TGF superfamily and \(MMP_2, MMP_9, TIMP_1\) genes expression in the endometrium of women with impaired reproduction

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Abstract: During the putative "implantation window", a period of maximal endometrial receptivity that spans 7-9 days after ovulation, a series of changes on the structural and molecular level occur that render the endometrium susceptible to implantation for the human embryo. Many members of the TGF\(\beta\)s are expressed by human endometrium at different stages of menstrual cycle. Also studies regarding the MMPs gene expression and activity of MMPs in the implantation window have shown a higher expression and activity of MMP2 in women with impaired fertility. We have examined by RT-PCR the expression of TGF\(\beta_2\) and MMP2, MMP9 and TIMP1 in 28 patients with idiopathic infertility, 16 patients with unexplained recurrent miscarriage and 16 control women were enrolled in this study. Seven to nine days after ovulation endometrial biopsy by Pipelle or hysteroscopy was performed to assess the expression of TGF\(\beta_2\), MMP2, MMP9 and TIMP1. We found that in endometria from women with idiopathic infertility TGF\(\beta_2\) expression was 2.8 fold higher than in endometria from control group and 2.1 fold higher in endometrial samples from women with unexplained recurrent miscarriage compared to the control group. The MMP2, MMP9 and TIMP1 expression in endometrial samples revealed no significant differences between the study groups and control group. There was a statistically significant negative correlation between TGF\(\beta_2\) and MMP9 expression in endometria from women in control group. The present investigations suggest that dysregulated TGF\(\beta_2\), MMP2, MMP9, MMP9 and TIMP1 expression are associated with infertility and early pregnancy loss. However the exact mechanism of how overexpression of endometrial TGF\(\beta\)s and MMPs interferes with implantation may be more complex.

Key words: Impaired reproduction - Endometrium - TGF\(\beta_2\) - MMP2 - MMP9 - TIMP1

Introduction

During the putative "implantation window", a period of maximal endometrial receptivity that spans 7-9 days after ovulation, a series of changes on the structural and molecular level occur that render the endometrium susceptible to implantation for the human embryo [1]. There are number of cytokines, adhesion molecules and receptors that possibly might play a role in increasing the receptivity of the endometrium, however the exact "composition" needed for implantation to occur is not known [1-3].

The TGF\(\beta\) superfamily comprises at least 42 distinct mammalian dimeric proteins, that share a similar structure [4].

These are divided into two subfamilies, the TGF\(\beta\) (activin) nodal subfamily and second subfamily including bone morphogenetic protein, müllerian inhibitory substance, and growth and differentiation factor [2].

The TGF\(\beta\)s [1-3] are each synthesized as a large precursor molecule from which a propeptid must be cleaved. After secretion, most TGF\(\beta\) is stored bound to extracellular matrix components as a complex of
TGFβ, propeptide and a peptide called latent TGFβ-binding protein. Release of active TGFβ from the complex is a critical regulatory step [5]. Many members of the TGFβ3 are expressed by human endometrium at different stages of menstrual cycle. The three TGFβ isoforms have been localized to both epithelial and stromal cells [6] with TGFβ3 staining being more intense in the stroma while TGFβ-1 and -3 is of equal staining intensity in stromal and epithelial cells [7].

Cyclical changes in expression level are not evident for TGFβ1 and TGFβ2, only TGFβ1 varies across the cycle, with higher expression level during the late secretory phase [2].

The production and secretion of TGFβ by epithelial cells in the secretory phase suggests a role in the preparation of the endometrium for implantation. TGFβ may play role in human implantation via their stimulation of fibronectin or vascular endothelial growth factor production [8] or by promotion of adhesion of trophoblast cells to the [9].

More recently it has been shown, that both TGFβ1 and activin A enhance the production of the implantation cytokine, leukemia inhibitory factor in epithelial cells [10] and have profound effects on implantation cytokine, leukemia inhibitory factor in and activin A enhance the production of the pro-implantation cytokine, leukemia inhibitory factor in and activin A enhance the production of the pro-implantation cytokine, leukemia inhibitory factor in and activin A enhance the production of the pro-implantation cytokine, leukemia inhibitory factor in and activin A enhance the production of the pro-implantation cytokine, leukemia inhibitory factor in and activin A enhance the production of the pro-implantation cytokine, leukemia inhibitory factor in and activin A enhance the production of the pro-

Tissue samples. All women underwent serial ultrasound assessments to track follicular growth and the formation of corpus luteum. During the same cycle, 7-9 days after ovulation (the putative implantation window) endometrial biopsy by Pipelle or hysteroscopy was performed to assess the expression of TGFβ isoforms have been localized to both epithelial and stromal cells [6] with TGFβ3 staining being more intense in the stroma while TGFβ-1 and -3 is of equal staining intensity in stromal and epithelial cells [7].

Our current study is based on working model hypothesis that disturbances in endometrial extracellular matrix is associated with disturbances of implantation and functional bleeding. Studies regarding the MMP2 gene expression and activity of MMP2 in the implantation window have shown a higher expression and activity of MMP2 in women with impaired fertility [13,14].

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Finland). The reaction profile and the mix content was done according to manufacturers instructions. Primers for the qPCR reaction were designed with Primer3 software. The specificity of oligonucleotides was confirmed using NCBI BLAST. To assess the efficacy of the qPCR reaction 6 subsequent 10 times dilutions of DNA was used, that was a specific reaction from the PCR of each studied transcript.

The qPCR was done on Rotor-Gene3000 thermocycler (CorbettResearch, Australia). During the qPCR, after each cycle the increase in amount of generated product was assessed, and at the end of a reaction the thermocycler program assigned the fluorescence level (Ct) at which the pace of increase of generated product was logarithmic.

To assess the expression levels and statistical analysis following programs were used:
- MedCalc (http://www.medcalc.be/) [15].

Results

**TGFβ2 expression in human endometrium during the implantation window**

The RT-PCR used to analyse TGFβ2 expression in endometrial RNA samples revealed that in endometria from women with idiopathic infertility TGFβ2 expression was 2.8 fold higher than in endometria from control group (Fig. 1).

Similarly TGFβ2 expression was 2.1 fold higher in endometrial samples from women with unexplained recurrent miscarriage compared to the control group (Fig. 2). Overexpression of TGFβ2 was found in 59% of endometria from women with infertility and in 64% of endometria from women with recurrent pregnancy

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**Fig. 1.** The relative TGFβ2 expression in idiopathic infertility group. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

**Fig. 2.** The relative TGFβ2 expression in unexplained miscarriage group. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.
loss compared to the results achieved in control group. Unfortunately there was no statistically significant differences.

**MMP₂, MMP₉, TIMP₁ expression in human endometrium during the implantation window**

The RT-PCR studies of MMP₂, MMP₉ and TIMP₁ expression in endometrial samples revealed no significant differences in their expression between the study groups and control group (Fig. 1 and 2).

However MMP₂, MMP₉ and TIMP₁ expression in endometria harvested from women suffering from idiopathic infertility compared to endometria from women without reproductive disorders was 1.3, 5.2 and 3.9 fold higher, respectively. Higher MMP₂, MMP₉, and TIMP₁ expression than in control group was observed in endometria harvested from women with infertility (58%, 75% and 92% higher, respectively). Also the expression of MMP₉ and TIMP₁ in endometrial samples collected from women with unexplained recurrent miscarriage compared to endometrial samples in control group was 2.4 and 3.8 higher, respectively. We found overexpression of MMP₉ and TIMP₁ in 63% and 88%, respectively, in endometrial samples from women with recurrent pregnancy loss compared to control group.

Although MMP₂ expression in endometrial samples didn't differ between unexplained miscarriage and control group, there was a higher MMP₂ expression in 43% of endometrial samples from unexplained miscarriage group.

**The correlation between TGFβ₂, MMP2, MMP9 and TIMP1 expression**

There was a statistically significant negative correlation between TGFβ₂ and MMP₂ expression in endometria from women in control group (Fig. 3), which means, that a higher TGFβ₂ expression was accompanied by lower MMP₂ expression. In both studied groups we have not been able to show any correlation between expression of TGFβ₂ and MMP₂, nor between MMP₉ and TIMP₁.

**Discussion**

While many techniques have been developed that are used to diagnose different causes of impaired fertility still in the etiology of about 50% of recurrent miscarriages and 10% of infertility remains unexplained. Many authors tried to explain the etiologies of idiopathic infertility and recurrent miscarriage by endometrial disturbances both at the tissue and also molecular level [16-18].

It is becoming increasingly clear, that transforming growth factor beta (TGFβ) is involved in cellular proliferation and differentiation, extracellular matrix modification, tissue remodeling, angiogenesis and decidualization of uterine endometrium during the implantation [19].

All three isoforms of TGFβ have been localized in rodent and human endometrial epithelium and stroma. It has been shown, that TGFβs modulate maternal immunotolerance during implantation and regulate in vitro several molecules related to implantation as VEGF, IGFBP₁, LIF and MMP₉ [2,20]. A role for TGFβ in the modulation of endometrial receptivity in monkey has also been reported [21].

Our RT-PCR investigation showed trend for higher expression of TGFβ₂ in endometrial samples from women with idiopathic infertility and unexplained recurrent miscarriages compared to endometria from control group, but we didn't found any statistically significant differences. Kothapalli et al. [22] identified ebafl/lefly - a novel human gene of the TGFβ superfamily during a search for genes highly expressed during the non-receptive phase in human endometrium. Lefty proteins were present in low amounts during the receptive phase in the endometria and sera of normal fertile women, but were abundant in the endometria of a number of infertile patients subjects during the receptive phase of the cycle [23]. The authors speculated, that successful implantation occurs in the presence of a low level of ebafl protein in human endometrium, and that a high level of ebafl would be associated with infertility. In their study in over 50% of endometria from infertile patients, a mRNA that hybridized with full-length ebafl cDNA was up-regulated during the endometrial receptivity period. The infertility in these women was associated with endometriosis, polycystic ovary syndrome, bilateral tube occlusion, anovulatory cycle, luteal phase defect.
premature ovarian failure and habitual abortion. In some women, the underlying reason of infertility remained unknown.

In our study only patients with unexplained infertility were included. In 59% of their endometria we have observed higher TGFβ2 expression than in control group. Also in 64% of endometria from women with unexplained recurrent miscarriages we have noticed a higher TGFβ3 expression than in endometria from women in control group.

It has been known, that nearly 50% of early pregnancy losses occur when implantation occurs after postovulatory day 10, when the ebf protein is relatively abundant in endometrium [24]. These data and our observation can suggest, that dysregulated TGFβs gene expression might be a contributing factor leading to the impairment of implantation.

Lately much attention was paid to the role of MMPs for endometrial receptivity and the implantation process. Expression of genes taking part in the production and degradation of endometrial extracellular matrix in women with unexplained infertility and recurrent miscarriages was the subject of studies from Jokimaa et al. [14]. These authors have observed higher mRNA level for collagen type 1, MMP2 and cathepsin H in studied patients compared to control group. Compared to normal endometria, from control group, in our own RT-PCR study we have observed higher MMP2 expression in 58% endometria from women with idiopathic infertility and only in 43% endometria from women with unexplained recurrent miscarriages. These data can lead to the conclusion, that the extracellular matrix participate in the endometrial receptivity and that the imbalances in the extracellular matrix might offer explanation for same case of unexplained infertility and recurrent miscarriages.

Our results regarding MMP2 and TIMP1 mRNA expression in endometrium during the mid-luteal phase in patients with idiopathic infertility differ from results in Wu and Zhon study [25]. While these authors have noticed significantly lower expression of MMP9 and TIMP1 mRNA in endometrium from women with idiopathic infertility, we have observed a trend for higher expression of these molecules, however without statistical significance. We have also found in endometria from women with unexplained recurrent miscarriage a higher expression of endometrial MMP9 and TIMP1 (63% and 88%, respectively) compared to control group. Bany et al. [26] have shown significantly higher content of MMP9 mRNA in stimulated decidualization compared with non-stimulated one in the horn of mouse uterus. In our previous study [27] we have observed a lower MMP9 and TIMP1 concentration in uterine fluid collected from women with idiopathic infertility and women with unexplained cause for recurrent miscarriage than in controls. These discordance can be explained by hypothetic disturbances between modules expression and their secretion and transport.

Fact, that decasualization also occurs in the presence of inhibitors, points to a conclusion, that MMPs are not indispensable able for endometrial transformation. It has already been mentioned above, that TGFβ3 has profound effect on ECM production and degradation of enzymes [11]. To support this hypothesis we have found statistically negative correlation between the TGFβ2 and MMP9 expression but only in endometria from control group.

Also Tabibzadeh et al. [23] have found, that ebf up-regulates the expression of matrix metalloproteinase -3 and -7, therefore they my be involved in tissue shedding.

The present investigations suggest that dysregulated TGFβ3, MMP2, MMP9 and TIMP1 expression are associated with infertility and early pregnancy loss. However the exact mechanism of how overexpression of endometrial TGFβ3 and MMPs interferes with implantation may be more complex.

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