplantation 1976; 22: 595-603.
10. Faulk WP, Sanderson AR, Temple A: Distribution of MHC antigens in human placental chorionic villi. Transplant. Proc. 1977; 9: 1379-1384.
11. Chumbley G, King A, Gardner L, Howlett S, Holmes N, Loke YW: Generation of an antibody to HLA-G in transgenic mice and demonstration of the tissue reactivity of this antibody.

J. Reprod. Immunol. 1994; 27: 173-186.

12. McMaster MT, Librach CL, Zhou Y, Lim KH, Janatpour MJ, DeMars R, Kovats S, Damsky C, Fisher SJ: Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. J. Immunol. 1995; 154: 3771-3778.
13. Fujii T, Ishitani A, Geraghty DE: A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. J. Immunol. 1994; 153: 5516-5524.
14. Trowsdale J, Travers P, Bodmer WF, Patillo RA: Expression of HLA-A, -B, and -C and $\beta$2-microglobulin antigens in human choriocarcinoma cell lines. J. Exp. Med. 1980; 152: 11s-17s.
15. Ellis SA, Palmer MS, McMichael AJ: Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA class I molecule. J. Immunol. 1990; 144: 731-735.
16. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R: A class I antigen, HLA-G, expressed in human trophoblasts. Science 1990; 248: 220-223.
17. Chumbley G, King A, Robertson K, Holmes N, Loke YW: Resistance of HLA-G and HLA-A2 transfectants to lysis by decidual NK cells. Cell. Immunol. 1994; 155: 312-322.
18. Zhou Y, Damsky CH, Chiu K, Roberts JM, Fisher SJ: Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. J. Clin. Invest. 1993; 91: 950-960.
19. Khong TY, Wolf FD, Robertson WB, Brosens I: Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. Br. J. Obstet. Gynaecol. 1986; 93: 1049-1059.
20. Robertson WB, Khong TY, Brosens I, Wolf FD, Sheppard BL, Bonnar J: The placental bed biopsy: Review from three European centers. Am. J. Obstet. Gynecol. 1986; 155: 401-412.
21. Gerretsen G, Huisjes HJ, Elema JD: Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. Br. J. Obstet. Gynaecol. 1981; 88: 876-881.
22. Roberts JM, Redman CWG: Pre-eclampsia: more than pregnancy-induced hypertension. Lancet 1993; 341: 1447-1451.
23. Kupferminc MJ, Peaceman AM, Wigton TR, Rehnberg KA, Socol ML: Tumor necrosis

factor-$\alpha$ is elevated in plasma and amniotic fluid of patients with severe preeclampsia. Am. J. Obstet. Gynecol. 1994; 170: 1752-1759.

24. Sunder-Plassmann G, Derfler K, Wagner L, Stockenhuber F, Endler M, Nowotny C, Balcke P: Increased serum activity of interleukin-2 in patients with pre-eclampsia. J. Autoimmun. 1989; 2: 203-205.
25. King A, Loke YW: Human trophoblast and JEG choriocarcinoma cells are sensitive to lysis by IL-2-stimulated decidual NK cells. Cell. Immunol. 1990; 129: 435-448.
26. Soubiran P, Zapitelli JP, Schaffar L: IL-2 like material is present in human placenta and amnion. J. Reprod. Immunol. 1987; 12: 225-234.
27. Lee N, Malacko AR, Ishitani A, Chen MC, Bajorath J, Marquardt H, Geraghty DE: The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous peptides but differ with respect to TAP association. Immunity 1995; 3: 591-600.
28. Sasagawa M, Watanabe S, Ohmomo Y, Honma S, Kanazawa K, Takeuchi S: Reactivity of two monoclonal antibodies (Troma 1 and CAM 5.2) on human tissue sections: Analysis of their usefulness as a histological trophoblast marker in normal pregnancy and trophoblastic disease. Int. J. Gynecol. pathol. 1986; 5: 345-356.
29. Bulmer JN, Smith J, Morrison L, Wells M: Maternal and fetal cellular relationships in the human placental basal plate. Placenta 1988; 9: 237-246.
30. Bulmer JN, Wells M, Bhabra K, Johnson PM: Immunohistological characterization of endometrial gland epithelium and extravillous fetal trophoblast in third trimester human placental bed tissues. Br. J. Obstet. Gynaecol. 1986; 93: 823-832.
31. Wells M, Bulmer JN: The human placental bed: histology, immunohistochemistry and pathology. Histopathology 1988; 13: 483-498.
32. Khong TY, Lane EB, Robertson WB: An immunocytochemical study of fetal cells at the maternal-placental interface using monoclonal antibodies to keratins, vimentin and desmin. Cell Tissue Res. 1986; 246: 189-195.
33. Todt JC, Yang Y, Lei J, Lauria MR, Sorokin Y, Cotton DB, Yelian FD: Effects of tumor necrosis factor-alpha on human trophoblast cell adhesion and motility.

Am. J. Reprod. Immunol. 1996; 36: 65-71.

34. King A, Birkby C, Loke YW: Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. Cell. Immunol. 1989; 118: 337-344.

35. Morales P, Corell A, Martinez-Laso J, Martin-Villa JM, Varela P, Paz-Artal E, Allende LM, Arnaiz-Villena A: Three new HLA-G alleles and their linkage disequilibria with HLA-A. Immunogenetics 1993; 38: 323-331.

36. Yamashita T, Fujii T, Watanabe Y, Tokunaga K, Tadokoro K, Juji T, Taketani Y: HLA-G gene polymorphism in a Japanese population. Immunogenetics 1996; 44: 186-191.

37. Van der Ven K, Ober C: HLA-G polymorphisms in African Americans. J. Immunol. 1994; 153: 5628-5633.

Table 1. Clinical Profile of Preeclamptic Patients

| Case No. | Age (yrs.) | Parity (times) | Onset of disease (weeks) | Blood pressure at delivery (sys/dias)(mmHg) | Proteinuria at delivery (mg/dl) | Edema at delivery | Weeks of delivery (weeks) | Method of delivery | Baby's weight (g) | Apgar score (1min) | Signs of infection |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 32 | 0 | 39 | 180-126/110-78 | 30 | Pretibial, moderate | 41 | Vaginal | 3030 | 8 | - |
| 2 | 33 | 0 | 37 | 169-137/97-71 | 30 | - | 40 | Vaginal | 3114 | 9 | - |
| 3 | 28 | 0 | 28 | 162/114 (Hydralazine prescribed) | 260 | Pretibial, mild | 30 | Caesarean (Fetal distress) | 972 | 4 | - |
| 4 | 29 | 1 | 33 | 162-134/95-76 (Hydralazine prescribed) | - | - | 37 | Vaginal | 2195 | 9 | - |
| 5 | 29 | 0 | 35 | 186-120/107-66 (Hydralazine prescribed) | 1000 | - | 37 | Forceps (Fetal distress) | 2785 | 8 | - |
| 6 | 26 | 0 | 38 | 162-110/103-60 | - | Pretibial, mild | 38 | Vaginal | 3162 | 9 | - |

Table 2. Clinical Profile of Normal Pregnant Women

| Case No. | Age (yrs.) | Parity (times) | Blood pressure at delivery (sys/dias)(mmHg) | Weeks of delivery (weeks) | Method of delivery | Baby's weight (g) | Apgar score (1min) | Signs of infection |
|---|---|---|---|---|---|---|---|---|
| 1 | 29 | 2 | 120-118/75-55 | 38 | Vaginal | 3378 | 10 | - |
| 2 | 35 | 1 | 112-100/70-63 | 40 | Vaginal | 3572 | 9 | - |
| 3 | 33 | 1 | 129-118/72-64 | 39 | Vaginal | 3130 | 10 | - |
| 4 | 25 | 1 | 125-108/82-70 | 39 | Vaginal | 3024 | 9 | - |
| 5 | 34 | 1 | 125-110/80-74 | 39 | Vaginal | 3024 | 9 | - |
| 6 | 25 | 1 | 124-108/79-68 | 41 | Vaginal | 3584 | 9 | - |
| 7 | 25 | 1 | 127-106/80-68 | 40 | Vaginal | 3344 | 9 | - |
| 8 | 32 | 0 | 113-106/76-60 | 39 | Vaginal | 2410 | 8 | - |
| 9 | 30 | 0 | 123-117/78-71 | 37 | Vaginal | 2782 | 9 | - |
| 10 | 34 | 0 | 125-102/74-64 | 40 | Vaginal | 3316 | 9 | - |
| 11 | 42 | 1 | 128-112/71-60 | 37 | Caesarean (Post-myomectomy) | 2902 | 9 | - |
| 12 | 34 | 2 | 127-111/79-65 | 38 | Vaginal | 3680 | 10 | - |
| 13 | 29 | 0 | 120-105/75-58 | 39 | Vaginal | 2910 | 9 | - |
| 14 | 26 | 0 | 126-117/78-60 | 38 | Vaginal | 2752 | 8 | - |

Table 3. Results of Immunohistochemical Staining of Preeclamptic Patients

| Case No. | Presence of IL-2 in extravillous cytotrophoblasts | Count of HLA-G positive cells per 1000 extravillous cytotrophoblasts |
|---|---|---|
| 1 | Yes (#1) | 95/1000 (#2) |
| 2 | Yes | 166/1000 |
| 3 | Yes | 420/1000 |
| 4 | Yes | 502/1000 |
| 5 | Yes | 580/1000 |
| 6 | No | not examined |

#1, #2 Pictures were shown.

Table 4. Results of Immunohistochemical Staining of Normal Pregnant Women

| Case No. | Presence of IL-2 in extravillous cytotrophoblasts | Count of HLA-G positive cells per 1000 extravillous cytotrophoblasts |
|---|---|---|
| 1 | No (#3) | 991/1000 (#4) |
| 2 | No | 992/1000 |
| 3 | No | 905/1000 |
| 4 | No | 964/1000 |
| 5 | No | 967/1000 |
| 6 | No | 830/1000 |
| 7 | No | 850/1000 |
| 8 | No | not examined |
| 9 | No | not examined |
| 10 | No | not examined |
| 11 | No | not examined |
| 12 | No | not examined |
| 13 | No | not examined |
| 14 | No | not examined |

#3, #4 Pictures were shown.

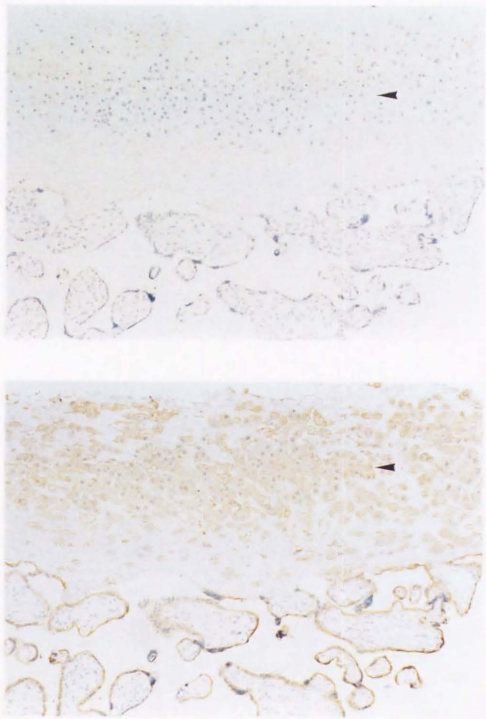


Figure 1. Immunohistochemical staining of IL-2 (Upper) or cytokeratin (Lower) molecule in the placenta of uncomplicated pregnancy ($\times$100). The serial sections from fresh-frozen placental tissue were reacted with mouse anti-IL-2 or anti-cytokeratin monoclonal antibody, and then stained by the labeled streptavidin biotin method (See the 'Materials and Methods'). (The areas indicated by the arrows were high-magnified in Figure 2.)

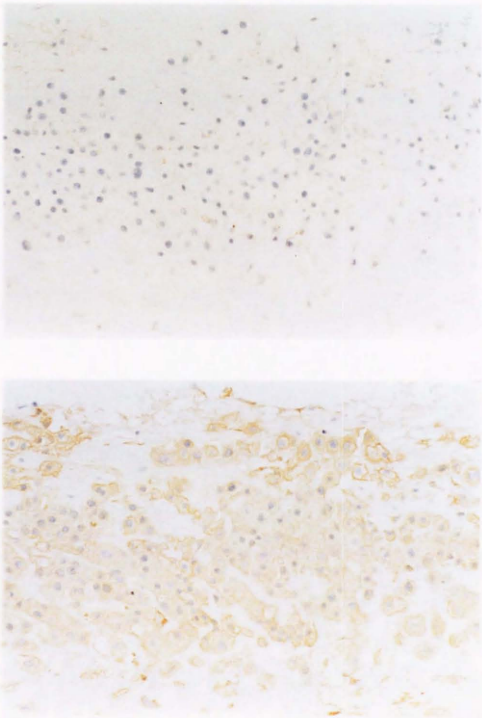


Figure 2. Immunohistochemical staining of IL-2 (Upper) or cytokeratin (Lower) molecule in the placenta of uncomplicated pregnancy ($\times$200).

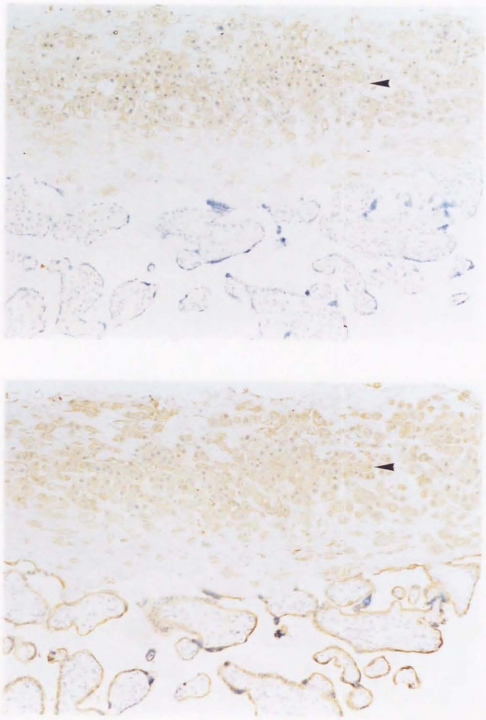


Figure 3. Immunohistochemical staining of HLA-G (Upper) or cytokeratin (Lower) molecule in the placenta of uncomplicated pregnancy ($\times$100). The serial sections from fresh-frozen placental tissue were reacted with mouse anti-HLA-G or anti-cytokeratin monoclonal antibody, and then stained by the labeled streptavidin biotin method (See the 'Materials and Methods'). (The areas indicated by the arrows were high-magnified in Figure 4.)

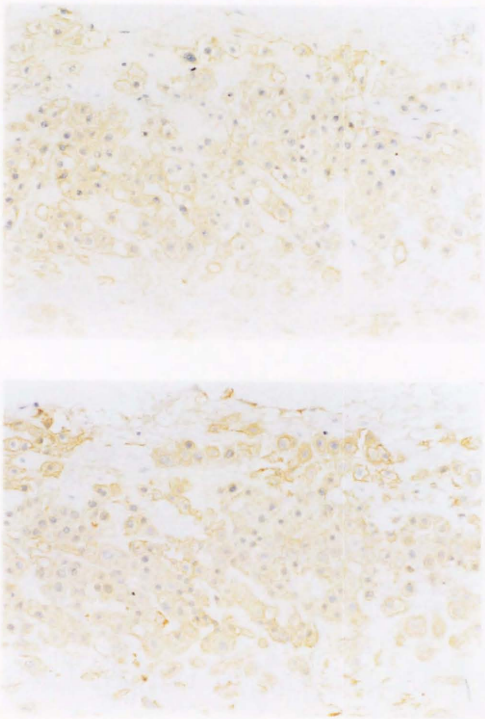


Figure 4. Immunohistochemical staining of HLA-G (Upper) or cytokeratin (Lower) molecule in the placenta of uncomplicated pregnancy ($\times$200).

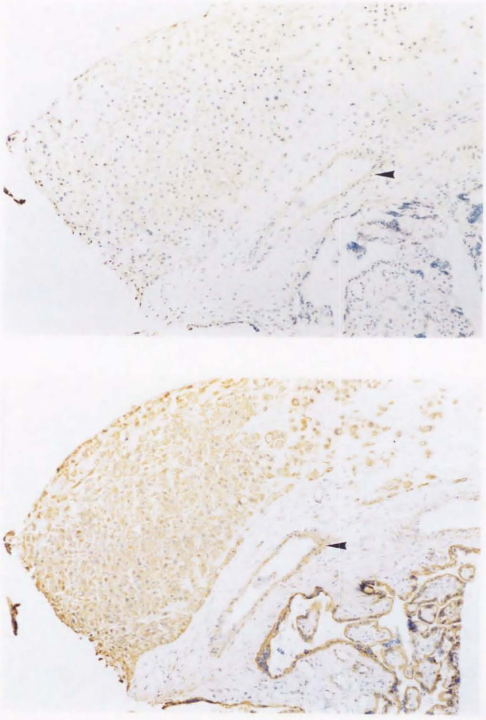


Figure 5. Immunohistochemical staining of IL-2 (Upper) or cytokeratin (Lower) molecule in the placenta of preeclamptic pregnancy (×100). The serial sections from fresh-frozen placental tissue were reacted with mouse anti-IL-2 or anti-cytokeratin monoclonal antibody, and then stained by the labeled streptavidin biotin method (See the 'Materials and Methods'). (The areas indicated by the arrows were high-magnified in Figure 6.)

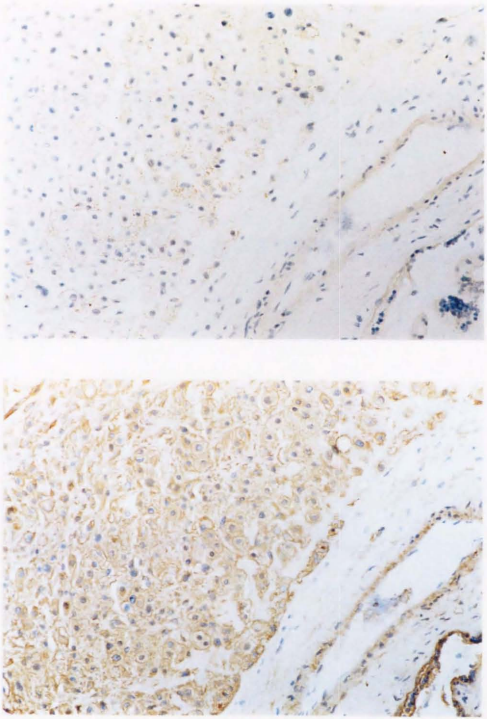


Figure 6. High magnitude (×200) figures of immunohistochemical staining of IL-2 (Upper) or cytokeratin (Lower) molecule in the placenta of preeclamptic pregnancy.

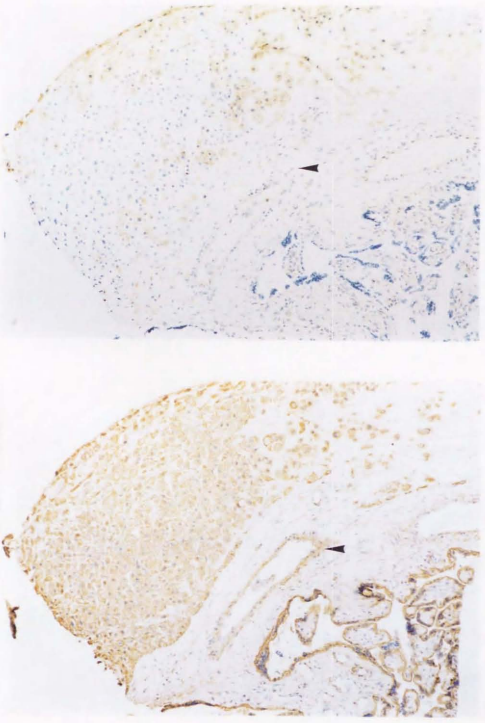


Figure 7. Immunohistochemical staining of HLA-G (Upper) or cytokeratin (Lower) molecule in the placenta of preeclamptic pregnancy ($\times$100). The serial sections from fresh-frozen placental tissue were reacted with mouse anti-HLA-G or anti-cytokeratin monoclonal antibody, and then stained by the labeled streptavidin biotin method (See the 'Materials and Methods'). (The areas indicated by the arrows were high-magnified in Figure 8.)

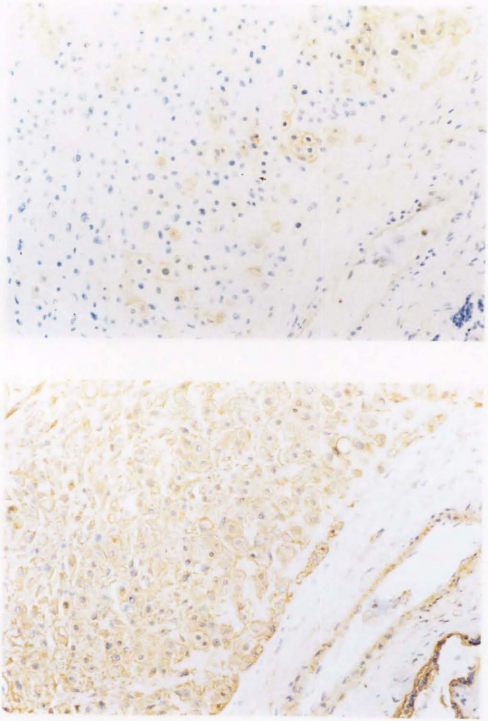


Figure 8. High magnitude (×200) figures of immunohistochemical staining of HLA-G (Upper) or cytokeratin (Lower) molecule in the placenta of preeclamptic pregnancy.

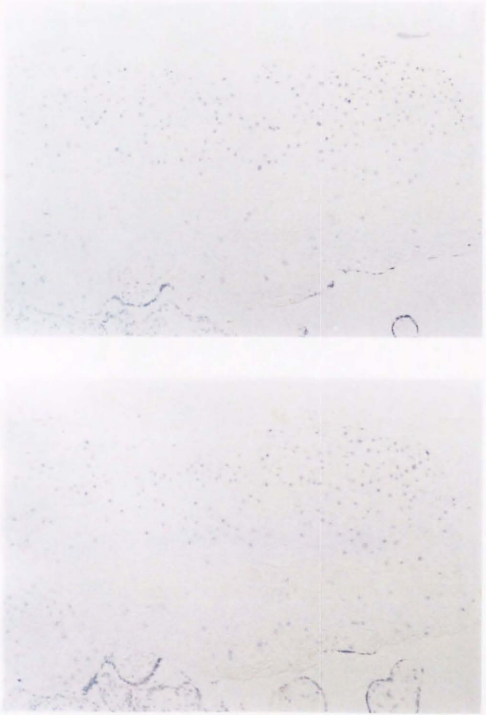


Figure 9. Immunohistochemical staining with $IgG_1$ negative control (Upper) or TBS (Lower) instead of the anti-IL-2 monoclonal antibody in the placenta of uncomplicated pregnancy ($\times 100$). Fresh-frozen placental tissue was reacted with mouse $IgG_1$ negative control or TBS, and then stained by the labeled streptavidin biotin method (See the 'Materials and Methods').

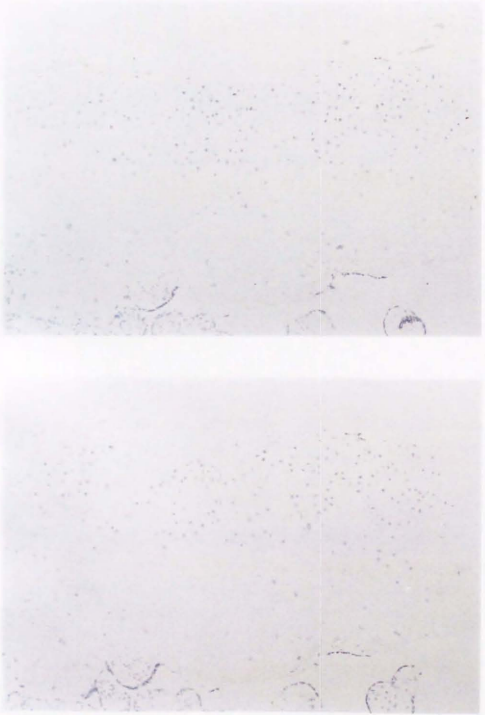


Figure 10. Immunohistochemical staining with IgG2a (Upper) or IgG2b (Lower) negative control instead of the anti-cytokeratin and anti-HLA-G monoclonal antibody in the placenta of uncomplicated pregnancy ($\times$100).

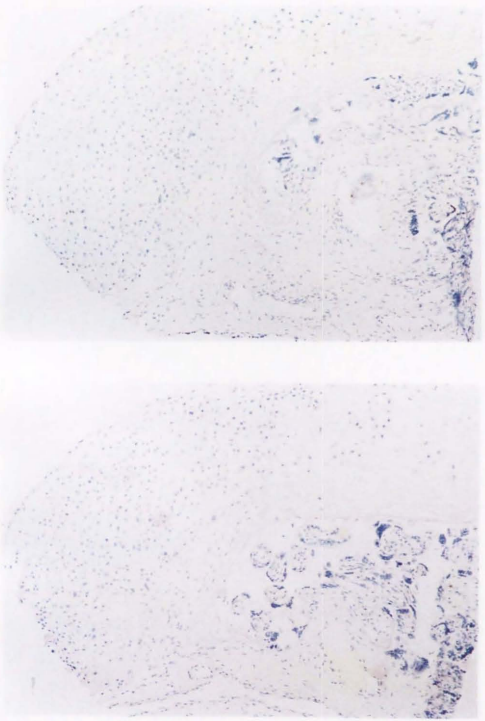


Figure 11. Immunohistochemical staining with IgG1 negative control (Upper) or TBS (Lower) instead of the anti-IL-2 monoclonal antibody in the placenta of preeclamptic pregnancy (×100). Fresh-frozen placental tissue was reacted with mouse IgG1 negative control or TBS, and then stained by the labeled streptavidin biotin method (See the 'Materials and Methods').

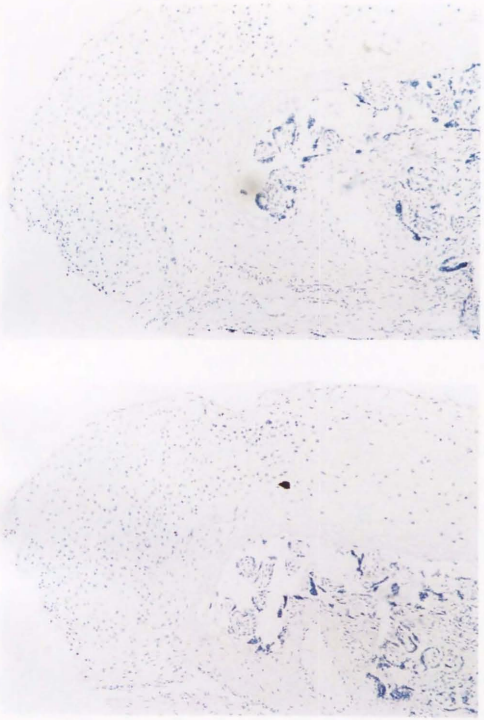
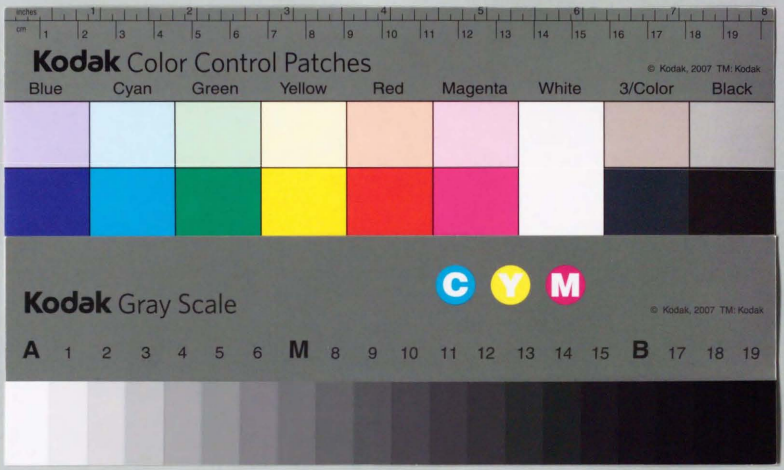


Figure 12. Immunohistochemical staining with IgG2a (Upper) or IgG2b (Lower) negative control instead of the anti-cytokeratin and anti-HLA-G monoclonal antibody in the placenta of preeclamptic pregnancy ($\times$100).


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19