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Research Article

Expression of Angiogenic Proteins in Chorionic Villi of Normal Human Placentas

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Abstract

Objective: We have explored the interactions of three placental angiogenic proteins at each trimester of normal human pregnancy: Vascular endothelial growth factor (VEGF) $VEGF_{165}$, $VEGF_{165}b$ and matrix metalloproteinase (MMP) MMP-9. We have additionally determined the relationship of $VEGF_{165}b$ and MMP-9 proteins with $VEGF_{165}$, the potential mediator of placental angiogenesis in human.

Methods: 195 placentas were obtained from normotensive pregnancies. Chorionic villi were isolated and $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 proteins expression were analyzed by ELISA. The kits were purchased from R&D Systems, Minneapolis, MN. Placentas were grouped by trimesters and non-parametric tests were performed. Mediation test was carried out. $P < 0.05$ was considered significant.

Results: 61 placentas were collected from the 1st trimester, 42 from the 2nd trimester and 92 from the 3rd trimester of normotensive pregnancies. Kruskal Wallis and Mann Whitney U tests revealed significant differences ($p = 0.001$) in all three proteins among the trimester groups. The protein profiles throughout gestation showed that in the 2nd trimester, $VEGF_{165}$ was comparatively lower, while $VEGF_{165}b$ protein showed a peak. MMP-9 protein progressively increased with an increase in gestational age. $VEGF_{165}b$ and MMP-9 proteins were correlated with gestational age in days (GAD) in the 1st trimester of human pregnancy (rho = 0.280, p = 0.029, rho = 0.290, p = 0.023 for $VEGF_{165}b$ and MMP-9, respectively). No correlation was seen in the 2nd trimester. In the 3rd trimester, both isoforms of $VEGF_{165}$ were significantly correlated (rho = 0.368, p = 0.0001). The correlation between $VEGF_{165}$ and MMP-9 proteins was significant but negative (rho = -0.306, p = 0.003). Mediation test results revealed that $VEGF_{165}b$ and MMP-9 both exerted independent effects on $VEGF_{165}$ but the effect of $VEGF_{165}b$ on $VEGF_{165}$ was not mediated via MMP-9.

Conclusion: The study demonstrates a temporal variation in $VEGF_{165}b$ protein expression throughout gestation, suggesting that $VEGF_{165}b$ protein is more stringently controlled at each phase of human gestation, and may be actively participating in placental development. The correlation of the anti-angiogenic protein $VEGF_{165}b$ with $VEGF_{165}$ throughout gestation, and the negative association between MMP-9 and $VEGF_{165}$ proteins in the 3rd trimester suggest that both $VEGF_{165}b$ and MMP-9 proteins in human pregnancy could contribute in restraining over expression of $VEGF_{165}$; which if left un-checked, could lead to pregnancy-related complications.

Keywords: *Gestational Age-Specific Protein Expressions; Normal Human Pregnancy; $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 Proteins; Hispanic and Black Ethnic Groups; C-Section and Vaginal Methods of Delivery*

Abbreviations

$VEGF_{165}$: Vascular Endothelial Growth Factor $(VEGF)_{165}$; $VEGF_{165}b$: Vascular Endothelial Growth Factor $(VEGF)_{165}b$; MMP-9: Matrix Metalloproteinase-9; GAD: Gestational Age in Days

Introduction

Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis [1]. Gene knockout studies revealed a central role of VEGF in placental angiogenesis. Targeted homozygous null mutations of VEGF receptors in mice demonstrated failure in hematopoiesis and formation of blood islands and blood vessels that resulted in embryonic death by day 8 of pregnancy [2,3]. In humans, there are nine different isoforms of VEGF but the 165 amino acid form is most abundant *in vivo* and is well-studied [4]. $VEGF_{165}b$ is a sister isoform of $VEGF_{165}$, formed by alternate splicing of $VEGF_{165}$ mRNA [5,6]. $VEGF_{165}$ and $VEGF_{165}b$ proteins contain equal number of amino acids but six amino

acids at the C-terminus of the two proteins are different. In $VEGF_{165}$, they are CDKPRR, whereas in the $VEGF_{165}$b protein they are SLTRKD [5]. The switch in these six amino acids alters the tertiary structure of the $VEGF_{165}$b protein [7]. Investigators have shown that $VEGF_{165}$b protein inhibits angiogenesis *in vivo* in different tumor models [8,9].

During human pregnancy, cytotrophoblasts proliferate, migrate and invade the pregnant uterus for successful implantation and placentation [10]. The invasive property of the trophoblasts is dependent on their ability to secrete proteases such as matrix metalloproteinases (MMPs) which are capable of degrading the basement membrane and extracellular matrix [11]. Of the MMPs, MMP-2 and MMP-9 are the most studied. Reciprocal embryo transfer experiments have demonstrated the presence of MMP-9 at embryo implantation site and additionally reports that MMP-9 also contributes in embryonic trophoblast development [12].

For a number of years we have focused our attention on delineating the expression patterns of $VEGF_{165}$, $VEGF_{165}$b and MMP-9 angiogenic proteins, throughout gestation in normotensive pregnancies that had normal fetal outcomes. What distinguishes our studies from those cited in the literature are: 1) the majority of previous studies investigated placental expression of $VEGF_{165}$ and MMP-9 proteins using either first and/or third trimester human placentas, omitting the 2nd trimester placental samples; and 2) report on placental expression of $VEGF_{165}$b protein in human pregnancy is practically nonexistent. There is only one study reported in the literature on placental expression of $VEGF_{165}$b protein, which investigated the protein expression in the third trimester of normal and preeclamptic term placentas [13]. In our previous separate studies investigating placental angiogenic proteins, we embraced the whole spectrum of human gestational period, and have included sizeable number of placentas from the 2nd trimester [14-17]. Importantly, our studies are the only ones cited in the literature that have reported noteworthy expression of $VEGF_{165}$b protein in the first, second and third trimesters of normal human pregnancy [15,17]. In the present study, placental expression of $VEGF_{165}$, $VEGF_{165}$b and MMP-9 proteins were investigated simultaneously, throughout gestation, in normal human pregnancy. The interest was in determining the association between the three placental proteins at each trimester of human gestation. In the present study, we have also included a sizable number of term delivered placentas (n = 92), obtained from women with normotensive pregnancies, with a goal to understand the association between these placental proteins when the fetal demand and growth and placental development are the highest, prior to parturition.

Methods

Women were not enrolled for the study. Placental tissues after clinical care, following elective termination of first and second trimester pregnancies, and placental tissues following term deliveries that would otherwise have been discarded, were collected under a protocol approved by the Institutional Human Subject Committee of the BronxCare Health System, Bronx, New York, without informed consent. The protocol allowed certain clinical information to be collected at the time of tissue collection without the identification of the patients' names and their medical record numbers. The clinical information collected included: maternal age, race, gestational age as determined by ultrasound and/or by the initial date of the last menstrual period, and method of delivery. Since the focus of the study was to understand the gestational age-specific changes in protein expression in normal human pregnancy, placentas were collected from mothers who had opted for elective termination of pregnancies, only if the mothers had normal systolic and diastolic blood pressure on the day the elective termination of pregnancy was performed. Placentas collected following term deliveries were from mothers who were normotensive throughout gestation. Placentas from elective termination of pregnancy or from term delivery were not collected as well if the mother had a missed abortion or had pregnancies complicated with any infection, diabetes, hypertension, chronic renal disease, chronic peripheral vascular disease, multifetal gestation or with major fetal anomalies. Notably, the placental samples were collected prior to COVID-19 pandemic. Placental tissues were collected within 30 minutes of elective first or second trimester pregnancy terminations (7 - 24 week gestation) and after vaginal or cesarean deliveries (37 - 42 week gestation). Placentas delivered overnight or when placenta sample collection could not be done within the 30-minute window, the placentas were not collected to keep the sample collection protocol consistent.

Placental tissues were thoroughly washed in cold saline and were then dissected in saline to collect free floating chorionic villi, not anchored to the basal plate nor emerging from the chorionic plate surface vessels [14]. Sections of chorionic villi samples from the same placenta were placed in separate cryovials bearing identical study number and were transported to the laboratory on ice. The chorionic villi samples from the same placenta were stored in individual freezer boxes at -80^{0}C until assay.

Commercially available Enzyme Immunoassay (EIA) kits were purchased from R&D Systems Minneapolis, MN to determine the chorionic villi protein expressions of $VEGF_{165}$, $VEGF_{165}$b and MMP-9. The three proteins were not analyzed from the same homogenate. $VEGF_{165}$

protein assays of all placental samples were carried out first. On the day of the assay, chorionic villi samples were taken out of the -80^{0}C freezer in chronological order the placental tissues were collected. The tissues were homogenized, then centrifuged at 13,000 rpm for 2 minutes and the supernatants were used for direct-sandwich EIA. When $VEGF_{165}$ protein assays were completed, $VEGF_{165}b$ protein expression was carried out following the same chronological order as stated above, followed by MMP-9 protein assays. Sensitivity of the assay kit used was 31.3 pg/ml for $VEGF_{165}$, 62.5 pg/ml for $VEGF_{165}b$ and 31.3 pg/ml for MMP-9, respectively. The MMP-9 ELISA kit measured the 92 kDa Pro-MMP-9 and the 82kDa active MMP-9. Samples were analyzed in duplicates and had an intra assay and inter assay variations < 10% for all proteins.

Statistical analyses

Statistical evaluation of the data was carried out using SPSS^R statistical package version 26 (IBM Corporation, Armonk, NY). Normal distribution of the data was first tested and was found to be skewed. Hence, non-parametric analyses were used for the study. Chorionic villi tissue samples were grouped by trimester. Kruskal Wallis test was performed to compare the differences among three trimester groups followed by Mann Whitney U test for inter-group comparisons. Spearman Rank correlation coefficient test was applied to summarize the strength and direction of a relationship between the protein variables and gestational age in days (GAD). $P < 0.05$ was considered significant.

Mediation test

To determine, whether the interactions between $VEGF_{165}$ and $VEGF_{165}b$ proteins, was mediated via MMP-9, the Mediation test was performed. The model for the test is presented in figure 1A, which considered $VEGF_{165}b$ protein as the Independent variable; $VEGF_{165}$ protein as the Dependent variable; and MMP-9 protein as the Mediator. The test was carried out, in 3 steps. First, the Total Effect of the Independent variable on the Dependent variable was determined using Bivariate Regression Analysis. In the 2nd step, a second Bivariate Regression Analysis was carried out with $VEGF_{165}b$ predicting MMP-9. The unstandardized coefficient and unstandardized standard error values obtained are referred to as "a" (Figure 1A). Next, a Multiple Regression Analysis was carried out with $VEGF_{165}b$ and MMP-9, predicting $VEGF_{165.}$ The unstandardized coefficient and unstandardized standard error values obtained are referred to as "b" and "c, respectively (Figure 1A). However, getting the values for "a", "b" and "c" is not enough to determine whether the effect mediated by MMP-9, was significant. For this, we needed to carry out the Sobel test, which is a specialized t test. For the Sobel test, the raw regression and standard error values for "a" and "b" were populated in the Sobel calculator. A p value of < 0.05 if obtained would indicate that the effect of $VEGF_{165}b$ on $VEGF_{165}$ was mediated via MMP-9. The important thing that needs to be underscored at this point is, unless the result of the first Bivariate Regression Analysis happens to be significant; the remaining portion of the Mediation test cannot be carried out and the test needs to be annulled.

Results

The demographic characteristics of women from whom placental samples were obtained showed that there was no significant difference in maternal age among the three trimester groups (26.5 ± 6.4, 26.1 ± 6.0 and 28.1 ± 6.5, respectively). Race/ethnicity was self-reported and the distribution pattern showed that women in the study were 62% Hispanic, 26% Black, 3% Caucasian and 9% of other ethnic origins. A total of 61 placental tissues were from women in the first trimester with median gestational age (GA) of $8^{3/7}$ weeks; 42 were from the second trimester with median GA of $16^{6/7}$ weeks; and 92 were from the third trimester with median GA of $39^{4/7}$ weeks.

Variables	1st Trimester	2nd Trimester	3rd Trimester	P value
	Mean Rank	Mean Rank	Mean Rank	
N	61	61	61	
$VEGF_{165}$ (pg/100 mg tissue)	85.57	75.05	116.72	0.0001
$VEGF_{165}b$ (pg/ 100 mg tissue)	67.28	143.85	97.44	0.0001
MMP-9 (ng/ 100 mg tissue)	65.10	85.71	125.42	0.0001

Table 1: *Results of Kruskal Wallis test.*

Variables		2nd Trimester		3rd Trimester	
$VEGF_{165}$ (pg/100 mg tissue)		Test Score	P value	Test Score	P value
	1st Trimester	1107.5	0.244	1874.5	0.001
	2nd Trimester	-	-	1141.5	0.0001
$VEGF_{165}b$ (pg/100 mg tissue)	1st Trimester	313.5	0.0001	1899.5	0.001
	2nd Trimester	-	-	974.0	0.0001
MMP-9 (ng/100 mg tissue)	1st Trimester	956.5	0.029	1123.5	0.0001
	2nd Trimester	-	-	1091.5	0.001

Table 2: *Results of Mann Whitney U test.*

The three proteins, $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 were detected in all 195 chorionic villi samples that were analyzed. Kruskal-Wallis test carried out showed that each of the three proteins differed significantly ($p < 0.0001$) among the three trimester groups (Table 1). Mann Whitney U test which compared the protein expressions between two trimester groups at a time, also showed significant differences between the groups ($p < 0.001$) (Table 2).

Spearman Rank correlation coefficient test showed that in the first trimester, there was no correlation between the studied proteins. However, $VEGF_{165}b$ and MMP-9 proteins were significantly correlated to GAD (rho = 0.280, p = 0.029, rho = 0.290, p = 0.023, respectively). In the second trimester, no correlation was seen either between the proteins or between the proteins and GAD. In the third trimester, a significant correlation was noted between $VEGF_{165}$ and $VEGF_{165}b$ isoforms (rho = 0.368, p = 0.0001); while a significant but negative correlation was seen between $VEGF_{165}$ and MMP-9 protein (rho = -0.306, p = 0.003). In the third trimester, the proteins were not correlated with gestational age in days. When all three trimesters were combined, Spearman's correlation showed significant correlation between $VEGF_{165}$ and $VEGF_{165}b$ (rho = 0.200, p = 0.005). The results additionally showed that all three proteins $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 were significantly correlated with GAD (rho = 0.236, p = 0.001; rho = 0.158, p = 0.028; and rho = 0.475, p = 0.0001, respectively) (Table 4).

Spearman's Correlation	N			$VEGF_{165}$	$VEGF_{165}b$	MMP-9
	92	$VEGF_{165}$	Correlation Coefficient	1.000	0.368	-0.306
			Sig. (2-Tailed)	-	0.0001	0.003
	92	$VEGF_{165}b$	Correlation Coefficient	0.368	1.000	-0.115
			Sig. (2-Tailed)	0.0001	-	0.274
	92	MMP-9	Correlation Coefficient	-0.306	-0.115	1.000
			Sig. (2-Tailed)	0.003	0.274	-

Table 3: *Correlation of chorionic villi proteins in the 3rd trimester.*

Spearman's Correlation	N		$VEGF_{165}$	$VEGF_{165}b$	MMP-9
	195	Correlation Coefficient	0.236	0.158	0.475
		Sig. (2-Tailed)	0.001	0.028	0.0001

Table 4: *Correlation of chorionic villi proteins with gestational age in days (GAD) when three trimester data were merged together.*

Majority of the women in the study were Hispanic (62%) and 26% were Black. When the means ± SD protein expression data were compared between Hispanic (n = 120) and Black (n = 50) ethnic groups by T test; expression of all three proteins were found to be comparable (Table 5). Cesarean section (n = 53) or vaginal delivery (n = 39) did not affect the expression patterns of the three proteins (Table 6). It needs to be pointed out, that the higher number of cesarean sections seen in the study was because it was easier to collect placental samples in a timely manner from a scheduled cesarean section, than from spontaneous vaginal delivery.

	Ethnic Groups	N	Mean	Std. Dev	P value
$VEGF_{165}$ (pg/100 mg tissue)	Black	50	116.99	73.97	
	Hispanic	120	141.05	107.24	.095
$VEGF_{165}b$ (pg/100 mg tissue)	Black	50	313.81	207.88	
	Hispanic	120	303.30	217.87	.768
MMP-9 (ng/ 100 mg tissue)	Black	50	19.43	17.50	
	Hispanic	120	20.19	14.53	.790

Table 5: *Chorionic Villi protein expressions among ethnic groups.*

	Delivery Type	N	Mean	Std. Deviation	P value
$VEGF_{165}$ (pg/100 mg tissue)	C-Section	59	153.87	109.78	
	Vaginal	33	183.23	99.75	0.196
$VEGF_{165}b$ (pg/100 mg tissue)	C-Section	59	285.38	196.63	
	Vaginal	33	315.72	240.95	0.539
MMP-9 (ng/100 mg tissue)	C-Section	59	25.10	15.11	
	Vaginal	33	28.19	14.45	0.337

Table 6: *Chorionic Villi protein expressions as affected by method of delivery.*

The Mediation test was carried out with the 3rd trimester protein data in three steps. In step one, the Bivariate Regression Analysis was carried out to determine the Total effect of $VEGF_{165}b$ on $VEGF_{165}$, which was found to be significant (p = 0.001) (Figure 1B). This allowed the remaining steps of the Mediation test to be carried through. In the 2nd step, a second Bivariate Regression Analysis was carried out with $VEGF_{165}b$ predicting MMP-9. The unstandardized coefficient β (-0.005) and unstandardized standard error (0.007) values obtained were populated in the Mediation Test model as values for "a" (Figure 1C). In the third step, a Multiple Regression Analysis was carried out with both $VEGF_{165}b$ and MMP-9, predicting $VEGF_{165.}$ The unstandardized coefficient β value of -1.785 and unstandardized standard error value of 0.662 for "b"; and unstandardized coefficient β value of 0.207 and unstandardized standard error value of 0.046 for "c" as obtained, were populated in the Mediation test model (Figure 1D and 1E). The Sobel test was then carried out by populating the raw regression and standard error values for "a" and "b" in the Sobel calculator. The Sobel test calculator of K J Preacher was used [18] and the test revealed a p value of 0.4898989 (p > 0.05) (Figure 1F) which confirmed, that the effect of $VEGF_{165}b$ on $VEGF_{165}$ was not mediated via MMP-9. The results of the Multiple Regression Analysis, however, revealed a significant p value (p = 0.0001) for $VEGF_{165}b$ and a significant p value (p = 0.008) for MMP-9 (Figure 1E), which indicated that both $VEGF_{165}b$ and MMP-9 proteins exerted independent effects on $VEGF_{165}$ protein.

Discussion

In this study, the three proteins that we have selected were$VEGF_{165}$, $VEGF_{165}b$ and MMP-9. The rationale in selecting these three proteins was because the multistep process of placental angiogenesis begins with a rise in local angiogenic growth factors, followed by a breakdown of decidual basement membrane that facilitates cytotrophoblast migration and proliferation [19]. $VEGF_{165}$ and $VEGF_{165}b$ are both angiogenic molecules generated from the same transcript. While $VEGF_{165}$ is recognized as a proangiogenic molecule, $VEGF_{165}b$ is recognized as antiangiogenic [5]. Breakdown of decidual basement membrane, and cytotrophoblast migration and proliferation are

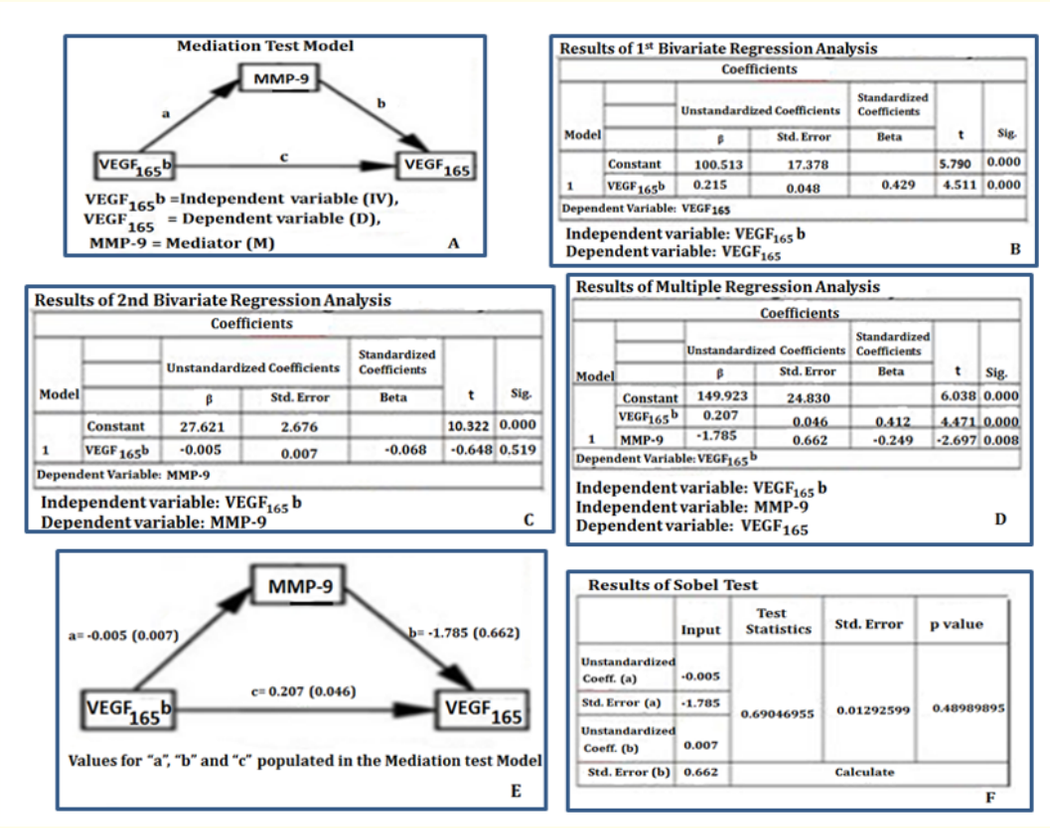

Figure 1: *Mediation test model and mediation test model results.*

integral part of human pregnancy. MMP-9 is recognized as a key effector of ECM remodeling [20]. Moreover, reciprocal embryo transfer experiments have demonstrated the presence of MMP-9 at embryo implantation site and have also reported that MMP-9 contributes to embryonic trophoblast development [12].

Spearman's Rank correlation coefficient test performed, using the combined protein expression values of the three trimester groups revealed, that all three proteins were significantly correlated with GAD (rho = 0.236, p = 0.001, rho = 0.158, p = 0.028, rho = 0.475, p = 0.0001 for $VEGF_{165}$, $VEGF_{165}$b, and MMP-9, respectively). The 195 placental samples in the study were collected from mothers throughout gestation in the first, second or third trimester of pregnancy. The ubiquitous presence of all three proteins throughout gestation and the significant correlation seen between the angiogenic proteins and GAD, emphasize that the three proteins may have regulatory roles in placental development and in normal human pregnancy.

One of the goals of the study was in determining the association between the three proteins in each trimester of normal human pregnancy. Results revealed that in the 1st trimester of normal human pregnancy, there was no correlation between the studied proteins; suggesting that the proteins manifest their regulatory actions independent of each other. In the first trimester, significant correlations were however seen between $VEGF_{165}$b protein and GAD (rho = 0.280, p = 0.029), as well as between of MMP-9 protein and GAD (rho = 0.290, p = 0.023) but not with $VEGF_{165}$ protein and GAD. The presence of $VEGF_{165}$ protein in 61 chorionic villi samples collected from the 1st trimester supports the angiogenic role of $VEGF_{165}$ in human pregnancy [1-3,21,22]. Aside from the angiogenic role of $VEGF_{165}$, Alfaidy., *et al*. have suggested that in the 1st trimester of human pregnancy, $VEGF_{165}$ is involved in the formation and maintenance of the trophoblastic plugs that block the spiral arteries [23].

A failure in the upregulation of $VEGF_{165}$b protein in the plasma, in first trimester of human pregnancy, has been reported in a study to be a predictive marker of preeclampsia, a disorder of pregnancy with vascular dysfunction [24]. The significant positive correlation seen

between chorionic villi $VEGF_{165}b$ protein and GAD, seen in the first trimester of this study, may therefore supports a potential involvement of $VEGF_{165}b$ protein in the development of villous vascular network in human pregnancy. Likewise, the significant positive correlation seen between MMP-9 protein and GAD in the first trimester of this study supports its potential role in the degradation of ECM [25].

In the 2nd trimester, there was no correlation seen either between the proteins or between the proteins and GAD, indicating that all three proteins act independently at this particular phase of human gestation. Our results on the protein expression of $VEGF_{165}$ in the 2nd trimester are consistent with a finding of an immunohistochemical study that reported $VEGF_{165}$ antigen staining to be weaker in mid-gestational placental tissues, compared to the intensity of staining for the protein in the first or third trimester placental samples [26]. To our knowledge, report on $VEGF_{165}b$ protein expression in the second trimester of human pregnancy has not been previously reported. In the present study, a significant up-regulation of $VEGF_{165}b$ protein was noted in the second trimester which can be explained as follows. It is well known that placental development occurs in a relatively low oxygen concentration [27,28]. This environment protects the developing embryo from free radical damage. For normal pregnancy to progress efficiently, the transition from a hypoxic to a normoxic environment is vitally important; and occurs when the trophoblast plugs blocking the spiral arteries are removed, and the maternal-placental circulation becomes established which occurs towards the end of the first trimester [29]. In an *in vitro* study it was shown that placental explants that were subjected to hypoxia-reoxygenation, showed an increase in the concentration and release of proteins, compared to tissues that were maintained under hypoxia alone [30]. In an earlier study we have demonstrated that switching of the placental oxidative status from hypoxic to a normoxic state is a naturally occurring phenomenon that occurs in the second trimester of normal human pregnancy [31]. The increase in $VEGF_{165}b$ protein expression observed in the 2nd trimester of this study could be a consequence of this shift. The increase in MMP-9 protein expression in the second trimester of this study may reflect once again the involvement of MMP-9 in ECM degradation.

In the third trimester of normal pregnancy the upregulation in expression of $VEGF_{165}b$ protein seen in the second trimester no longer prevails, rather a downregulation of $VEGF_{165}b$ protein expression occurs. In this study, 92 term delivered placentas were analyzed for the two isoforms of $VEGF_{165,}$ and the results show a significant positive correlation between the two proteins (rho = 0.368, p = 0.0001). This finding is in agreement with a report that showed simultaneous increase in both isoforms of placental VEGF in 18 human placental samples [13]. The increase in fetal growth and uterine blood flow during the last half of gestation is dependent on a dramatic growth of placental vascular beds [32,33]. The positive correlation seen in this study between the two $VEGF_{165}$ isoforms in the third trimester, may suggest that angiogenic modification of placental vasculature, that allows maximum blood to flow through, may depend on the synergistic action of both VEGF isoforms.

Our findings also accentuate a notable difference between tumor and placental angiogenesis. In human tumors, an up-regulation of $VEGF_{165}$ protein occurs with a proportional drop in $VEGF_{165}b$ levels [5,8,34,35]. The findings of the present study however show that in normal human pregnancy, $VEGF_{165}$ protein expression is maintained at a comparable level throughout gestation. However, the expression of $VEGF_{165}b$ protein shows temporal variations, waxing and waning at different phases of human gestation, yet maintaining a significant positive correlation with $VEGF_{165}$ in the third trimester, when maximum angiogenesis is required. This gestational age-specific expression of both VEGF isoforms could imply that the balance between the two isoforms of $VEGF_{165}$ is more critical for a successful pregnancy outcome.

The third trimester of human pregnancy is a unique phase when fetal and placental developments reach their peaks. However, this well-orchestrated angiogenic event is obligated to come to a halt and the resilient fetal membrane needs to be programmed for rupture, prior to parturition. In this study, MMP-9 protein expression was highest in the third trimester of pregnancy; and the findings are consistent with other investigators who have reported MMP-9 protein levels in the human fetal membrane to be significantly higher at the time of labor [36]. The assay kit that we have used in this study measured both the (92 kDa) pro-MMP-9 and (82 kDa) its active form. We question whether the findings of progressive increase in MMP-9 protein expression with an increase in gestational age as seen in this study would have been different, had we analyzed each form individually, in early pregnancy and at the time of labor.

Our study has limitations: In the study, mRNA expressions of the chorionic villi proteins were not measured. Given the wide variations in the three protein expressions seen in the study, linking mRNA and protein expression would have been desirable. Furthermore, inclusion of other placental cell types e.g. extravillous cytotrophoblasts or Hofbauer cells in the study, and evaluation of other potential

contributors of placental angiogenesis would have been advantageous as well. Moreover, ethnic groups in the study were not uniformly represented. The strengths of our study are as follows: (1) we have isolated chorionic villi samples of the placenta, free of the chorionic and the basal plates, particularly to focus primarily on the cytotrophoblasts. Since the majority of placental cellular mass consists of trophoblast cells, the increase in protein expressions observed could be attributed largely to trophoblast cells. (2) The sample size of the study is large (n = 195) and we have included 42 placental samples from the second trimester. (3) The EIA methods used provided objective quantification of the protein levels, as opposed to previous studies which relied on histochemical and/or cell culture methods. (4) The EIA methods applied monoclonal antibodies to human $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 proteins that did not cross react with other human proteins based on manufacturer's suggestions. The methods used were sensitive that allowed the detection of $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 protein as low as 31.3 pg/ml for $VEGF_{165}$, 62.5 pg/ml for $VEGF_{165}b$ and 31.3 pg/ml for MMP-9, respectively.

Sobel test results showed that the effect of $VEGF_{165}b$ on $VEGF_{165}$ was not mediated via MMP-9 (Figure 1F). However, the results of Multiple Regression Analysis revealed that both $VEGF_{165}b$ and MMP-9 exert independent effects on $VEGF_{165}$ (Figure 1D). The significant negative correlation seen between $VEGF_{165}$ and MMP-9 proteins (rho = -0.306, p = 0.003) suggests that the contribution of MMP-9 protein in placental angiogenesis may include other functions in addition to degradation of ECM.

Conclusion

The various events of human pregnancy**:** the angiogenic process, the invasion of the decidua and the invasive characteristics of the cytotrophoblasts are precisely regulated; and the boundaries of invasion are also strictly defined [37]. The constant modification in protein expression of $VEGF_{165}b$ protein throughout gestation as seen in the present study indicates that $VEGF_{165}b$ protein is more stringently controlled and is an essential component of normal human pregnancy and placental development. The significant negative correlation seen between $VEGF_{165}$ and MMP-9 proteins in the 3rd trimester suggests that the contribution of MMP-9 protein in human pregnancy may include other functions in addition to degradation of ECM. The correlation seen between the anti-angiogenic protein $VEGF_{165}b$ with $VEGF_{165}$ throughout gestation; and the negative association seen between MMP-9 and $VEGF_{165}$ proteins in the 3rd trimester suggest that both $VEGF_{165}b$ and MMP-9 proteins in human pregnancy may have a physiological role in restraining over expression of $VEGF_{165}$; which if left un-checked, could lead to pregnancy-related complications.

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Conflict of Interest

The authors declare no conflicts of interest with respect to the research, authorship and/or publication of this article.

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