

Cycle dependent expression of endometrial metallothionein

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Abstract

OBJECTIVES: Molecular changes observed in endometrium with respect to menstrual cycle changes seem to be crucial for the reproductive function. Accumulation of cytotoxic cells increases the exposure of endometrial cells to apoptosis. Protection against apoptosis may be reached by endometrial cells by self molecular regulation. Metallothionein was suggested to participate in this process. The aim of our study was to evaluate endometrial MT immunoreactivity with respect to the menstrual cycle phases.

MATERIALS AND METHODS: MT expression was assessed using immunohistochemistry method in 47 endometrial tissue samples derived from randomly selected patients with respect to the menstrual cycle phases – proliferative and secretory with distinguishing early, mid and late subphases in each.

RESULTS: MT expression changes were observed respectively to hormonal fluctuations with the highest level during mid secretory phase and its respective decrease during the early, late secretory and mid proliferative menstrual cycle phases. The lowest MT immunoreactivity level was disclosed during early proliferative phase.

CONCLUSION: Significant differences in MT expression observed in endometrium with respect to menstrual cycle changes might suggest MT participation in endometrial cells protection against apoptosis.

Introduction

Molecular changes concerning endometrial and immune cells present in decidua observed respectively to menstrual cycle changes seem to be crucial for the reproductive function. During decidualization the number of immune cells grow and endometrium is ready for the ovum implantation (implantation window). Epithelial endometrial cells are surrounded then by a high number of mononuclear cells including NK, macrophages, T

lymphocytes and other cells [9,17]. Accumulation of cytotoxic cells increases the exposure of endometrial cells to apoptosis. The integrity of endometrial tissue can be preserved owing to its ability to regulate the cytotoxic immune response. Many factors accumulated in endometrium at the time of implantation window possess immunomodulating activity such as: Galectin, VEGF, RCAS1 and other [5,14,15,16,24]. Protection against unwanted

apoptosis may be also reached by endometrial cell self molecular regulation. Metallothionein was suggested to participate in this process [6,7,26].

Metallothionein (MT) is a metal-binding low molecular weight protein with functional roles in cell growth, repair and differentiation [1,21]. It is also known that perinuclear MT location is important for protective function of MT against DNA damage and apoptosis induced by external stress stimuli [11,22]. According to this study it was suggested that MT over-expression might protect the tumor cells from entering the apoptotic process and thereby to contribute to tumor expansion. The relation between MT expression and primary invasive breast cancer progression was observed [8]. MT seems to be a biomarker of tumor differentiation and aggressiveness [3]. MT expression was increased in endometrial carcinoma cases compared with benign hyperplastic endometrial lesions [13].

The aim of our study was to evaluate MT immunoreactivity in normal endometrium with respect to the menstrual cycle phases – proliferative and secretory with distinguishing early, mid and late subphases in each.

Materials and Methods

Human subjects

Eutopic human endometrium tissues were obtained from non-menopausal fertile women, aged 25–45 years. These patients underwent hysterectomy because of benign gynecological indication (leiomyomas), laparoscopic or hysteroscopic diagnostic procedures. Patients' agreement was obtained in all cases. The approval for the research program of the Jagiellonian University Ethical Committee was obtained prior to the study. No patient included in our study received any hormonal treatment. The surgical procedure was performed in Gynecology and Infertility Department of the Jagiellonian University in Krakow, Poland. 450 women with the symptoms of benign gynecological lesion were referred to the Gynecology and Infertility Department between March 2004 and March 2005. Finally, 47 out of 450 women were randomly selected to our study. All tissue samples were verified by the standard histology using hematoxylin and eosin staining technique following formalin fixation. Tissue samples were classified

according to the menstrual cycle phases, with division of the cycle into seven phases: early proliferative (7 cases), mid proliferative (10 cases), late proliferative (4 cases), early secretory (5 cases), mid secretory (12 cases), late secretory (5 cases) and menstrual bleeding (4 cases).

Immunohistochemistry method

In all cases immunohistochemistry was performed applying Envision method using Dako Autostainer. For metallothionein immunostaining the monoclonal mouse antibody anti-metallothionein (Stressgen Bioreagents, Canada, dilution 1:40) was used, without any previous unmasking procedure, with 60-min-incubation at the room temperature. The positive control for this protein was a tissue taken from breast carcinoma. MT expression was evaluated in the entire slide (stromal and epithelial endometrial cells), as follows:

- 0 – lack of any expression,
- 1+ – weak/strong expression in up to 10% of the cells,
- 2+ – weak expression in 11–50% of the cells or strong (also nuclear) expression in 11–30% of the cells,
- 3+ – stronger expression than 2+.

The used method was described in details in our previous reports [26].

Statistical analysis

The distribution of the data was analyzed using Shapiro-Wilk's test. The results were compared with the use of Student's t-test for normally distributed data and Mann-Whitney U test if non normal distribution was found. Significance of differences between the groups was set at $p < 0.05$.

Results

MT expression was evaluated in 47 endometrial tissue samples, from which 19% (9 cases) were MT expression negative. (Table 1)

The highest MT expression was observed in endometrium in mid secretory menstrual cycle phase and was statistically significantly higher than in early, mid and late proliferative phases (respectively $p = 0.003$, $p < 0.001$, $p < 0.001$). Significant differences in MT

Table 1. Analysis of MT immunoreactivity in endometrial tissue samples with respect to menstrual cycle changes.

Variables	MT immunoreactivity			
	0	+1	+2	+3
Endometrium in early proliferative cycle phase (n=7)	28 (2)	72 (5)	–	–
Endometrium in mid proliferative cycle phase (n=10)	–	40 (4)	60 (6)	–
Endometrium in late proliferative cycle phase (n=4)	75 (3)	–	25 (1)	–
Endometrium in early secretory cycle phase (n=5)	20 (1)	–	40 (2)	40 (2)
Endometrium in mid secretory cycle phase (n=12)	–	–	50 (6)	50 (6)
Endometrium in late secretory cycle phase (n=5)	–	40 (2)	40 (2)	20 (1)
Menstrual bleeding (n=4)	–	75 (3)	25 (1)	–

expression were also observed between mid secretory endometrium and endometrium during menstruation ($p=0.002$). The level of MT immunoreactivity was higher in endometrium during mid secretory phase than in endometrium during early and late secretory cycle phases ($p=0.17$, $p=0.58$). MT expression in endometrium during the early and late secretory cycle phases was at the level comparable to the expression in endometrium during the mid proliferative phase. Significant differences were identified in MT endometrial immunoreactivity level between the mid proliferative and early proliferative cycle phases ($p=0.01$). Similarly, significant differences in MT expression were found between mid proliferative and late proliferative menstrual cycle phases ($p=0.08$). MT expression was significantly higher during the early and late secretory cycle phases than in early proliferative phase ($p=0.002$, $p=0.008$). Additionally MT endometrial level was found to be higher in early and late secretory menstrual cycle phases than during the late proliferative one ($p=0.001$, $p=0.006$). The drop of MT expression during menstruation was significantly lower only in comparison to the MT level in endometrium during mid secretory menstrual cycle phase.

Discussion

In the present study MT expression changes were observed respectively to hormonal fluctuations with the highest level during mid secretory phase and its respective decrease during the early, late secretory and mid proliferative menstrual cycle phases. The lowest MT immunoreactivity level was disclosed during early proliferative phase.

Ioachin reported MT expression growth in normal endometrium during secretory cycle phase in comparison to proliferative one and an inverse correlation between MT and ER receptor [7]. MT gene is potentially down-regulated by estrogen receptor alpha, although MT expression in invasive ductal breast cancers was independent of ER status [20]. MT-I and MT-II genes expression seems to be controlled by progesterone, what was confirmed in vitro in endometrial cancer cell line (Ishikawa cells) [18]. Contrary to these findings Ioachin observed an inverse correlation between MT values in normal endometrium and progesterone receptor content [7]. In our study MT immunoreactivity was significantly higher in secretory than in proliferative cycle phase.

MT expression was recently demonstrated to be stimulated by INF- α in vitro [4]. In eutopic endometrium INF- α concentration alters respectively to menstrual cycle phases and was at the highest level during mid secretory cycle phase [12]. INF- α is secreted by immune cells, concentration of which grows during secretory cycle phase and induces IL-2 and other cytokines secretion by immune cells, stimulating the cytotoxic response [2,19]. Receptors for INF- α are located in endometrial cells [23]. The increase of MT endometrial immunoreactivity during the implantation window seems to be secondary to the

growing cytotoxic activity. It was suggested that MT expression might lead to induction of resistance against apoptosis [18]. Implantation window is the moment of IL-15 accumulation, which is crucial for dNK cells infiltration and activation [10]. MT expression results from an interaction between immune cells and endometrial cells. In our previous report MT expression alterations were observed respectively to changes of number and activity of CD56 positive cells in eutopic endometrium, ovarian endometriosis and endometrial adenocarcinoma [25,26]. The protection of endometrial tissue function (ovum implantation, decidualization) requires the interaction between endometrial cells and immune cells, as the endometrial tissue integrity has to be preserved from the growing cytotoxic activity.

In summary, significant differences in MT expression disclosed in endometrium with respect to menstrual cycle changes might suggest MT participation in endometrial cells protection against apoptosis.

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REFERENCES

- 1 Cherian MG, Jayasurya A, Bay BH: Metallothioneins in human tumors and potential roles in carcinogenesis. *Mutat Res* 2003; **533**:201–209.
- 2 De Castro FA, Palma PV, Morais FR, Simoes BP, Carvalho PV, Ismael SJ, Lima CP, Voltarelli JC: Immunological effects of interferon-alpha on chronic myelogenous leukemia. *Leuk Lymphoma* 2003; **44**:2061–2067.
- 3 Gallicchio LM, Flaws JA, Fowler BA, Ioffe OB: Metallothionein expression in invasive and in situ breast carcinomas. *Cancer Detect Prev* 2005; **29**:332–337.
- 4 Guevara-Ortiz JM, Omar-Castellanos V, Leon-Chavez BA, Achanzar WE, Brambila E: Interferon alpha induction of metallothionein in rat liver is not linked to interleukin-1, interleukin-6, or tumor necrosis factor alpha. *Exp Mol Pathol* 2005; **79**:33–38.
- 5 Hackmon R, Hallak M, Krup M, Weitzman D, Sheiner E, Kaplan B, Weinstein Y: HLA-G antigen and parturition: maternal serum, fetal serum and amniotic fluid levels during pregnancy. *Fetal Diagn Ther* 2004; **19**:404–409.
- 6 Ioachin E: Immunohistochemical tumor markers in endometrial carcinoma. *Eur J Gynaecol Oncol* 2005; **26**:363–371.
- 7 Ioachim EE, Kitsiou E, Carassavoglou C, Stefanaki S, Agnantis NJ: Immunohistochemical localization of metallothionein in endometrial lesions. *J Pathol* 2000; **191**:269–273.
- 8 Ioachim E, Tsanou E, Briasoulis E, Batsis Ch, Karavasilis V, Charchanti A, Pavlidis N, Agnantis NJ: Clinicopathological study of the expression of hsp27, p52, cathepsin D and metallothionein in primary invasive breast cancer. *Breast* 2003; **12**:111–119.
- 9 King A, Burrows T, Loke YW: Human uterine natural killer cells. *Nat Immun* 1996; **15**:41–52.
- 10 Kitaya K, Yamaguchi T, Honjo H: Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16 (-) natural killer cells into human endometrium. *J Clin Endocrinol Metab* 2005; **90**:2932–2940.
- 11 Levadoux-Martin M, Hesketh JE, Beattie JH, Wallace HM: Influence of Metallothionein-1 localization on its function. *Biochem J* 2001; **355**:473–479.
- 12 Li Q, Zhang M, Kumar S, Zhu LJ, Chen D, Bagchi MK, Bagchi IC: Identification and implantation stage-specific expression of an

- interferon-alpha-regulated gene in human and rat endometrium. *Endocrinology* 2001; **142**:2390–2400.
- 13 McCluggage WG, Maxwell P, Hamilton PW, Jasani B: High metallo-thionein expression is associated with features predictive of aggressive behaviour in endometrial carcinoma. *Histopathology* 1999; **34**:51–55.
 - 14 Merviel P, Carbillon L, Challier JC, Rabreau M, Beauvils M, Uzan S. Pathophysiology of preeclampsia. links with implantation disorders. *Eur J Obstet Gynecol Reprod Biol* 2004; **115**:134–147.
 - 15 Nardo LG. Vascular endothelial growth factor expression in the endometrium during the menstrual cycle, implantation window and early pregnancy. *Curr Opin Obstet Gynecol* 2005; **17**:419–423.
 - 16 Popovici RM, Krause MS, Germeyer A, Strowitzki T, Von Wolff M. Galectin-9: A New Endometrial Epithelial Marker for the Mid and Late Secretory and Decidual Phase in Humans. *J Clin Endocrinol Metab.* 2005 Aug 16; [Epub ahead of print].
 - 17 Saito S, Sasaki Y, Sakai M: CD4⁺CD25^{high} regulatory T cells in human pregnancy. *J Reprod Immunol* 2005; **65**:111–120.
 - 18 Smid-Koopman E, Kuhne LC, Hanekamp EE, Gielen SC, De Ruiter PE, Grootegoed JA, Helmerhorst TJ, Burger CW, Brinkmann AO, Huikeshoven FJ, Blok LJ. Progesterone-induced inhibition of growth and differential regulation of gene expression in PRA- and/or PRB-expressing endometrial cancer cell lines. *J Soc Gynecol Investig* 2005; **12**:285–292.
 - 19 Sotnikowa N, Antsiferova I, Malyskhina A. Cytokine network of eutopic and ectopic endometrium in women with adenomyosis. *Am J Reprod Immunol* 2002; **47**:251–255.
 - 20 Surowiak P, Matkowski R, Materna V, Gyorffy B, Wojnar A, Pudelko M, Dziegiel P, Kornafel J, Zabel M. Elevated metallothionein (MT) expression in invasive ductal breast cancers predicts tamoxifen resistance. *Histol Histopathol* 2005; **20**: 1037–1044.
 - 21 Theoharis SE, Margeli AP, Klijanienko JT, Kouraklis GP: Metallothionein expression in human neoplasia. *Histopathology* 2004; **45**:103–118.
 - 22 Tsangaris GT, Tzortzatou-Stathopoulou F. Metallothionein expression prevents apoptosis; a study with antisense phosphorothioate oligodeoxynucleotides in a human T cell line. *Anticancer Res* 1998; **18**:2423–2433.
 - 23 Wang B, Xiao Ch, Goff AK. Progesterone-Modulated Induction of Apoptosis by Interferon-Tau in Cultured Epithelial Cells of Bovine Endometrium. *Biol Reprod* 2003; **68**:673–679.
 - 24 Wicherek L, Dutsch-Wicherek M, Mak P, Klimek M: The role of RCAS1 and oxytocinase in immune tolerance during pregnancy. *Fetal Diagn Ther* 2005; **20**:420–425.
 - 25 Wicherek L, Galazka K, Wojtys A, Tetlak T, Dutsch-Wicherek M, Klimek M. Metallothionein expression in human ectopic endometrium. *Eur J Obstet Gynecol Reprod Biol* 2005; **123** (Suppl 1): S47.
 - 26 Wicherek L, Popiela TJ, Galazka K, Dutsch-Wicherek M, Oplawski M, Basta A, Klimek M. Metallothionein and RCAS1 expression in comparison to immunological cells activity in endometriosis, endometrial adenocarcinoma and endometrium according to menstrual cycle changes. *Gynecol Oncol* 2005; **99**:622-630.