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Lack of varied endometrial expression of proprotein convertase 6 in infertile women with minimal grade endometriosis and idiopathic infertility

Brak różnic w ekspresji konwertazy proproteinowej 6 u kobiet niepłodnych z endometriozą minimalnego stopnia i u kobiet z niepłodnością idiopatyczną

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Abstract

Objective: *Proprotein convertase 6(PC6) is known to be the key enzyme involved in the transformation of many hormones, cytokines and their receptors into their active forms. Experimental in vitro studies have also proven that lack of PC6 in the endometrium prevents decidualisation. Therefore in our study we have aimed at determining whether infertility in some patients might be attributable to decreased expression of PC6.*
Material and methods: *With the use of RealTime PCR we have studied the expression level of PC6 in receptive phase endometria from 36 idiopathic infertile patients, 26 infertile patients with minimal grade endometriosis and compared those results with fertile, age-matched controls. The endometria were collected 7-9 days after ovulation.*
Results: *There were no statistically significant differences regarding the expression of PC6 in endometria from patients with idiopathic infertility, infertile patients with endometriosis and controls.*
Conclusions: *Since there is no detectable difference in PC6 expression, the decreased expression of PC6 is unlikely to cause infertility.*

Key words: **infertility** / **endometriosis** / **proprotein convertase** / **endometrium** /

Streszczenie

Cel pracy: *Konwertaza proproteinowa 6 (PC6) jest kluczowym enzymem biorącym udział w przekształceniu wielu prohormonów, cytokin i ich receptorów w aktywne formy. Badania eksperymentalne in vitro dowiodły, iż brak PC6 uniemożliwia przemianę doczesnową w endometrium. Naszym celem była ocena czy u pacjentek niepłodnych czynnikiem wywołującym niepłodność może być zaburzona ekspresja PC6.*
Materiał i metoda: *Stosując RT-PCR zbadaliśmy poziom ekspresji PC6 w fazie receptywnej endometrium u 36 kobiet z niepłodnością idiopatyczną, 26 pacjentek z endometriozą minimalną oraz porównaliśmy te wyniki z płodnymi pacjentkami dobranymi pod względem wieku. Endometrium zostało pobrane 7-9 dni po owulacji.*

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Wyniki: *Nie stwierdziliśmy statystycznie znamiennych różnic w ekspresji PC6 w endometrium z grupy z niepłodnością idiopatyczną, niepłodnymi pacjentkami z endometriozą a grupą kontrolną.*
Wnioski: *Wydaje się, że zaburzona ekspresja PC6 nie jest przyczyną niepłodności.*

Słowa kluczowe: **niepłodność** / **endometrioza** / **konwertaza proproteinowa** / / ***endometrium*** /

Introduction

Idiopathic infertility and infertility associated with minimal grade endometriosis present physicians with a diagnostic, as well as therapeutic dilemma [1]. Idiopathic infertility occurs in about 15% of patients suffering from infertility, while different stages of endometriosis affect about 30% of them [2, 3].

Since the cause of idiopathic infertility remains unknown, the treatment tends to be empirical at best, including ovarian stimulation, intrauterine inseminations, and finally assisted reproductive techniques [4]. In case of patients with minimal endometriosis there have been numerous possible causes of the accompanying infertility, including immunologic imbalances, hormonal changes and anomalies within the eutopic endometrium [5, 6]. Therefore, the treatment choices are similar to those of idiopathic infertility and include in-vitro fertilization-embryo transfer (IVF-ET) cycles, though in patients with minimal endometriosis the results of IVF cycles are invariably poorer than those with e.g. male factor infertility [7]. The success of IVF cycles depends on a whole range of factors, mainly the ovum and embryo quality atraumatic transfer of an embryo and finally, the receptivity of the endometrium [7, 8, 9]. While there are ways to assess the quality of the oocytes and the embryo, including pre-implantation genetic diagnostics, the implantation rate remains quite low [10].

Thus, there exists an obvious need to study the endometrial receptiveness. Routine histological assessment is no longer recommended as part of diagnostic tests for infertility [11]. Attention has been drawn to molecular and genetic aspects of endometrial receptivity [12]. One study, aimed at finding new endometrial targets for contraception, concluded that among many enzymes present in the human endometrium there is one that seems to be indispensable for a proper decidualisation of stromal cells [13], namely proprotein convertase 6 (PC6). It is a serine protease that is structurally related to bacterial subtilisins [14]. Their function is the conversion of various inactive peptide hormones, enzymes and growth factors into their active forms [15]. Since PC6 acts directly within the endometrium, it regulates the timely and orderly conversion of many substances, a process which is critical for organized events that occur during the creation of window of implantation [16]. There are two forms of PC6 in mice: membrane-bound and soluble, while in humans the presence of only the soluble form has been confirmed [17, 18]. Researchers found that in mice PC6 is up-regulated at the time of the embryo implantation primarily in cells surrounding the embryo [19]. Blocking of PC6 production by antisense oligonucleotides resulted in total inhibition of the implantation [16]. In human endometrium PC6 is present throughout the cycle, with increased expression during the midsecretory phase [13]. Similarly, in humans the blocking of PC6 activity stops the decidualisation process, thus preventing the preparation of the endometrium for the embryo attachment [20, 21]. These studies are also supported by work from Kao et al. who have shown an increase in the expression of PC6 around the time of implantation, revealing its vital role in human reproduction [12]. That is why we have decided to check whether patients with both idiopathic infertility and minimal endometriosis associated infertility exhibit local, i.e. endometrial, decreased PC6 expression which might explain the cause of infertility in those patients and, if so, how much decreased expression of PC6 might be attributed to infertility.

Materials and methods

The study was conducted in the Division of Reproduction, Department of Obstetrics, Gynecology and Gynecological Oncology of K. Marcinkowski Medical University in Poznan, Poland, between January 2006 and February 2007. Only infertile patients with all diagnostic tests negative or those patients who had minimal endometriosis were included in the study. Each couple underwent the following tests: semen analysis, ovulation tracking, hysterosalpingography, hormonal studies and laparoscopy with hysteroscopy. We had 36 patients with idiopathic infertility and 26 infertile patients with minimal grade endometriosis diagnosed by means of laparoscopy and histology, following the American Fertility Society recommendations [22]. The mean duration of infertility in both groups was 3.4 years (1-5 years) and 3.2 years (1.2-6 years), respectively. Furthermore, 24 patients from the same age group, with at least one child, without miscarriages and with negative history of infertility and endometriosis were enrolled in the study. Those patients were admitted to the hospital due to non-endometrial diseases, and were asked to donate endometrium for the purpose of the research. The study protocol was approved by the local ethical committee and the patients signed informed consent forms. None of the patients in all groups, including the controls, had taken any hormonal preparations for at least three months prior to the study.

Collection of samples and RNA isolation

All patients had a biopsy sample obtained 7-9 days after ovulation, confirmed by ultrasound follicular tracking. The endometrial sample was placed in RNAlater™ buffer from Qiagen (Hilden, Germany) and frozen until extraction.

The isolation of total RNA was done with the use of RNAeasy Mini Kit (Qiagen, Germany). QiaShredder columns were used for homogenization (Qiagen) following the manufacturers instructions. The total mRNA was treated with QantiTect Reverse Transcription (Qiagen) to acquire cDNA.

Primers design and qPCR reaction

RNA specific primers for RealTime PCR were created with the Primer3 software (http://frodo.wi.mit.edu/primer3) based on mRNA (NM_006200) sequence from NCBI Gene database (http://www.ncbi.nlm.nih.gov/). The thermodynamic features of the designed primers were analyzed with the use of OligoAnalyzer1.2 software first, followed by the specificity of constructed primer check against BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained results confirmed the specificity of the designed primers for the human PC6 gene transcript: GAPDH - forward: ACAGTCAGCCGCATCTTCTT and reverse: ACGACCAAATCCGTTGACTC and for PC6 - forward: TGCAGTGACAATACACATCC and reverse: TCTCTCAATTCCGTCATCC.

The resulting DNA was checked on agarose gel for the confirmation of the set primers specificity and additionally, secondly derivative analysis melting curve product of these primers set Real Time PCR reaction was used to confirm the specificity.

The resulting cDNA samples were used as matrix for RealTime PCR which was conducted in duplicate, on RotorGene 3000 RealTime thermocycler (Corbett Research). The optimized protocol was used with the mastermix including HotStart polymerase (DyNAmo HS SYBRGreen qPCR Kit from Finnzymes, Espoo, Finland). Thermal profile was 95°C for 15min; 94°C for 10 sec; 55°C for 25 sec; 72°C for 30 sec and at 79°C first data acquisition. The thermocycler was set for 40 runs. After all runs completed, 72°C for 10min cycle was accomplished and next the melting curve was established by changing the temperature from 72°C to 95°C, increasing by 0,5°C with each step, which in turn allowed for calculation of the second derivative to confirm uniformity of the resultant products of amplification. The expression of PC 6 was compared to a reference gene that is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whose expression in cells is universally considered to be constant throughout the whole menstrual cycle.

In order to determine the efficiency of the qPCR reaction for both investigated transcripts, 10 subsequent dilutions of the linear DNA molecule were used. Triplicate qPCR reaction was performed for each dilution. On the basis of the results the thermocycler PCR analysis software automatically determined the efficiency of both reactions and constructed the standard curve which, in turn, was used to determine the transcript level in the tests. They were analyzed in duplicate to correct the standard curve.

The transcription level of PC6 was shown in relation to the GAPDH and calculated using REST®2005 analyzing software v.1.9.9 from Corbett Research, which also automatically calculates the differences between studied groups. The mean GAPDH expression levels did not differ significantly among the groups.

Results

There was no difference in the expression of PC6 between all infertile patients (idiopathic infertility and endometriosis) and controls. The results are presented in Table I.

There was also no statistically significant difference in PC6 expression in endometria from patients with idiopathic infertility and controls, minimal endometriosis and controls. The results are presented in Tables II and III.

Furthermore, there was no difference between the expression of PC6 in patients with idiopathic infertility vs. minimal endometriosis. (Table IV).

Discussion

To the best of our knowledge this is the first study in the literature comparing the in vivo expression of PC 6 in fertile and infertile patients. Infertile patients require advanced diagnostic and therapeutic methods to improve their chances for pregnancy.

In natural cycles the monthly fecundability remains at the level of 30% [23]. We know that a large number of these early loses might be attributed to embryo defects [10]. However, one needs to bear in mind that even with the use of Preimplantation Genetic Screening which effectively rejects abnormal embryos, the implantation rate still remains low [10].

Part of the blame for such poor results might be attributed to the faulty implantation caused by poor endometrial receptivity at the time of embryo transfer [9]. Any disturbances in the window of implantation might lead to the rejection of the normal embryo and result in infertility. In our paper we have chosen to assess the role of PC6 as the potential cause of infertility. The choice was based on two major facts. Firstly, that PC6 is involved in the transformation of many substances into their active form. Many enzymes, cytokines, growth factors and hormones are dependant on the proper expression and function of PC6 [15]. Potentially, many of the factors taking part in the creation and maintenance of receptivity period of the endometrium might be influenced by aberrant expression of PC 6. Secondly, that it has been proven in a mouse that inhibiting the expression of PC6 leads invariably to a complete block of implantation [16]. Patients with idiopathic infertility and those with minimal grade endometriosis may have, as yet undiscovered, defects within the eutopic endometrium that lead to implantation failures. Therefore, in our present study we decided to assess the expression profiles of PC6 in those two subgroups of patients to determine whether it might be due to infertility.

In our current study we were unable to demonstrate any differences between the expression level for PC6 between patients with idiopathic infertility, minimal grade endometriosis and fertile population. Based on our research we came to the conclusion that infertility caused by decreased expression of PC6 is quite an uncommon phenomenon for several reasons. Conclusively, PC6 is a very important enzyme in humans. It regulates many biological pathways, including those in the gastrointestinal tract, aorta, kidneys, lungs and many others; therefore, lack of PC6 might be incompatible with life [16], though a local (endometrial) decrease in the expression might be expected.

Table I. The reaction efficiencies, expression results in endometria and statistical evaluation of proprotein convertase 6 (PC6) differences between the entire infertile group and controls.

Gene	Type	Reaction Efficiency	Expression	Standard Error	95% C.I.	P(H1)
GAPDH	REF	0.87	1.000	0.103 - 9.349	0.001 - 8 369.628	1.000
PC6	TRG	0.85	0.500	0.075 - 3.236	0.014 - 72.313	0.081

P(H1) - Probability of an alternative hypothesis claiming that the difference between study and control groups is merely coincidental.
GAPDH-glyceraldehyde- 3-phosphate dehydrogenase. TRG – Target; REF – Reference; C.I, - Confidence Intervals

Table II. The reaction efficiencies, expression results in endometria and statistical evaluation of proprotein convertase 6 (PC6) differences between idiopathic infertility and controls groups.

Gene	Type	Reaction Efficiency	Expression	Standard Error	95% C.I.	P(H1)
GAPDH	REF	0.87	1.000	0.111 - 10.792	0.001 - 9 809.380	1.000
PC6	TRG	0.85	0.604	0.094 - 3.898	0.016 - 74.233	0.407

P(H1) - Probability of an alternative hypothesis claiming that the difference between study and control groups is merely coincidental.
GAPDH-glyceraldehyde- 3-phosphate dehydrogenase. TRG – Target; REF – Reference; C.I, - Confidence Intervals

Table III. The reaction efficiencies, expression results in endometria and statistical evaluation of proprotein convertase 6 (PC6) differences between infertile patients with minimal endometriosis and controls.

Gene	Type	Reaction Efficiency	Expression	Standard Error	95% C.I.	P(H1)
GAPDH	REF	0.87	1.000	0.090 - 8.155	0.017 - 6 419.122	1.000
PC6	TRG	0.85	0.331	0.049 - 1.954	0.009 - 45.169	0.204

P(H1) - Probability of an alternative hypothesis claiming that the difference between study and control groups is merely coincidental.
GAPDH-glyceraldehyde- 3-phosphate dehydrogenase. TRG – Target; REF – Reference; C.I, - Confidence Intervals

Table IV. The reaction efficiencies, expression results in endometria and statistical evaluation of proprotein convertase 6 (PC6) differences between patients with idiopathic infertility and minimal endometriosis group..

Gene	Type	Reaction Efficiency	Expression	Standard Error	95% C.I.	P(H1)
GAPDH	REF	0.87	1.000	0.114 - 4.769	0.023 - 1 3643.088	1.000
PC6	TRG	0.85	0.548	0.106 - 2.570	0.026 - 9.868	0.789

P(H1) - Probability of an alternative hypothesis claiming that the difference between study and control groups is merely coincidental.
GAPDH-glyceraldehyde- 3-phosphate dehydrogenase. TRG – Target; REF – Reference; C.I, - Confidence Intervals

Until now there is no gene deletion model for PC6 in a mouse, which seems to confirm our earlier hypothesis. Since mouse and human PC6 proteins have 95% homology, it indicates that this gene is highly conserved across species [16]. This might explain the lack of difference in the expression between control and study groups in our research. Also, since the only form that has been identified in humans is soluble, it is possible to speculate that total effects exerted by this enzyme might be correlated with posttranslational processes.

Conclusion

Since PC6 is involved in the activation of many molecules associated with either initial embryo attachment (like integrins) or the preparation of the endometrium for the embryo invasion (like epidermal growth factor and vascular endothelial growth factor), it would be worthwhile to look for possible substrates that could affect the local expression of PC6 within the endometrial cavity [15, 23, 24]. However, studies have demonstrated that PC6 expression, despite being more prominent in the second half of the cycle, so called 'progesterone dependant', does not increase with neither estrogens nor progesterone stimulation [19].

The endometrium has a natural state of receptiveness [12]. In vivo studies will help determine which of the genes that are up- or down-regulated in the implantation window are truly the cause of infertility and which are redundant in this process. Therefore, a functional study of PC6 action in the endometrial fluid might explain whether infertility could be caused by altered PC6 function.

References

1. Practice Committee of the American Society for Reproductive Medicine: Effectiveness and treatment for unexplained infertility. *Fertil Steril.* 2006, 86 (Suppl), S111-114.
2. Kennedy S, Bergqvist A, Chapron C, [et al.]. on behalf of the ESHRE Special Interest Group for Endometriosis and Endometrium Guideline Development Group: ESHRE guideline for the diagnosis and treatment of endometriosis. *Hum Reprod.* 2005, 20, 2698-2704.
3. Adamson G, Baker V. Subfertility: causes, treatment and outcome. *Best Pract Res Clin Obstet Gynaecol.* 2003, 17, 169-185.
4. Cahill D, Wardle P. Management of infertility. *BMJ.* 2002, 325, 28-32.
5. Homburg R. The case for initial treatment with intrauterine insemination as opposed to in vitro fertilization for idiopathic infertility. *Hum Fertil.* 2003, 6, 122-124.
6. Zeitoun K, Bulun S. Aromatase: a key molecule in the pathophysiology of endometriosis and a therapeutic target. *Fertil Steril.* 1999, 72, 961–969.
7. Andersen A, Gianaroli L, Felberbaum R, [et al.]. The European IVF-monitoring programme (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2002. Results generated from European registers by ESHRE. *Hum Reprod.* 2006, 21, 1680-1697.
8. Barnhart K, Dunsmoor-Su R, Coutifaris C. Effect of endometriosis on in vitro fertilization. *Fertil Steril.* 2002, 77, 1148-1155.
9. Hourvitz A, Lerner-Geva L, Elizur S, [et al.]. Role of embryo quality in predicting early pregnancy loss following assisted reproductive technology. *Reprod Biomed Online.* 2006, 13, 504-509.
10. Zenke U, Chetkowski R. Transfer and uterine factors are the major recipient-related determinants of success with donor eggs. *Fertil Steril.* 2004, 82, 850-856.
11. Shahine L, Cedars M. Preimplantation genetic diagnosis does not increase pregnancy rates in patients at risk for aneuploidy. *Fertil Steril.* 2006, 85, 51-56.
12. Coutifaris C. Histological dating of secretory endometrium: what controversy? *Fertil Steril.* 2004, 82, 1301-1302.
13. Kao LC, Tulac S, Lobo S, [et al.]. Global gene profiling in human endometrium during the window of implantation. *Endocrinology.* 2002, 143, 2119-2138.
14. Nie G, Findlay J, Salamonsen L. Identification of novel endometrial targets for contraception. *Contraception.* 2005, 71, 272-281.
15. Seidah N, Day R, Marcinkiewicz M, [et al.]. Precursor convertases: an evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. *Ann N Y Acad Sci.* 1998, 839, 9-24.
16. Seidah N, Chretien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res.* 1999, 848, 45-62.
17. Nie G, Li Y, Wang M, [et al.]. Inhibiting uterine PC6 blocks embryo implantation: an obligatory role for a proprotein convertase in fertility. *Biol Reprod.* 2005, 72, 1029-1036.
18. Nakagawa T, Murakami K, Nakayama K. Identification of an isoform with an extremely large Cys-rich region of PC6, a kex2-like processing endoprotease. *FEBS Lett.* 1993, 327, 165-171.
19. Miranda L, Wolf J, Pichuantes S, [et al.]. Isolation of the human PC6 gene encoding the putative host proteases for HIV-1 gp160 processing in CD4+ T lymphocytes. *Proc Natl Acad Sci USA.* 1996, 93, 7695-7700.
20. Nie G, Li Y, Minoura H, [et al.]. Specific and transient up-regulation of proprotein convertase 6 at the site of embryo implantation and identification of a unique transcript in mouse uterus during early pregnancy. *Biol Reprod.* 2003, 68, 439-447.
21. Okada H, Nie G, Salamonsen L. Requirement for proprotein convertase 5/6 during decidualization of human endometrial stromal cells in vitro. *J Clin Endocrinol Metab.* 2005, 90, 1028-1034.
22. Revised American Society for Reproductive Medicine Revised classification of endometriosis: 1996. *Fertil Steril.* 1997, 67, 817-821.
23. Gnoth C, Goderhardt D, Goderhardt E, [et al.]. Time to pregnancy: results of the German prospective study and impact on the management of infertility. *Hum Reprod.* 2003, 18, 1959-1966.
24. Siegfried G, Basak A, Cromlish J, [et al.]. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest.* 2003, 111, 1723-1732.