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Distribution of Notch family proteins in intrauterine growth restriction and hypertension complicated human term placentas

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ABSTRACT

Members of the Notch family have been detected in many developmental and cell specification processes during placental development. However, Notch protein expression in Intrauterine Growth Restriction (IUGR) and Pregnancy Induced Hypertension (PIH) is not clear. In this study we aimed to clarify the immunolocalization of Notch proteins in full-term placentas after IUGR and PIH in comparison with normal placentas. Formalin-fixed, paraffin-embedded term placentas obtained by caesarean operations were processed for immunohistochemical localization of Notch 1, 2, 4 and Jagged 2. Transmission electron microscopy was also performed. In normal term placentas, all Notch proteins were intensely immunostained in the brush border of cells of the syncytiotrophoblast layer of the basal (maternal) side and the chorionic plate (fetal) side. The endothelial cells were also intensely immunostained in both sides for Notch 1. However, in IUGR and PIH placentas, the immunoreactivities of all Notch proteins were decreased significantly in the brush border of cells of the syncytiotrophoblast layer and the reaction was generally observed in the cytoplasm of syncytiotrophoblast cells in the basal and chorionic plate sides. The reactivity in endothelial cells was also significantly decreased. Our results have shown that the immunoreactivity and localization of Notch proteins is altered in pathologic placentas. Therefore, we propose that deregulated expression of Notch proteins may contribute to the disruption of trophoblast differentiation, endothelial cell function and/or feto-maternal traffic down-regulation during pregnancy or *vice versa* in such pathologic conditions.

Introduction

The developing fetus primarily depends on the function of placenta, which supplies nutrients and oxygen and which also synthesizes and secretes various hormones and molecules needed for the well-being and development of the fetus in the uterus. The successful development and functioning of the placenta requires several different highly regulated processes throughout pregnancy including trophoblast cell type differentiation, branching of the fetal angiogenesis, as well as formation of a maternal blood circulation (Rossant and Cross, 2001; Demir et al., 1989; 1995; 2004). Alterations of maternal blood pressure and nutrition are also extremely important for normal fetal development and growth and may cause pregnancy-related pathologies.

Pregnancy-induced hypertension (PIH) and intrauterine growth restriction (IUGR) are candidates for pregnancy-related pathologies, though the etiology of these disorders has not been fully elucidated despite intensive research. Since there is no unique definition and classification of these complications in pregnancy, IUGR is mainly described as the failure of the fetus to achieve its intrinsic growth potential due to an adaptation to unfavourable intrauterine environmental conditions in the feto-placental-maternal unit (Rosenberg, 2008). Hypertensive disorders are described as a response to defective uteroplacental perfusion (Olofsson et al., 1993) and similar mechanisms may operate in both disorders. In general, defective trophoblast invasion and incomplete remodelling of spiral arteries is considered to cause these pathologies (Pijnenborg, 1998; Brosens et al., 2002).

Notch proteins constitute a family of transmembrane receptors conserved in evolution across species from invertebrates to humans. Mammals have four Notch receptors, Notch 1–4, which are activated by cell surface ligands called Jagged 1 and Jagged 2 or Delta 1, Delta 3 and Delta 4, which are homologues of Serrate and Delta in *Drosophila*, respectively. Following activation by ligand binding, the Notch receptor undergoes proteolytic processing, and the functional Notch protein fragment is translocated to the nucleus where it interacts with a transcription factor complex to express Notch target genes (Artavanis-Tsakonas et al., 1991, 1995, 1999; Lai, 2004).

Members of the Notch signalling pathway have been detected in the developing placenta and have been shown to play an

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important role in the normal development and functioning of the placenta (De Falco et al., 2007; Gasperowicz and Otto, 2008). Notch members are activated in subsets of trophoblasts (Nakayama et al., 1997), the essential cells in contact with the uterine wall, invading the uterus and producing hormones necessary for initiating maternal recognition of pregnancy epithelium at implantation (Cross et al., 1994). They are also involved in the formation of the circulatory system throughout pregnancy (Xue et al., 1999; Rossant and Howard, 2002; Iso et al., 2003; Karsan, 2005). Mouse embryos deficient in Jagged 1, Notch 1, Notch 1/4 die between E9.5 and E10.5 and display a severely disorganised vasculature (Shawber and Kitajewski, 2004).

If Notch signalling plays an important role during development of the placenta, then deregulated expression of the Notch signalling pathway might be expected to induce phenotypes that affect placental development. Notch protein members have been shown to be altered in pre-eclamptic placentas compared to normal ones (Cobellis et al., 2007).

Although the mechanisms involved in IUGR and PIH still remain unknown, the involvement of the Notch pathway in multiple aspects of placental development indicates a role for Notch signalling in these pathologies. Therefore, the aim of this study was to detect the immunolocalization of Notch family proteins in IUGR and PIH placentas and to compare their expression to normal human placentas at full-term of gestation.

Materials and methods

Term placentas from healthy women were obtained immediately after caesarean section and were used as control group (n=6). Placentas from women with PIH (n=6) and IUGR (n=6) were obtained immediately after caesarean deliveries. The gestational age of control placentas ranged from 36 to 40 weeks. The gestational age of PIH and IUGR specimens ranged from 35 to 40 weeks. The samples were supplied from the Department of Obstetrics and Gynaecology at Akdeniz University, Medical Faculty after informed consent of patients. The Ethical Committees of Medical Faculty of Akdeniz University approved the consent forms and protocols.

The criteria for PIH and IUGR placentas were as previously described (Report of National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (2000)). Briefly, hypertensive pregnant patients were defined as those with persistent hypertension (blood pressure of $\geq$140–90 mm Hg as an absolute reading, developing after 20 weeks of pregnancy), and criteria for pregnant patients with IUGR were defined as a birth weight $\leq$ 2 standard deviation (SD), corresponding to the 2.5 percentile for gestational age.

Tissue processing

The villous tree samples were observed from normal and complicated human term placentas of basal plate (maternal side in close vicinity to the *Decidua basalis*) and chorionic plate (fetal side in close vicinity to the amnion). All samples were immediately fixed in 10% formalin for immunohistochemistry. Formalin was removed by several washings with tapwater. This was followed by rehydration; washing with 70% ethanol then 80%, 90% and 100% ethanol, respectively for 5 min each and finally embedding in paraffin wax. For the immunohistochemical assays, 5–6 μm thick tissue sections were mounted on slides coated with poly-L-lysine (Sigma-Aldrich, St.Louis, MO, USA).

Immunohistochemistry

For immunohistochemical assays, sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. To unmask antigens, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer, pH 6.0, in a microwave oven at 750 W for 5 min, twice. After cooling for 20 min at room temperature, the sections were washed in phosphate buffered saline (PBS; pH 7.4). Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 for 30 min and washed with PBS three times. Afterwards, sections were incubated in a blocking solution (Ultra UV Block, LabVision Corporation, Fremont, CA, USA;TA-125UB) for 7 min at room temperature in order to block non-specific binding. Goat polyclonal anti-Notch 1 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-6014), rabbit polyclonal anti-Notch 2 (Santa Cruz, sc-5545), goat polyclonal anti-Notch 3 (Santa Cruz, sc-7424), goat polyclonal anti-Notch 4 (Santa Cruz, sc-8643), rabbit polyclonal anti-Jagged 1 (Santa Cruz, sc-8303), goat polyclonal anti-Jagged 2 (Santa Cruz, sc-8157), and anti-PECAM-1 antibodies (Santa Cruz, sc-1506) were applied overnight at +4 °C as 1:100, 1:100, 1:50, 1:100, 1:50, 1:100, 1:500 (X μg IgG/mL) dilution, respectively. Negative controls were performed by replacing the primary antibodies with their appropriate non-immune IgG. Sections were rinsed three times in PBS and incubated with biotinylated secondary antibody (Dako, Glostrup, Denmark; LSAB-2 system HRP K0679-ready to use) and a peroxidase-labelled streptavidin (Dako, LSAB-2 system HRP K0679-ready to use), respectively, for 30 min with each of the steps being followed by three rinses in PBS. Peroxidase activity was visualized with diaminobenzidine (DAB) chromogen (BioGenex, San Ramon, CA, USA) as 1 drop chromogen in 1 ml substrate for 3 min. Sections were counterstained with Mayer's Hematoxylin (Dako) for 30 s, dehydrated with an increasing series of ethanol (70%, 80%, 90% for 5 min each and 100% ethanol and xylenes for 10 min each), mounted and examined by light microscopy. All samples were treated with the same protocol.

Transmission electron microscopy (TEM)

Samples of human placenta tissues were processed as described previously (Demir 1980; Acar et al., 2004). Tissues were fixed by immersion in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 4 h and were postfixed in 1% phosphate-buffered osmium tetroxide for 2 h. The specimens were dehydrated in a graded series of ethanol and embedded in Araldite epoxy resin (SPI Structure Probe, West Chester, PA, USA). Semi-thin (1 mm) and ultrathin sections were cut using a Nova ultramicrotome. Ultrathin sections were collected on coated grids and stained with uranyl acetate (1 g uranyl acetate in 100 ml absolute ethanol) and lead citrate (sodium citrate 2.137 g and lead nitrate 1.33 g, dissolved in 30 ml water and then added 8 ml 1 N sodium hydroxide and made to 50 ml with distilled water) (Demir et al, 2005) prior to examination using a transmission electron microscope (TEM; Philips 300, Zeiss EM 10 and Leo 910).

H-SCORE and semiquantitative evaluations

The evaluations of the immunohistochemical staining of control, PIH and IUGR groups were done utilizing H-SCORE (Sahin et al., 2005). Briefly, stained sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Three randomly selected slides, each with five different fields at $\times$200 magnification were evaluated for the analysis of immunohistochemical staining of the antibodies. The

staining was scored in a semi-quantitative fashion that included the intensity of specific staining in sections. The evaluations were recorded as percentages of positive stained cells of all types in each of four intensity categories which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), 3+ (intense). The evaluation was made by two blinded observers and for each tissue, an H-SCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [H-SCORE=S P_i (i+1), where i is the intensity score and P_i is the corresponding percentage of the cells]. The H-SCORE values were graphed. The distribution of immunoreactive cells in all experimental groups was also determined semi-quantitatively: 0=negative; (+)= weak positive;+= positive; ++=strong positive; +++=very strong positive.

Statistical analysis

Statistical analyses of Notch 1, 2, 4 and Jagged 2 were evaluated by One-Way Anova tests followed by Post hoc Tukey tests for parametric data. Comparisons were made with control group placentas. Probability values of less than 0.05 were considered significant; values are presented as mean $\pm$ SEM.

Results

Immunohistochemical findings

The labelled intensity of Notch 1, 2, 4 and Jagged 2 were analyzed in control, PIH and IUGR placenta tissues and results are summarized in Table 1.

Notch 1 immunolocalization in control and pathologic placentas

In the control term placenta, we observed that Notch 1 was localized intensely and continuously in the brush border of the syncytiotrophoblast layer both in the basal plate and chorionic plate sides (Fig. 1a, b). It was also stained intensely in the vascular endothelial cells of vessels in the basal plate side where it was weak in the chorionic plate side (Fig. 1a, b). Notch 1 immunostaining was not seen in the stroma cells of the villous core in the chorionic plate or basal plate sides.

In PIH (Fig. 1c, d) and IUGR (Fig. 1e, f) placentas, we observed that the immunostaining of Notch 1 was localized discontinuously in the syncytiotrophoblast layer. In PIH placenta, the expression of Notch 1 was mainly seen in the brush border of the syncytiotrophoblast layer (Fig. 1c, d) whereas in the IUGR placenta it showed a granular localization in the cytoplasm of syncytiotrophoblast cells (Fig. 1e, f). The endothelial cells were negative for Notch 1 in PIH and IUGR placentas (Fig. 1 d–f) except for the basal plate side of PIH placenta (Fig. 1c).

When comparisons were made between the control and pathologic groups, H-SCORE analysis revealed that there was a significant decrease of Notch 1 protein staining in both pathologic groups versus control group ($^*p < 0.05$) (Fig. 1 g).

Notch 2 immunolocalization in control and pathologic placentas

In control term placenta, Notch 2 immunoreaction was also stained intensely and continuously similar to Notch 1 reaction. Notch 2 was densely localized in the brush border of the cells of the syncytiotrophoblast layer and also in the basal compartment of the syncytiotrophoblast cells in basal and chorionic plate sides (Fig. 1h, i). Notch 2 immunostaining was not detected in either the endothelial cells or the stroma cells of the villous core in the basal or the chorionic plate sides of control placenta.

In PIH (Fig. 1j, k) and IUGR (Fig. 1l, m) placentas, Notch 2 presented granular cytoplasmic staining of the syncytiotrophoblast cells on both basal and chorionic plate sides. None of these groups had an immunoreaction for Notch 2 in the brush border region of the syncytiotrophoblast of the placental villi and in endothelial cells.

Comparisons made between control group and the pathologic groups; H-SCORE analysis revealed that there was a significant decrease of Notch 2 protein immunostaining in both pathologic groups versus control group ($^*p < 0.05$) (Fig. 1n).

Notch 4 immunolocalization in control and pathologic placentas

In control placenta, Notch 4 immunoreaction was intense and continuous in the brush border region of the syncytiotrophoblast layer on both sides (Fig. 2a, b). Endothelial cells of vessels stained weakly and the stroma cells of the villous core were negative on both sides.

In PIH placenta of the basal plate side, Notch 4 protein was generally localized segmentally in the cytoplasm of the syncytiotrophoblast cells, especially around the nuclear membrane (Fig. 2c). There was no staining in the chorionic plate side (Fig. 2d). The endothelial cells were negative on both sides of PIH

Table 1
Semi-quantitative analysis of Notch 1, 2, 4 and Jagged 2 immunostaining in control and pathological placentas.

Groups	Marker	Basal Plate Side			Chorionic Plate Side		
		SN	VE	ST	SN	VE	ST
CONTROL	N1	+++	+++	0	+++	+	0
	N2	++	0	0	++	0	0
	N4	+++	(+)	0	++	(+)	0
	J2	+++	0	0	++	0	0
PIH	N1	++	+	0	++	0	0
	N2	+	0	0	(+)	0	0
	N4	(+)	0	0	0	0	0
	J2	(+)	(+)	0	(+)	0	0
IUGR	N1	+	0	0	+	0	0
	N2	(+)	0	0	(+)	0	0
	N4	+	+	0	+	0	0
	J2	(+)	0	0	(+)	0	0

PIH: Placenta induced with hypertension, **IUGR:** Intrauterine growth restriction; **N1**: Notch 1, **N2:** Notch 2, **N4:** Notch 4, **J2:** Jagged 2; **SN:** Syncytium, **VE:** Vascular endothelium, **ST:** Stroma. **0:** negative, **(+):** weak positive, **+:** positive, **++:** strong positive, **+++:** very strong positive.

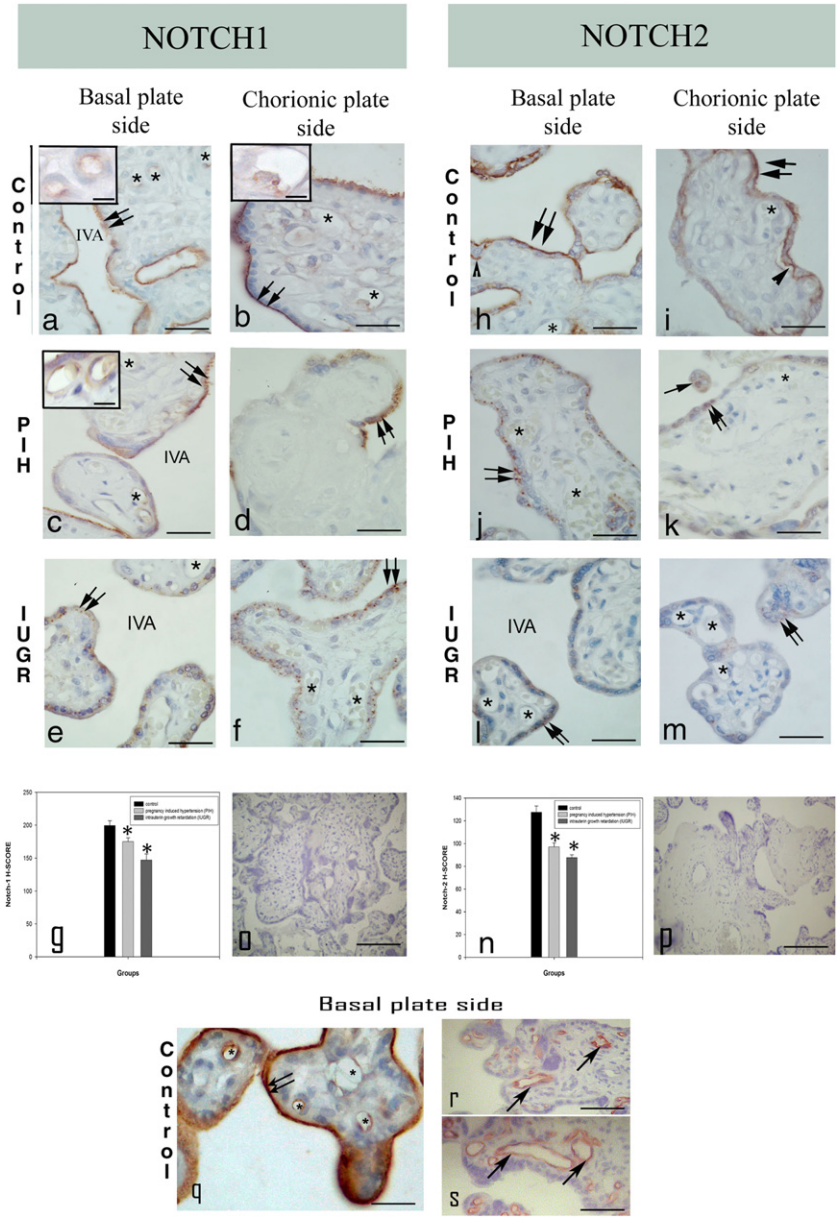

Fig. 1. Notch 1 and 2 immunostaining in the control group (a, b, h, i,), in the PIH group (c, d, j, k,) and in the IUGR group (e, f, l, m). Notch 1 immunostaining in control group (a, b), was seen in the brush border line of the syncytiotrophoblast layer in both sides (double arrows). The endothelial cells were densely immunopositive in basal plate side (a, asterisks) and weakly immunopositive in chorionic plate side (b, asterisk). In PIH and IUGR placenta (c–f), Notch 1 expression was seen discontinuously in the syncytiotrophoblast layer (double arrows). The endothelial cells were immunonegative (d–f) except for the weak staining in the basal-plate side of PIH placenta (c, asterisk). The stromal elements of the villous core were negative for this antibody. The Notch 2 immunostaining in the control group (h, i) was seen in the brush-border line of the syncytiotrophoblast layer (double arrows) and also in the basal compartment of the syncytiotrophoblast cells in both sides (arrow head). Endothelial cells were stained negatively in both sides (h, i, asterisk). In PIH and IUGR placenta (j–m), Notch 2 immunoexpression was seen segmentally and stained granularly in the syncytiotrophoblast cells (double arrows). Endothelial cells were negative (asterisks). The stromal elements of the villous core were negative for this antibody. H-SCORE analysis of Notch 1 and Notch 2 (g, n respectively) in the control, PIH and IUGR groups. IVA: Intervillous area; Scale bars = 75 μm; Black bar: control group, White bar: PIH group, Gray bar: IUGR group. $^{*}p < 0.05$.

placenta (Fig. 2c, d). In IUGR placenta, Notch 4 localization was seen granularly in the cytoplasm of the syncytiotrophoblast cells on both sides (Fig. 2e, f). The endothelial cells were also weakly positive on the basal plate side for Notch 4 (Fig. 2e). There was no immunostaining in the stroma cells of the villous core of IUGR and PIH placentas (Fig. 2c–f).

When comparisons were made between control group and the pathologic groups; H-SCORE analysis revealed that there was a significant decrease of Notch 4 protein immunostaining in both pathologic groups versus control group ($^{*}p < 0.05$) (Fig. 2g).

Jagged 2 immunolocalization in control and pathologic placentas

In control term placenta, an intense and continuous Jagged 2 expression was present in the brush-border region of the syncytiotrophoblast layer on both sides (Fig. 2h, i). There was no immunopositivity either in the endothelial cells or the stroma cells of the villous core.

In PIH placenta, Jagged 2 immunoreaction was observed discontinuously in the cytoplasm of the syncytiotrophoblast cells especially around the nuclear membrane (Fig. 2j, k). Endothelial

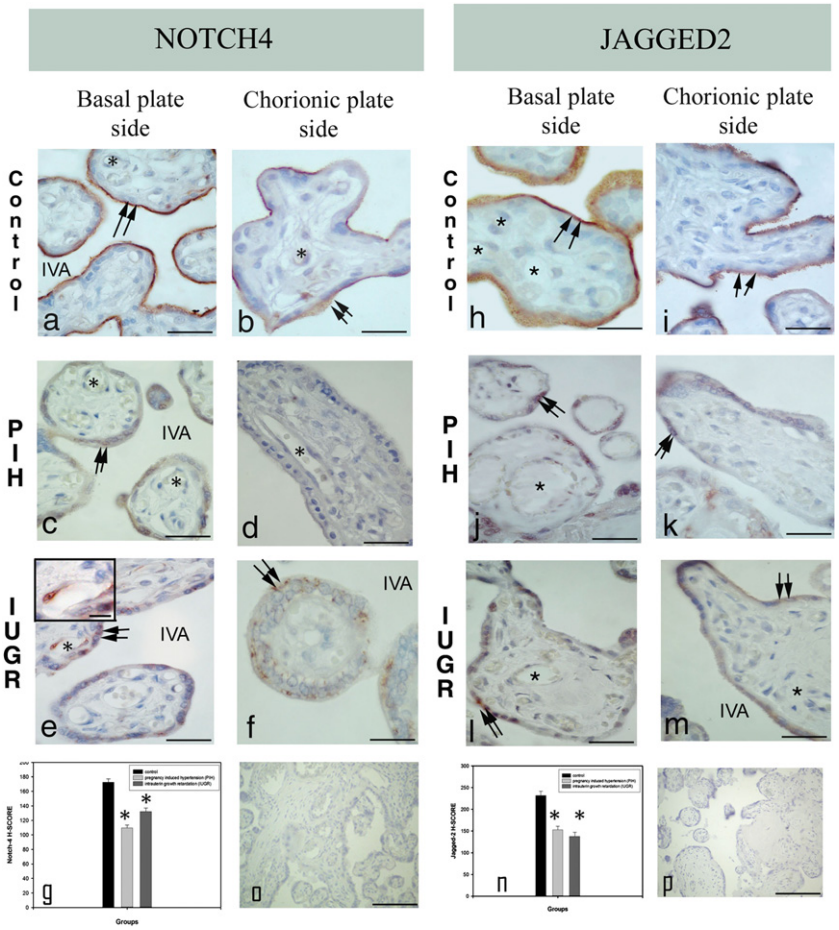

Fig. 2. Notch 4 and Jagged 2 immunostaining in the control (a, b, h, i), PIH (c, d, j, k) and IUGR (e, f, l, m) groups. The Notch 4 immunostaining in the control group (a, b) was seen continuously in the brush-border line of the syncytiotrophoblast layer (double arrows). Endothelial cells were very weakly immunopositive (asterisks). In the PIH and IUGR placentas (c–f), Notch 4 was localized segmentally in the syncytiotrophoblast cells (double arrows). There are no immunoreaction on the brush-border line of the syncytiotrophoblast layer and stromal cells. Endothelial cells were immunostained weakly in the basal-plate side of IUGR placenta (e) but not in chorionic plate side (f) or in PIH (c, d). The Jagged 2 immunolocalization in the control group (h, i) was seen in the brush-border line of the syncytiotrophoblast layer (double arrows). No immunostaining was seen in the endothelial cells of control placenta (h, i, asterisk). In the PIH and IUGR placentas (j–m), Jagged 2 was localized segmentally in the syncytiotrophoblast cells (double arrows). There was a weak staining of Jagged 2 on the endothelial cells of the PIH placenta in the basal plate side (j, asterisk). There was no immunopositivity for Jagged 2 in the stromal cells of basal and chorionic plate sides. H-SCORE analysis of Notch 4 and Jagged 2 (g, n, respectively) in the control, PIH and IUGR groups. IVA: Intervillous area. Scale bars=75 μm. Black bar:control group, White bar: PIH group, Gray bar: IUGR group. $^{*}p < 0.05$.

cells showed a very weak immunopositivity on the basal-plate side of the PIH placenta (Fig. 2j). In IUGR placenta, Jagged 2 was localized in the cytoplasm of the syncytiotrophoblast cells and showed granular staining on the basal and the chorionic plate sides (Fig. 2l, m).

When comparisons were made between the control and the pathologic groups; H-SCORE analysis revealed that there was a significant decrease of Jagged 2 protein expression in both pathologic groups versus control group ($^{*}p < 0.05$) (Fig. 2n).

In general, the immunoreactions of the above mentioned proteins were much stronger in the basal-plate side when compared to the chorionic plate side. There were no immunopositivity for Notch 3 and Jagged 1 proteins in the control or in the pathological placentas.

TEM observations

In normal term placenta, many microvilli of different sizes were observed on the surface of the syncytiotrophoblast layer. Many pinocytotic vesicles were clearly seen at depth in the microvilli. Many vacuoles and mitochondria were dispersed throughout the cytoplasm and heterochromatic nuclei of synsytiotrophoblast were also observed (Fig. 3a).

The degenerative structures of the syncytiotrophoblast layer were similar in PIH and IUGR placentas (Fig. 3b, c, respectively). In PIH placenta, very dense cytoplasm with degenerated subcellular elements were increased (Fig. 3b). The apical microvilli had deformed structures with considerably reduced density. In IUGR placenta, pycnotic nuclei and advanced destruction of syncytiotrophoblast cells including various vacuoles and degenerated subcellular elements were observed in the syncytial layer (Fig. 3c). Areas devoid of microvilli were also observed on the surface of the syncytiotrophoblast layer. Moreover, the underlying basal lamina was not uniform and some areas was thicker than that of normal basal lamina of syncytiotrophoblasts.

Discussion

The study demonstrates the immunolocalization of Notch proteins, Notch 1, 2, 4 and Jagged 2, in IUGR and PIH complicated human placentas compared to normal at the term of gestation. Our results suggest that in IUGR and PIH complicated placentas, the down-regulated immunostaining of Notch proteins and its

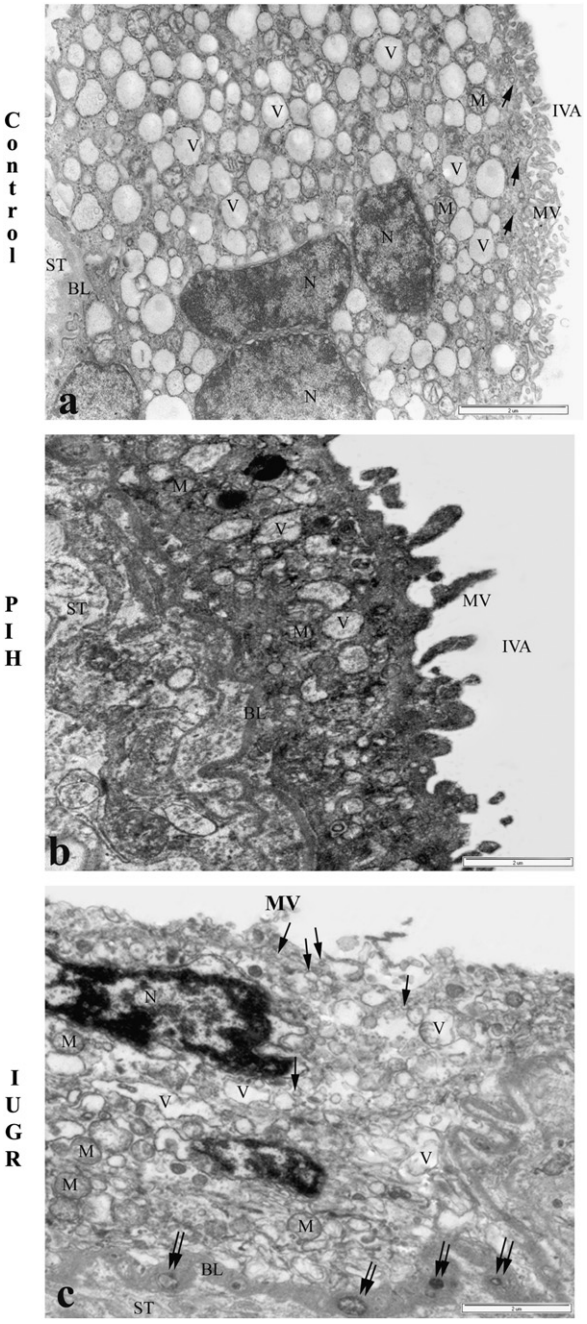

Fig. 3. This figure shows the ultrastructural features of syncytiotrophoblasts of normal (a), PIH (b) and IUGR (c) human placentas. A significant decrease in the density of microvilli, pycnotic nuclei and structural degeneration of syncytiotrophoblasts are dominantly seen in the pathological placentas, as seen in b and c. The non-uniform basal lamina with electrondense granules, is also observable (double arrows). N; nuclei, BL; basal lamina, ST; stroma, M; mitochondrion, V; vacuoles, Scale bar=2 μm.

shifting from syncytiotrophoblast membrane to the cytoplasm of syncytiotrophoblast cells may be due to the activation change of these proteins.

In this study, we found that the receptors Notch 1, 2 and 4 and the ligand Jagged 2 was densely localized in normal human term placenta, especially in the brush-border region of the syncytiotrophoblast layer. As Notch 1 and 4, have been described in previous studies (Cobellis et al., 2007), the aim of our study was to try and determine new insight into the role of Notch 2 and Jagged 2 in the maintenance of human term placenta The intense immunostaining of the ligand Jagged 2 was consistent with the hypothesis that high levels of ligand enhance the activation of the Notch pathway (Fiuza and Arias, 2007) which could induce or inhibit the differentiation potential of trophoblast cells in the organization of the placental villous tree. However, during the term of gestation the strong immunolocalizaton of Notch proteins on the syncytiotrophoblast layer led us to speculate that the Notch proteins may have an additional role, i.e. regulating feto-maternal trafficking which is crucial for the developing fetus.

The immunostaining of Notch 1 in the endothelial cells of normal term placenta was also confirmed by other studies with the requirement of Notch proteins for vascular morphogenesis in human term placenta. As indicated by other papers, the robustly expressed Notch genes in the vasculature suggest a role for Notch in guiding endothelial cells through the myriad of cell-fate decisions needed to form the vasculature (Shawber and Kitajewski, 2004). Moreover, the loss of Notch 1 function enhances the vascular remodelling defects (Krebs et al., 2000; MacKenzie et al., 2004).

IUGR and PIH have been reported to exert effects on the fetus through different placental and vascular mechanisms. Our TEM observations showed structural changes in the placenta of PIH and IUGR cases, such as the decreased apical density of microvilli and the disordered structure of the basal lamina, and these are in accordance with previous studies (Battistelli et al., 2004, Muntefering et al., 2004) and support the idea that there could be a disruption in feto-maternal trafficking in these pathologic cases.

In IUGR and PIH complicated placentas, we detected a significant decrease in the immunoreactivity of Notch proteins compared to normal term placenta. These observations are consistent with the data published by Cobellis et al. (2007) which demonstrated that Notch proteins were decreased significantly in pre-eclamptic placentas compared with controls in the term of gestation and this has been proposed as an ethiopathogenetic explanation for the onset of this pathology (Cobellis et al., 2007). Although, our results partly match the previous studies presented by Cobellis et al. (2007) some discrepancies regarding Jagged 1 immunostaining in normal human placenta may be due to differences in the experimental systems.

According to our results, decreased immunostaining of Notch proteins in pathologic placentas may be related, firstly, to the disturbed mechanism of differentiation state of trophoblast cells, which are crucial for a successful pregnancy outcome. Its ability to regulate cell differentiation and proliferation explains that deregulation of this pathway in PIH and IUGR placentas may disrupt the steady state of the proliferation or differentiation balance of trophoblast cells. Moreover, the decreased immunostaining of Notch proteins, coinciding with a reduction in placental weight, was more apparent in the IUGR placentas than in the PIH placentas. This suggests that Notch proteins may also play a role in cell proliferation within the placenta. Secondly, the localization of Notch proteins in IUGR and PIH placentas in the cytoplasm of the syncytiotrophoblast cells rather than the syncytiotrophoblast membrane as observed in the normal term placenta may also lead to the feto-maternal traffic down-regulation in these patients. Thirdly, the decreased immunoreaction of Notch 1 in vascular endothelial cells in PIH and IUGR placentas may indicate that the feto-placental vascular system could be disturbed as a result of the distracted role of Notch proteins in vasculogenesis and angiogenesis.

In conclusion, we hypothesize that in human term placenta, Notch signalling proteins, Notch 1, 2, 4 and Jagged 2 may be involved in the regulation of trophoblast fate decision, vasculogenesis and/or feto-maternal trafficking via unidentified mechanisms. In IUGR and PIH placentas, it is apparent that some morphological changes occur because of these poor developmental circumstances and affect both intensity of these proteins and their localization at the term of gestation. It has not yet been

established whether these morphological changes first occur and reduce the staining level and localization of these proteins or *vice versa* but, we can speculate that these abnormal changes may cause feto-maternal traffic down-regulation which is crucial for the onset of pregnancy. However, to resolve these questions further studies including placenta tissue culture with Notch inhibitor needs to be undertaken.

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