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Assessment of heparanase and heparin-binding growth and angiogenesis factors in the uterine cavity fluid in women with impaired reproduction

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ABSTRACT

Introduction. Numerous reports lead to conclusion that either the absence or insufficient amounts of heparanase and heparin binding growth factors on the luminal surface of the epithelium in the endometrium may be associated with impaired reproduction. The aim of this study was to assess the suitability of the fluid from the uterus to predict reproductive disorders.

Material and methods. The group consisted of 32 women with 2 or more consecutive unexplained miscarriages, and 33 idiopathic infertility patients; the control group comprised 22 women with normal reproductive potential. Concentration of the studied factors was assayed by ELISA in uterine fluid.

Results. The uterine flushings from women with two or more consecutive miscarriages showed significantly lower concentrations of HPA1 ($p < 0.001$) compared to the control group and infertile patients. In contrast, we didn't observe statistically significant differences of concentration of HB-EGF, VEGF, FGF2 in the studied groups. Statistically significant correlations were obtained between the levels of HPA1 and growth factors in all groups $p < 0.05$. The ROC curve was used to test the diagnostic value of HPA1. With a cut-off point of 8.56 U/L for HPA1 levels, we achieved 58.6% sensitivity and 84.6% specificity in the detection of women with recurrent miscarriage compared to fertile controls and infertile women combined. The area under curve (AUC) value was 0.751.

Conclusions. The procedure for determining the concentrations of HPA1, HB-EGF, VEGF, FGF2 by ELISA in fluids derived from the uterine cavity is insufficient to predict either success of reproduction or reproductive disorders.

Keywords: heparanase, HPA1, heparin binding growth factors, HB-EGF, VEGF, FBF2, recurrent miscarriage, infertility, uterine fluid.

Introduction

Implantation of the blastocyst and invasion of trophoblasts are connected with changes of the extracellular matrix (ECM). Components of the ECM structure and enzymes that enable its digestion are responsible for this process. The extracellular matrix not only maintains the tissue structure, but is also responsible for its remodeling, angiogenesis and establishment of links between the trophoblast and decidua [1].

One of the main components of polysaccharide-protein coat (glycocalyx) is heparan sulfate (HS). Its presence has been demonstrated in the endometrium, the decidua and trophoblast invasion area [2]. Heparan sulfate acts as an integrating molecule, not only bonding tissue and cells, but also acting as a binding site and releasing angiogenic growth factors, including heparin-binding EGF-like growth factor (HB-EGF), vascular endothelial growth factor (VEGF), the fibroblast growth factor family (FGF's), and bone morphogenetic proteins (BMP's). Binding of these factors with the HS as a coreceptor not only increases their concentration, but also modulates their effects on target cells [3, 4]. On the other hand, ECM stimulates both angiogenesis by the trophoblast and increased release of the factor-HS complex [5].

Heparanase (HPA1) – endo-β-D-glucuronidase is responsible for the HS degradation process, cell migration and release of heparan sulfate-binding agents. It cleaves the sugar chain at specific sites, generating short oligosaccharides consisting of 10–20 sugar moieties [6]. Reduction of heparanase expression *in vitro* by nonenzymatic methods affects angiogenesis and impairs hemostasis by reducing the expression of VEGF and tissue factor (TF) [7]. It is not known whether an analogous situation occurs in the endometrium.

There are relatively few reports about the expression and role of heparanase in processes related to endometrial receptivity and embedding. In mammals, including primates and human, HPA1 expression has been found in the endometrium and placenta, and a mouse model has demonstrated its central role in the process of embedding [6–12].

In studies of mouse blastocyst implantation, it has been shown that the synthesis of HB-EGF is closely related to the implantation site. In the mouse endometrium, production of HB-EGF occurs in the form of a protein associated with endometrial epithelium. This factor binds to the HS present on the surface of the blastocyst. *In vitro* studies have also shown that endometrial HB-EGF acts as a factor for blastocyst growth by binding to receptors for epidermal growth factors (EGF) – HER1, HER4 [13, 14]. It should be noted, however, that the direct comparison of test results of implantation in animal models to humans may lead to erroneous conclusions. This is due to the specificity of biochemical implantation and the physiology of pregnancy, especially in mammalian species.

Also, other well-known growth and angiogenesis factors, e.g. FGF2 and VEGF, represent HS binding domains. So far, numerous studies have demonstrated an important role of both factors in the process of implantation and pregnancy development, both in humans and in other mammals [15–18].

There is a general agreement that the synthesis of endometrial HB-EGF, FGF and VEGF is controlled by sex hormones. The expression of these factors has been repeatedly confirmed not only in the stroma, but also in the glandular and luminal epithelium of the endometrium. The proteins are localized in the uterus secretory phase [16, 19, 20]. These reports lead to conclusion that either the absence or insufficient amounts of growth factors on the luminal surface of the epithelium of the endometrium may be associated with impaired reproduction in humans.

A common mechanism for controlling the expression of growth factors, as well as our previous observations, led us to conclusion that a positive correlation is also noticeable with respect to the concentrations of the HPA1, HB-EGF, FGF2 and VEGF proteins in the uterus. Therefore, the aim of this study was to assess the suitability of the fluid from the uterus to predict reproductive disorders. We studied the uterine cavity fluid protein concentrations of HPA1, HB-EGF, FGF2 and VEGF, which are potential markers of endometrial receptivity, and explored the correlations between HPA1 and HB-EGF, and FGF2 and VEGF in both groups of women with impaired reproduction, and a group of healthy women, i.e. the control group.

Material and methods

Patients and controls

Uterine flushings have been collected from the women hospitalized in the Division of Reproduction, Department of Obstetrics and Gynecology, University of Medical Sciences in Poznań. The study included 87 reproductive-age women. The group consisted of 32 women with 2 or more consecutive unexplained miscarriages, and 33 idiopathic infertility patients; the control group comprised 22 women with normal reproductive potential. The women in the miscarriage group had had at least two consecutive unexplained miscarriages in the first trimester of pregnancy. This group included 20 women with diagnosed recurrent miscarriage (e.g. 3 consecutive miscarriages). The mean duration of infertility in idiopathic infertility patients was 3 years (range: 1–5 years). The control group consisted of women that had at least one child, regular menses, and were without anatomical or functional lesions within the endometrium. The study protocol was approved by the Karol Marcinkowski Medical University bioethical committee, and the patients signed an informed consent form. No patients in either the study or control group had taken any hormonal preparations for at least three months prior to the study. The exclusion criteria were: current use of hormonal contraception, and any serious diseases. The age of women, number of miscarriages and parity are presented in **Table 1**.

Uterine fluid collection

Seven to nine days after ultrasound confirmation of ovulation (the putative implantation window), women underwent uterine flushing followed by endometrial biopsy with a Pipelle. Endometrial tissue samples were sent to histopathology for hematoxylin and eosin staining and assessment according to Noyes and Hertig criteria. The procedure involved placing a sterile catheter in the os of the uterine cervix, which was connected to

Table 1. Clinical characterization of the study groups

	n	Age		Number of miscarriages		Infertility duration (years)		Parity	
		Median	Span	Median	Span	Median	Span	Median	Span
Miscarriage group	32	32	23–41	3	2–5	NA		0	
Two consecutive, unexplained miscarriages	12	33	27–41	2	2				
Clinically diagnosed recurrent miscarriage	20	32	23–40	3	3–5				
Idiopathic infertility	33	33	25–40	NA*		3	1–5	0	
Control group	22	39	19–43	NA		NA		1	1–3

** not applicable*

a 20 ml syringe filled with 3.5 ml of sterile isotonic solution of sodium chloride (0.9% NaCl). The fluid was slowly infused into the uterine cavity and then gently aspirated in a repetitive fashion creating a turbulent flow (to achieve homogenous distribution of soluble factors). Next, the fluid was drawn into the syringe, which was transported to the laboratory, centrifuged and the supernatant was frozen at –20°C for further examination.

Protein assessment

Total protein concentration was estimated with a Pierce™BCA Protein Assay Kit (Thermo Scientific, USA). For assessment of the VEGF, FGF2 was assayed by immunoenzymatic tests (enzyme-linked immunosorbent assay; ELISA) which are commercially available (R&D Systems, Minneapolis, USA). HPA1 was determined using an ELISA kit, USCN (Wuhan, China). For the determination of HB-EGF the Sigma Aldrich ELISA kit was applied (St Louis, USA). All assays were performed according to the manufacturers' instructions. For plate reading, a Dynex Technologies MRX reader was used, (Chantilly, USA). The results were normalized to total protein concentration. All analyses were performed in the Tissue Culture Laboratory in Gynecological and Obstetric Clinical Hospital in Poznan, Poland.

Statistical analysis

For statistical analysis, SigmaStat3.5 software was used. The analysis of the results was based on the Kruskal-Wallis One Way Analysis of Variance on Ranks. Correlation of the studied proteins in the uterine flushing was performed using the Spearman rank sum test. P value less than 0.05 was considered to be statistically significant.

To test whether the obtained statistically significant values of the studied factors could be used for diagnostic purposes, we used Receiver Operating Characteristic (ROC) curves (Statistica v.10). The ROC curve displays diagnostic accuracy expressed in terms of the true-positive rate against the false-positive rate for different cut-off points [21].

Results

The uterine flushings from women with two or more consecutive miscarriages showed significantly lower concentrations of HPA1 ($p < 0.001$) compared to the control group and infertile patients. Concentrations of VEGF, FGF2 and HB-EGF did not differ between the studied groups and healthy controls. A detailed comparison of the concentrations of the studied factors is summarized in **Table 2**.

Using the Spearman rank test, we analyzed the relationships between the concentrations of HPA1 and growth factors in the uterine flushing. Statistically significant correlations were obtained between the levels of HPA1 and HB-EGF ($p = 0.015$) and HPA1 and VEGF ($p = 0.04$) in the control group. In the group of women

Table 2. Concentrations of the studied proteins in uterine fluid

Group	Control group		Miscarriage group		Idiopathic infertility		p
	Mean (SD)	Median [Span]	Mean (SD)	Median [Span]	Mean (SD)	Median [Span]	
HPA1 [U/L]	13.45 (± 5.63)	13.21 [2.14–22.3]	9.24 (± 5.15)	8.06 [2.86–23.47]	15.97 (± 8.54)	12.85 [6.4–38.82]	< 0.001*
HB-EGF [pg/ml]	168.88 (± 106.27)	141.36 [38.73–420.6]	111.27 (± 48.63)	104.5 [39.85–264.14]	128.79 (± 93.44)	110.75 [0–408.71]	ns**
VEGF [pg/ml]	110.87 (± 170.78)	72.93 [12.07–850.0]	109.75 (± 206.48)	35.48 [0–1006.0]	167.58 (± 206.54)	66.52 [0–731.61]	ns
FGF2 [pg/ml]	126.03 (± 104.94)	100.72 [14.62–517.08]	104.52 (± 110.8)	82.37 [0–490.28]	254.63 (± 323.43)	124.17 [0–1174.71]	ns

** Kruskal-Wallis One Way Analysis of Variance on Ranks with All Pairwise Multiple Comparison Procedures (Dunn's Method)*
*** Not significant*

with two or more consecutive miscarriages, we obtained correlations between HPA1 and VEGF (p = 0.009). In the infertile women group, the concentration of those HPA1 positive correlated with HB-EGF (p = 0.043), but we obtained negative correlations between the concentrations of HB-EGF and VEGF (p = 0.011). Detailed results of the assessment of the correlations in the uterine flushing are summarized in **Table 3**.

As mentioned above, only the HPA1 concentration in the uterine fluid obtained from women with recurrent miscarriages was significantly lower than in women from the control group and infertile women. To test the diagnostic value of HPA1 measurements for the detection of states of recurrent miscarriages, we utilized ROC curves. With a cut-off point of 8.56 U/L for HPA1 levels, we achieved 58.6% sensitivity and 84.6% specificity in the detection of women with recurrent miscarriage compared to fertile controls and infertile women combined. The area under curve (AUC) value was 0.751.

Table 3. Correlations between the levels of the heparanase and heparane-related proteins in the uterine flushing

	Control group			Miscarriage group			Idiopathic infertility		
	VEGF	HB-EGF	FGF2	VEGF	HB-EGF	FGF2	VEGF	HB-EGF	FGF2
HPA1									
Correlation coefficient	0.44	0.512	-0.231	0.478	0.101	0.359	0.227	0.378	0.292
p	0.0402	0.0149	ns*	0.009	ns	ns	ns	0.0429	ns
VEGF									
Correlation coefficient		-0.0096	-0.163		0.0875	0.349		-0.445	0.236
p		ns	ns		ns	ns		0.011	ns
HB-EGF									
Correlation coefficient			0.115			-0.00384			-0.17
p			ns			ns			ns

** Not significant*

Discussion

Fluid from the uterine cavity contains cytokines, growth factors responsible for receptivity and implantation. It has been demonstrated that fluid obtained from healthy women during the implantation window stimulated the *in vitro* growth of blastocysts and endometrial epithelial cell adhesion to fibronectin and collagen IV, extracellular matrix components, while fluid derived in the same way within the follicular fluid phase of the menstrual cycle did not show such properties [22]. Aspiration of fluid from the uterine cavity represents a minimally invasive form of testing, during which there is no damage to the endometrium. This method allows for quantitative and qualitative assessment of soluble and deposited factors on the surface of the endometrium. Some researchers question the usefulness of diagnostic fluid from the uterus. Hamilton et al. showed that the fluid obtained by aspiration of saline into the uterus contains varying amounts of protein. He also demonstrated that without the assistance of ultrasound it is not possible to assess the extent to which introduced salt solution fills the cavity of the uterus. Moreover, the results may be unreliable if it comes to aspiration of blood or mucus [23]. However, numerous studies have indicated the usefulness of fluid from the uterine cavity in the diagnosis of reproductive disorders [24–27] and the monitoring of therapy [28].

The growth factors we studied are secreted on the surface of the endometrium and associated with the glycocalyx, where they are released with the participation of HPA1 [4]. Their impact on the processes associated with endometrial receptivity and decidualization and implantation has been repeatedly confirmed in mammals including humans. The primary mechanism of the action of heparanase is to change the structure of the glycocalyx by removing the HS. This process facilitates the mutual contact of the embryo and the decidua. It has also been reported that in the process of implantation heparanase molecules can perform adhesive functions and can even act as a transcription factor [29]. During the implantation window, HPR1 can assist embryo implantation, and during pregnancy HPR1 acts as an angiogenesis-stimulating factor, especially as a catalyst for ECM changes. One of the mechanisms that control the activity of heparanase may be a change in pH. It has been revealed that the maximum catalytic activity of HPR1 is achieved at pH 5.0, while the pH of the sexual cycle of the uterine cavity is between 6.6 and 7.6. Such a pH promotes the adhesive properties of heparanase, even in the form of proenzyme, which is catalytically inactive [30, 31]. *In vitro*, the presence of heparanase in the external environment stimulates the embryo implantation process [32].

Our results of the determinations in the uterine cavity fluid showed that in women with two or more miscarriages HPA1 concentrations are lower than both in the control group and in the group of infertile women. This observation suggests that in humans endometrial heparanase is not only a factor responsible for implantation, but above all for the maintenance and development of the pregnancy. We have also observed that in fluid from the uterine cavity in the control group heparanase concentrations correlated positively with the concentrations of HB-EGF and / or VEGF. This observation is consistent with previous observations that indicate that the expression of both HPA1 and HB-EGF and VEGF are under a common expression mechanism controlled by sex hormones, and the severity of the degree of their expression correlates with changes in the cycle of the sexual endometrium [33].

It has been proven that the maximum level of expression of HB-EGF in human endometrial epithelium occurs at implantation, and the secretion of HB-EGF protein has been confirmed in fluid from the uterine cavity [19, 34]. Lessey et al. demonstrated that the HB-EGF in an autocrine manner stimulates the production of endometrial epithelium integrins, LIF and HOXA10 [35]. In turn, Stavreus-Evers et al. reported a close relationship between the synthesis of HB-EGF in the epithelium of the endometrium and the maximum of pinopod development at implantation. Moreover, HB-EGF located on the surface of pinopods lends itself as a good marker for the implantation window [36]. In our previous studies using the WB method, endometrial biopsies have shown a lower level of expression of HB-EGF proteins in women with at least two unexplained miscarriages compared to women with normal reproductive potential. At present, although HB-EGF concentrations in fluid from the uterine cavity are at their highest values in the group of women without reproductive failures, these levels did not differ with respect to the HB-EGF concentrations in the groups of women with impaired reproduction.

The fluid from the uterine cavity has also been evaluated for the VEGF concentration. In an animal model, Zhang et al. demonstrated VEGF expression in the epithelium of the endometrium and during implantation, pointing to an important role for this agent in the process of implantation and decidualization [37]. Based on a large selection of studies, it may be seen that many, especially older publications present divergent views as to the expression of VEGF in the sexual cycle [18, 38]. Despite this, the authors seem to agree on the role of VEGF in the early stages of pregnancy. In new research, Lash et al. showed that, in women with recurrent miscarriage, levels of VEGF were significantly lower in the glandular and luminal epithelium and in the vessels of the endometrium in relation to control groups [20]. Based on their results, Seo et al. proposed the use of VEGF as a predictor for pregnancy success in In Vitro Fertilization Treatment (IVF) [39]. In turn, Hannan et al. reported a lower concentration of VEGF in fluid from the uterine cavity in women with infertility and showed that VEGF is a key component of the fluid from the uterus in implantation and that it is responsible for the adhesion of epithelial cells of the endometrium and the blastocyst [22]. Although in our study the median value was the lowest in the group of patients with two or more abortions, similar to the case of HB-EGF, we did not obtain any statistically significant differences. We did not observe any difference in the concentrations of VEGF in the group of infertile women and the control group. In the group of infertile women, we obtained an inverse correlation between the concentrations of HB-EGF and VGFA. It cannot therefore be ruled out that one of the causes of infertility may be affected by the relative proportions between factors potentially conducive to implantation.

It has been shown that expression of VEGF and FGF is also stimulated by hCG [16]. Zimmermann et al. showed that the endometrium is capable of synthesis of hCG [41, 42]. FGF in the epithelial cells of the endometrium is synthesized during the entire sexual cycle. The maximum of its expression falls in the second phase of the cycle and remains at a high level in the first trimester of pregnancy. On the basis of ERK 1/2 acting as a MAP kinase [42], FGF receptors increase the binding of epithelial cells to fibronectin and collagen IV with ECM components of blastocyst trophoectoderm [43, 44]. The studied uterine cavity fluid FGF concentrations did not differ between the two groups.

In our previous studies, we observed lower levels of HPA1 expression in endometrial biopsies and lower levels of HB-EGF protein in women with recurrent miscarriage. The molecular studies of the endometrial sections showed correlations between the expression of HPA1 and HB-EGF at both the mRNA and protein levels [45]. In contrast, the present study on the uterine cavity fluid from women with reproductive disorders showed that among the four factors only HPA1 concentration exhibited a significantly lower concentration in women with two or more abortions. During the study, the level of HPA1 also correlated with HB-EGF and / or VEGF. Based on the analysis of the ROC curves, we can conclude that the determination of the concentration of HPA1 fluid from the uterine cavity does not meet

the criteria required to be considered a good predictor for this procedure. It should also be noted that the investigated factors are only part of the biochemical mechanisms responsible for the receptivity of the endometrium. Therefore, we do not have information on the level of receptors for the growth factors studied, nor do we have expression profile information on the components of the embryo.

Conclusions

The aim of this study was to search for predictors of endometrial receptivity in material non-invasively acquired in the non-conceptional cycle, thus enabling the possible conception. The procedure for determining the concentrations of HPA1, HB-EGF, VEGF, FBF2 by ELISA in fluids derived from the uterine cavity is insufficient to predict either success or reproductive disorders.

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Conflict of interest statement
The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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