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ORIGINAL ARTICLE

Placental expression of insulin-like growth factor-I, fibroblast growth factor-basic and neural cell adhesion molecule in pregnancies with small for gestational age fetuses

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Objective: To investigate placental expression of insulin-like growth factor-I (IGF-I), fibroblast growth factor-basic (FGF-b) and neural cell adhesion molecule (N-CAM) regarding the pathogenesis of pregnancies with small for gestational age (SGA) fetuses.

Study Design: An immunohistochemical analysis using anti-IGF-I, anti-FGF-b and anti-N-CAM antibodies was carried out on 4% paraformaldehyde-fixed placental tissues of third trimester pregnancies complicated with SGA fetuses ($n = 12$) and subjects exhibiting appropriately grown fetuses ($n = 10$). Immunostaining patterns of chorionic villi and amniochorionic membranes were assessed.

Result: IGF-I, FGF-b and N-CAM immunostainings in chorionic villi demonstrated significantly increased immunoreactivities in cytotrophoblasts of SGA cases, whereas increased IGF-I immunostaining in syncitiotrophoblasts and increased N-CAM immunostaining in capillary endothelium were noted in the same group. IGF-I, FGF-b and N-CAM immunostainings in amniochorionic membranes revealed significantly decreased IGF-I immunoreactivities in extravillous trophoblasts and increased IGF-I immunoreactivities in decidual cells of SGA cases, while significantly decreased N-CAM immunoreactivities in both decidual cells and extravillous trophoblasts were noted. FGF-b immunostaining revealed no significant differences in both extravillous trophoblasts and decidual cells of SGA cases.

Conclusion: Increased placental expression of IGF-I, FGF-b and N-CAM may act in an autocrine and/or paracrine manner to restore the impaired trophoblastic proliferation, migration and metabolism at all gestational stages by means of a positive feedback mechanism.
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Introduction

Intrauterine fetal growth restriction (IUGR) appears to be a frequent cause of perinatal morbidity and mortality with an incidence of 15 to 20% in developing countries and 3% in industrialized countries.[1–3] Impaired uteroplacental perfusion leading to inadequate placental function may be responsible of restricted fetal growth.[4]

Several molecules are involved in cellular growth and differentiation during embryogenesis and serve as modulators of placental function.[3–6] Altered gene expression and proteolytic release from extracellular matrix provide those local growth factors which are shown to be clearly associated with regular progression of normal embryogenesis.[1]

The trophic effects of insulin exhibiting enhanced somatic fetal growth occur through some interactive mechanisms. Release of secondary hormones such as insulin-like growth factors (IGFs) or insulin-like growth factor binding proteins (IGFBP) points out to be one of those acting mechanisms of growth-promoting insulin effects.[1,5]

IGF-I is a small polypeptide of 70 amino acids with an approximate molecular weight of 7.6 kDa. It exerts its potent mitogenic activity through increased DNA synthesis and biological actions are regulated through IGFBPs by endocrine and paracrine mechanisms.[6,7] It has been shown to act in folliculogenesis, pre-implantation embryonic development, implantation and fetal-placental growth.[7]

Fibroblast growth factor (FGF) is a potent inducer of angiogenesis that is widely expressed during embryonic and fetal development and is able to act as a mitogen, a tissue morphogen and a modulator of tissue differentiation and placental angiogenesis.[5,8] Placental expression of FGF/FGF receptors has been identified in rodents, pigs, ovine and human.[9–12]

It mediates maintenance of a competent fetoplacental network provided by branching or nonbranching angiogenesis, which is mandatory for endometrial development, implantation and placentation.

The rapid progress regarding the molecular mechanisms playing role in branching demonstrated that abnormal placental branching noted in IUGR placenta was possibly due to a number of angiogenetic factors in the pathogenesis of IUGR.[13–16] Additional investigations on the role of FGF in embryonal development have indicated that FGF receptor 1 deficient mice displayed severe IUGR both *in vivo* and *in vitro*.[17]

Neural cell adhesion molecule (N-CAM) is an adhesion molecule that is involved in morphogenesis, cellular signaling, proliferation, polarization and differentiation.[18] N-CAM is responsible of maintaining tissue integrity, regeneration of nerve or muscle and healing wounds and enhanced tumor growth.[19] It is a cell-surface glycoprotein, a member of immunoglobulin superfamily that provides homophilic and heterophilic adhesions which are between neighboring cells and between cells and extracellular matrix proteins, respectively.[20] Altered tissue N-CAM expressions in fetuses with IUGR may point out its role in pathogenesis of growth restriction processes.

In the present study, we aimed to elucidate whether placental expressions of IGF-I, FGF-basic (FGF-b) and N-CAM are altered in pregnancies complicated with small for gestational age (SGA) fetuses and determine the presense of any impact of those molecules on the pathogenesis of pregnancies with SGA fetuses.

Methods

The present study was conducted on pregnant subjects ($n = 12$) complicated with SGA fetuses and pregnant women ($n = 10$) with appropriately grown fetuses (controls) without known maternal and fetal diseases with birth weights between 10th and 90th percentiles. All the subjects were in their third trimester. The last menstrual dates, first trimester ultrasonographic examinations with crown rump length, risk factors related with SGA fetuses, uterine fundal height measurements of the cases were used to determine the gestational age properly. The diagnosis of pregnancies with SGA fetuses was established by serial ultrasonographic examinations involving fetal biometry. Women with fetal biometric measurements less than 10th percentile for gestational age were accepted to have pregnancies complicated with SGA fetuses. Clinical data involving gestational ages, maternal ages, maternal complications, delivery route, APGAR scores, birth weights, admission to neonatal intensive care unit were recorded. Placental weight, shape and diameter, number of cotyledons, umbilical cord length and diameter, type of insertion to the disk were determined. Twin placentae, placentae of infants with congenital anomalies, fetal aneuploidy and those with suspected or proven intrauterine infections and gross placental anomalies, pregnancies with known vascular maternal disease (hypertension, diabetes mellitus and autoimmune diseases) were excluded.

Histoplanimetrical analysis

In each immunostained section, quantification of immunoreactive cells was performed in every 10 chorionic villi, 10 capillaries and 0.10 mm^2 fields of amniochorionic membranes with a $\times 40$ objective using an ocular micrometer system (Olympus, Tokyo, Japan). In this study, the slides were coded to enable blind counting of random slides. Immunoreactive cells were, therefore, counted by a microscopic observation of three investigators in a blinded fashion.

Immunohistochemical analysis

Human placental tissue and amniochorionic membranes were obtained from all pregnant subjects following delivery. Pieces of 1 to 3 cm fetal placental tissue and amniochorionic membranes were fixed for 24 to 48 h in neutral buffered 4% paraformaldehyde, dehydrated in increasing alcohol series (70, 80, 90 and 100%) and xylene, prior to embedding in paraffin wax. Sections (5 μm thick) were obtained using a sliding microtome. The following antibodies were used: Polyclonal anti-IGF-I antibody (National Hormone and Pituatory Programme, Torrance, CA, USA), anti-FGF-basic antibody (Sigma, St Louis, MO, USA) and monoclonal anti-N-CAM antibody (Sigma). For antigen retrieval, the sections were treated with 10 mM sodium citrate for 5 min in a microwave oven. For immunohistochemistry, avidin-biotin-peroxidase method was applied to the placental tissue sections. The rehydrated sections were pretreated with 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity. Sections were then washed in phosphate-buffered saline (PBS)-Triton X 100 (Tx). To eliminate the nonspecific binding, sections were pretreated with normal rabbit serum. Sections were incubated with IGF-I primary antibody (dilution 1:800), FGF-b antibody (dilution 1:2000) and N-CAM antibody (dilution 1:50) for 24 h at 4 °C in a humidified chamber. Following washing in PBS-Tx, biotinylated secondary antibody (Histostain Plus Kit; Zymed, San Fransisco, CA, USA) was applied to the sections for 15 min at room temperature. After washing in Tris 0.6% hydrogen peroxide, 0.02% diaminobenzidine was applied for approximately 5 min at room temperature. As control, the primary antibody was omitted and replaced with nonimmune serum. Immunoreactivity was examined by light microscopy (BX50F-3; Olympus).

Statistical analysis

Except for descriptives, data were expressed as median (range). Nonparametric tests (Mann–Whitney *U*-test) were used for statistical analysis (Statistical Package for Social Sciences for Windows (SPSS), Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

Demographic and clinical variables of pregnant subjects with SGA fetuses and control cases are demonstrated in Table 1. Birth

Table 1 Demographic and clinical data of the subjects with SGA fetuses and controls

	Control group (n = *10*) *Median* (*range*)	*SGA group* (n = *12*) *Median* (*range*)	P
Age (years)	28.5 (19–39)	29.0 (17–40)	NS
Parity	1 (0–3)	1 (0–3)	NS
Gestational age (weeks)	36.5 (32–39)	36 (32–40)	NS
Birth weight (g)	3175.0 (2575–4090)	2170.0 (1500–2680)	<0.001
APGAR scores	9.5 (8–10)	7 (5–9)	<0.001
Placental weight (g)	605.0 (470–730)	300 (240–400)	<0.001
Cord diameter (cm)	0.9 (0.8–1.6)	0.6 (0.4–0.9)	<0.001

Abbreviations: NS, nonsignificant; SGA, small for gestational age.
$P<0.05$ statistically significant.

Table 2 The number of IGF-I, FGF-b and N-CAM immunoreactive cells of chorionic villi obtained from the subjects with SGA fetuses and controls

	Control group (n = *10*) *Median* (*range*)	*SGA group* (n = *12*) *Median* (*range*)	P
IGF-I (capillary)	15.5 (6–31)	17.0 (8–61)	0.192 NS
IGF-I (cytotrophoblasts)	20.0 (6–40)	42.0 (14–96)	<0.001*
IGF-I (syncitiotrophoblasts)	7.5 (3–21)	11.5 (5–26)	0.006*
FGF-b (capillary)	17.5 (12–25)	18.5 (10–37)	0.779 NS
FGF-b (cytotrophoblasts)	14.5 (5–26)	45.0 (18–36)	<0.001*
FGF-b (syncitiotrophoblasts)	12.0 (6–25)	12.0 (5–27)	0.758 NS
N-CAM(capillary)	16.0 (12–25)	25.0 (10–66)	0.014*
N-CAM (cytotrophoblasts)	15.0 (8–29)	26.0 (8–110)	0.001*
N-CAM (syncitiotrophoblasts)	10.0 (4–26)	11.0 (6–19)	0.841 NS

Abbreviations: FGF-b, fibroblast growth factor-basic; IGF-I, insulin-like growth factor-I; N-CAM, neural cell adhesion molecule; NS, nonsignificant; SGA, small for gestational age.
*$P<0.05$ statistically significant.

weights, APGAR scores, placental weights and cord diameter appeared to be significantly different between groups while age, parity, gestational age, cord length, number of cotyledons and placental grade were not demonstrated to be significantly different. Delivery route was shown to be cesarean section in 10 SGA cases (83.3%) whereas 3 control subjects (30%) required abdominal delivery. Eight of the neonates (66.6%) born to mothers with SGA fetuses were admitted to neonatal intensive care unit while none of the appropriately grown fetuses required to be kept in intensive care unit.

Comparison of IGF-I, FGF-b and N-CAM immunoreactive cells through the chorionic villi and amniochorionic membranes of placentae belonging to pregnancies with SGA fetuses and control cases are demonstrated in Tables 2 and 3, respectively.

IGF-I, FGF-b and N-CAM immunostaining patterns through the chorionic villi of placentae of SGA and control cases are demonstrated in Figure 1.

Table 3 The number of IGF-I, FGF-b and N-CAM immunoreactive cells of amniochorionic membranes obtained from the subjects with SGA fetuses and controls

	Control group (n = *10*) *Median* (*range*)	*SGA group* (n = *12*) *Median* (*range*)	P
IGF-I (extravillous trophoblastic cells)	62.5 (45–81)	35.5 (22–40)	<0.001*
IGF-I (decidual cells)	14.5 (10–22)	44.5 (34–54)	<0.001*
FGF-b (extravillous trophoblastic cells)	33.0 (23–44)	31.5 (24–43)	0.697 NS
FGF-b (decidual cells)	40.0 (35–49)	40.5 (33–53)	0.812 NS
N-CAM (extravillous trophoblastic cells)	50.0 (45–58)	27.5 (19–35)	<0.001*
N-CAM (decidual cells)	49.0 (38–55)	27.0 (20–34)	<0.001*

Abbreviations: FGF-b, fibroblast growth factor-basic; IGF-I, insulin-like growth factor-I; N-CAM, neural cell adhesion molecule; NS, nonsignificant; SGA, small for gestational age.
*$P<0.05$ statistically significant.

In the capillaries of chorionic villi of SGA cases, the number of N-CAM immunoreactive cells was increased significantly ($P = 0.014$). However, this number of IGF-I and FGF-b immunoreactive cells did not reveal significant difference when compared to the controls (Table 2; Figure 1; $P = 0.192$, $P = 0.779$). In the cytotrophoblastic cells of chorionic villi obtained from SGA cases, significantly increased IGF-I, FGF-b and N-CAM immunoreactivities were noted when compared to the controls (Table 2; Figure 1; $P<0.001$, $P<0.001$, $P = 0.001$). Additionally, a significantly increased number of IGF-I immunoreactive cells was observed among the syncitiotrophoblastic cells of SGA subjects while FGF-b and N-CAM immunoreactivities were not found to be significantly different in the same group of cells in SGA pregnancies when compared to the controls (Table 2; Figure 1; $P = 0.006$, $P = 0.758$, $P = 0.841$).

IGF-I, FGF-b and N-CAM immunostaining patterns through the amniochorionic membranes of placentae of SGA and control cases are demonstrated in Figure 2.

In the amniochorionic membranes of SGA cases, significantly decreased IGF-I immunoreactivities in extravillous trophoblastic cells and significantly increased IGF-I immunoreactivities in decidual cells were demonstrated when compared to the controls (Table 3; Figure 2; $P<0.001$, $P<0.001$). Additionally, FGF-b immunostaining was not found to be significantly differing in both extravillous trophoblasts and decidual cells of SGA subjects (Table 3; Figure 2; $P = 0.697$, $P = 0.812$). N-CAM immunoreactivity was demonstrated to be significantly decreased in both extravillous trophoblasts and decidual cells of SGA placentae in comparison to the controls (Table 3; Figure 2; $P<0.001$, $P<0.001$).

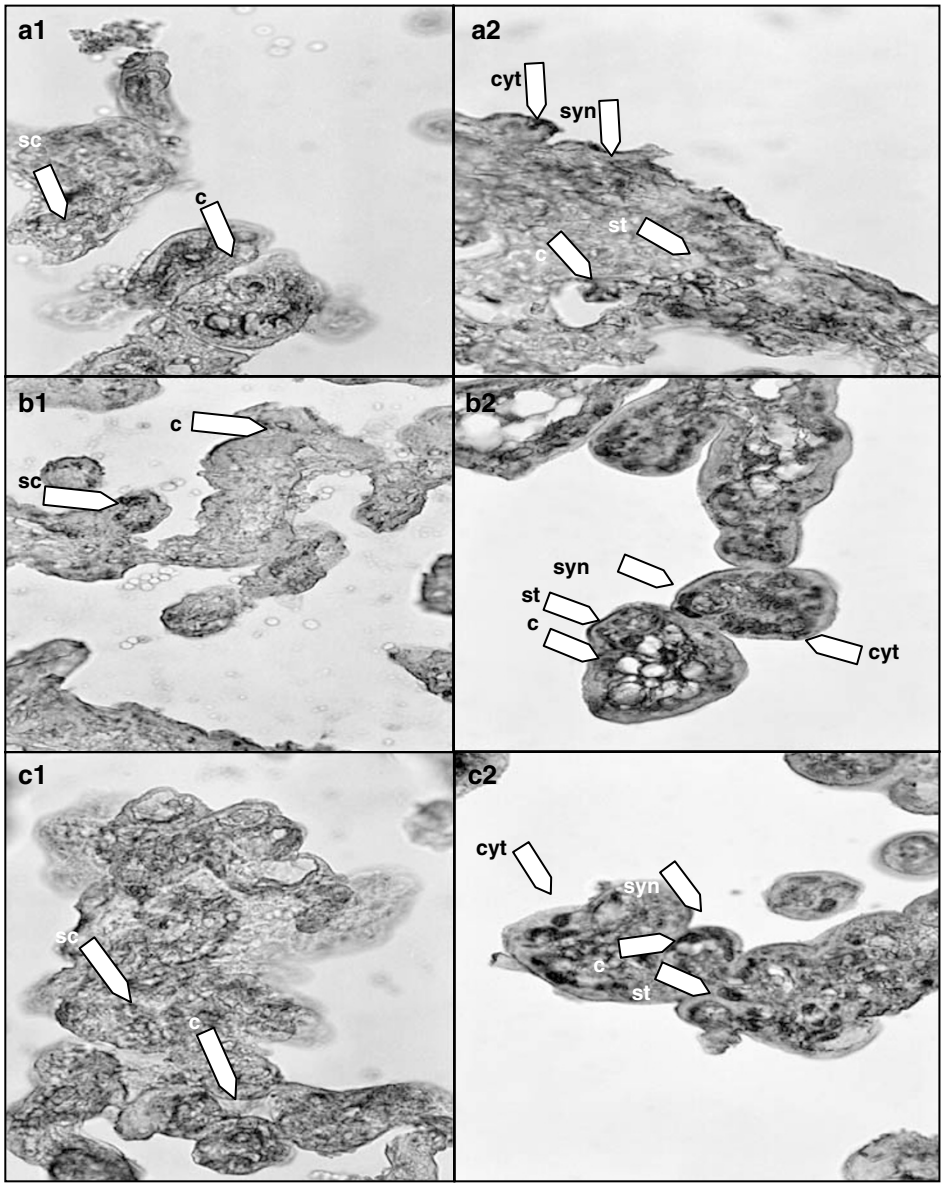


Figure 1 Photomicrographs showing IGF-I immunoreactivity from the placenta of the normal (**a1**) and SGA placental villus (**a2**). Note the IGF-I immunoreactivity both in (**a1**) and (**a2**). However intense IGF-I immunoreactivity was observed in the cytotrophoblast (cyt) and syncytiotrophoblast (syn) in the SGA placental villus (**a2**). Photomicrographs showing FGF-b immunoreactivity from the placenta of the normal (**b1**) and SGA villus (**b2**). Note the FGF-b immunoreactivity both in (**b1**) and (**b2**). However intense FGF-b immunoreactivity was observed in the cytotrophoblast (cyt) in the SGA placental villus (**b2**). Photomicrographs showing N-CAM immunoreactivity from the placenta of the normal (**c1**) and SGA villus (**c2**). Note the N-CAM immunoreactivity both in (**c1**) and (**c2**). However intense N-CAM immunoreactivity was observed in the cytotrophoblast (cyt) and capillary endothelium in the SGA placental villus (**c2**). Sc, stromal cells (fibroblasts and Hoofbauer cells); c, capillary endothelial cells. Scale bar: 50 μm.

Discussion

Several growth factors and adhesion molecules acting as modulators of placental function gain significance regarding the occurrence of IUGR.[2]

In accordance with previous findings, we determined significantly reduced fetal birth weights and placental size and weight in SGA group consistent with the definition of the IUGR. This demonstrated consequently that placental growth was correlated with fetal growth and development. Umbilical cord diameter was also decreased in SGA subjects pointing out that reduced blood flow through those cords might have led to fetal growth restriction.

A variety of previous studies demonstrated that significantly reduced maternal serum levels of IGF-I and additional mutations of IGF-I and IGF receptor genes were probably found to be associated with impaired fetal growth and development.[21] Some of

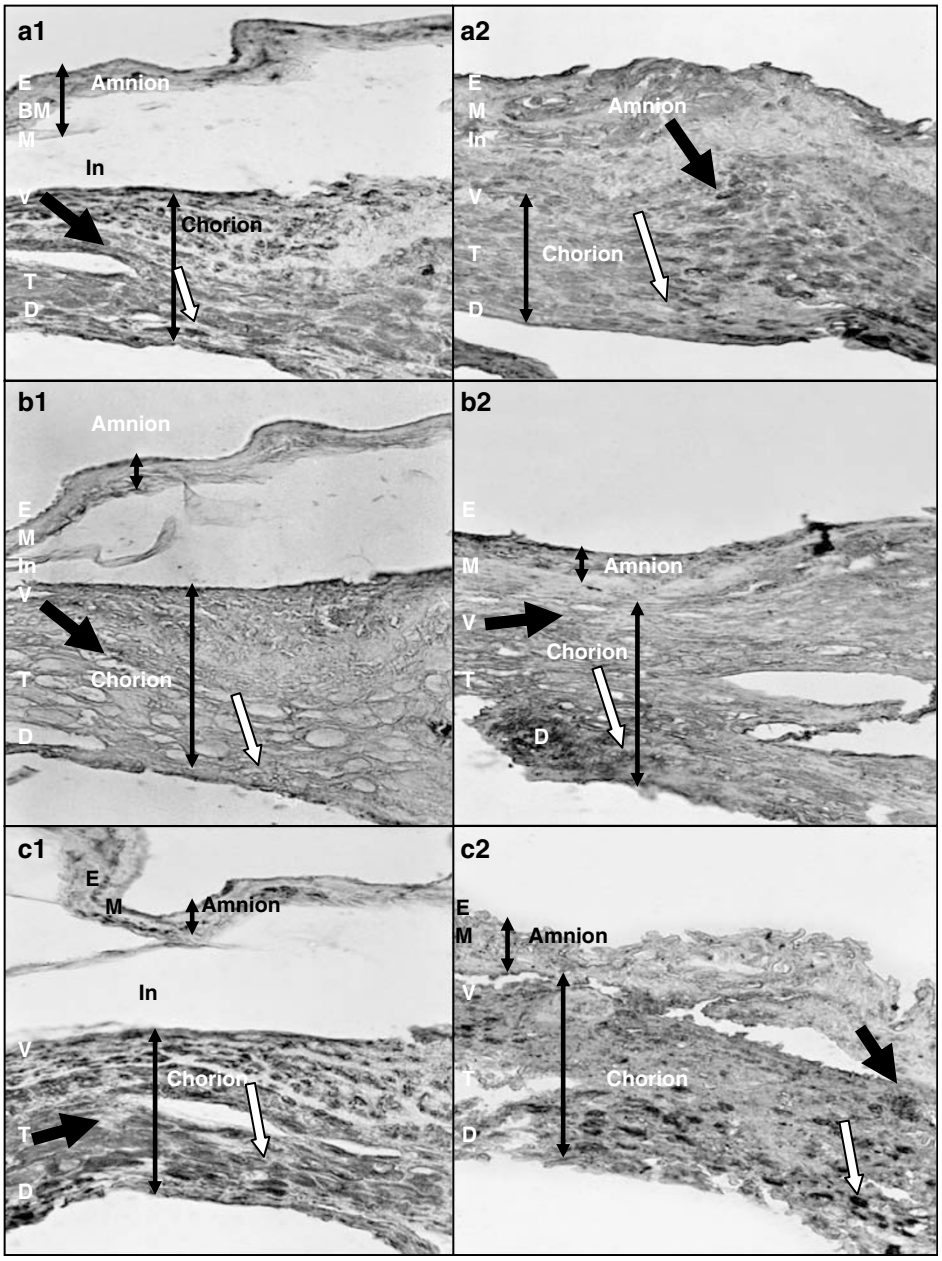


Figure 2 Photomicrographs showing IGF-I immunoreactivity from the term placenta of the normal (**a1**) and SGA amniochorionic membrane (**a2**). Note that the IGF-I immunoreactivity is decreased in extravillous trophoblastic cells and increased in decidual cells of SGA amniochorionic membranes. Photomicrographs showing FGF-b immunoreactivity from the term placenta of the normal (**b1**) and SGA amniochorionic membrane (**b2**). Note the low FGF-b immunoreactivity in (**b1**) and (**b2**). Photomicrographs showing N-CAM immunoreactivity from the term placenta of the normal (**c1**) and SGA amniochorionic membrane (**c2**). Note that N-CAM immunoreactivity is decreased in both extravillous trophoblasts and decidual cells of the SGA amniochorionic membranes. E, epithelial cells; BM, basement membrane; M, mesenchymal layer; V, vascular collagenous layer; In, intermediate zone seperates amnion from chorion; T, trophoblastic layer; D, maternal decidua capsularis. Black arrow, trophoblastic cells; white arrows, decidual cells. Scale bar: 150 μm.

the investigations pointed out a well-established correlation between increased placental expression of IGF-R and fetal growth that a decreased expression might be responsible of IUGR.[22] Demonstration of lower IGF-I concentrations and increased IGFBP-1 levels in human infants with IUGR and higher concentrations of IGF-I in macrosomic fetuses of diabetic women point out that fetal IGF-I expression may be closely associated with growth rate in the last trimester.[6] Experimental studies concluded similarly that tissue IGF-I mRNA and circulating IGF-I levels were diminished in animal models of IUGR established by uterine artery ligation or maternal fasting.[23,24] Additionally, Holmes *et al*.[25] indicated that distribution and intensity of IGF-I receptors in villous endothelium, stromal cells, trophoblasts and decidua of pregnancies with appropriately grown fetuses and

IUGR fetuses were similar. They suggested that anatomic distribution and intensity of trophoblastic IGF-I receptors were not correlated with functional capacity of receptors, namely, ability of binding and signal transduction. The findings of Sorem *et al*.[4] that were not in accordance with those demonstrated that IGF-I production and release appeared to be aberrant. Abu-Amero *et al*.[26] found significantly higher IGF-I and IGF-II receptor expression in IUGR and they suggested that a counter-regulatory mechanism is involved in response to growth restriction by means of elevated receptor expression. The results of our previous study were in accordance with the findings of Abu Amero (1998) as a distinct increase in IGF-I immunoreactive cells and intensity in decidua and umbilical veins of IUGR placentae was demonstrated.[3]

Based on our findings in this study, we conclude that increased immunoreactivity in placental chorionic villi of the SGA subjects may be due to a positive feedback mechanism by means of a paracrine/autocrine function in order to restore the impaired fetal growth. The other studies with different results showed that the release of placental IGF-I was obviously abnormal in certain cases of IUGR and might reflect deficiency of basal IGF-I production and aberrant regulation.

FGF-b seems to modulate tissue differentiation and placental angiogenesis and acts as a potent angiogenic agent. Placental development encompasses a number of processes namely villous angiogenesis, branching of chorionic villi and controlled invasion to maternal decidua. Since the placenta demonstrates a physiologic form of invasive growth, both placental and fetal tissues are sites of FGF-b expression.[5] The process of regulating angiogenesis is of utmost importance during placental development regarding subsequent increase in umbilical and uterine blood flow promoting sufficient blood supply to fetus. FGF–ligand interaction appears to be a crucial modulator of all those processes.[13] It reaches its serum peak values in the second trimester and serum concentrations are positively correlated with fetal size during both second and third trimesters. Serum FGF-b levels from second trimester onward positively correlating with fetal size and elevated FGF concentrations in pregnancies complicated with diabetes mellitus where especially fetal macrosomia is present further suggest a role of FGF in fetal growth.[5,27]

Although the precise role of FGF-b in IUGR remains to be elucidated, according to our findings, we postulate that FGF-b could be important in stimulating the proliferation of vascular smooth muscle and endothelial cells via cytotrophoblasts.

Cell adhesion is a key process in tissue formation and maintaining tissue integrity in early fetal development and adult life. Adhesion molecules are involved in epithelial migration, differentiation and topographic organization.[20] Of those, N-CAM is detected in neural and non-neural tissues. It provides junctional complexes and triggers intracellular signaling events involving correct positioning of cells in a tissue cell recognition and cell to cell contact.[18]

N-CAM expression is found to be abundant during development and becomes restricted in adults, which in turn impairs N-CAM expression may point out a key role in pathogenesis of fetal growth abnormalities.[19] Since the collected data regarding those issues with respect to pathogenetic mechanisms of IUGR are scarce, further investigation is mandatory to elucidate more precise and worthy conclusions.

The findings of the present study may again be attributed to a potential compensation mechanism in response to impaired N-CAM expression in order to correct disturbed fetal growth. Placental expression and recruitment of N-CAM may indicate a direct or indirect involvement of adhesion mechanisms and signaling in growth restriction processes.

Further studies are mandatory to clarify the precise implications of serum IGF-I levels and placental expression of IGF-I and IGF-I receptors, FGF-b and N-CAM on the pathogenesis of IUGR and SGA.

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