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# The Local Immune Mechanisms Involved in the Formation of Endometriotic Lesions

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## 1. Introduction

According to common view, endometriosis is characterized by the presence and growth of the endometrium-like glandular tissue and stroma outside the uterus. So, endometriosis would represent an autotransplant in which tissue is transplanted to an ectopic location in an organism. It is well known that the immune system plays the main role in regulation of tissue homeostasis in an organism. Thus, immunologists have a special interest in this disease as insights in the pathogenesis of endometriosis would help not only to understand how ectopic lesions grow but also allow searching new approaches to the medical treatment of this disease.

Numerous theories have been proposed to explain the endometriosis pathogenesis till date. The most widely accepted Sampson's transplantation theory proposes that, during menstruation, there is reflux of endometrial tissue via fallopian tubes into abdominal cavity where endometrium could attach to peritoneal surfaces, proliferate, invade, and become the disease known as endometriosis. However, this theory does not account for the fact that most of women of reproductive age exhibit some degree of reflux of endometrial debris, but only some patients develop endometriosis (Harada et al., 2004; Vinatier et al., 2001). There are two main suggestions explaining this contradiction. The first explanation propose that there are some changes in the endometrium of women with endometriosis and these changes can promote the resistance of endometrial cells to normal peritoneal cleaning (Vinatier et al., 2001). The second theory suggests that impaired immune recognition in peritoneal cavity due to abnormalities of the cellular and humoral immunity can promote the endometriosis development (Kyama et al., 2003).

The suggestion that the primary defect in endometriosis is located in the eutopic endometrium, was proposed many years ago and supported now by many investigators (Vinatier et al., 2001; Kyama et al., 2003; Harada et al., 2004), though the eutopic endometrium of women with and without endometriosis is histologically similar. This hypothesis is confirmed by different studies revealing that there are many fundamental differences between these two tissues. Invasive properties, decreased apoptosis, and increased steroid hormones and cytokine production have been identified in eutopic

endometrium of women with endometriosis compared to that in women without disease (Ulukus 2006). Impairment of some genes expression also was found in women with endometriosis. Aberrant expression of several genes such as matrix metalloproteinases, Hox genes, integrins, anti-apoptotic genes Bcl-2 was demonstrated in endometrium of women with endometriosis (Bondza et al., 2009). The significant increase of aromatase expression was shown in eutopic endometrium from endometriosis patients (Weiss et al., 2009). A constitutional or acquired anomaly in the nature of antigens, expressed by endometrium, such as transplantation antigens HLA-DR and HLA-A, B, C antigens, can explain the resistance of endometrium of women with endometriosis to the cytotoxicity of T-lymphocytes (Vinatier et al., 2001). Increased proliferative activity of endometrial cells due to altered expression of c-myc, TGF-β1 and bax genes was also found in patients with endometriosis (Jonson et al., 2005). Eutopic endometrium from endometriosis patients showed increased expression of midkine and pleiotropin mRNA expression compared with endometrium from normal women (Chung et al., 2002). It is known that midkine and pleiotropin function as tumor growth factors positively regulating tumor angiogenesis and metastasis of solid tumors. So these results evidence that eutopic endometrium from endometriosis patients may be more invasive and prone to implantation than that from women without endometriosis (Chung et al., 2002). All these findings let us to propose that cells and tissue elements derived from such an altered eutopic endometrium shed into peritoneal cavity would have a higher potential of implantation and growth on peritoneal surfaces. And many differences, observed between eutopic endometrium and ectopic tissue of patients with endometriosis, can be explained as the direct consequence of the specific environment of peritoneal fluid (Vinatier et al., 2001; Harada et al., 2004).

The local environment that surrounds the endometriotic implants in the peritoneal cavity is a dynamic one. Histologically, the peritoneum consists of a thin layer of loose connective tissue covered by a layer of mesothelium and is the most extensive serous membrane in the body, with a rich supply of subperitoneal blood vessels and lymphatics (Gazvani & Templeton, 2002). The peritoneal cavity is normally empty except for a thin film of fluid that keeps surfaces moist. The peritoneal fluid arises primarily from two sources: plasma transudate and ovarian exudate (Koninckx et al., 1998; Gazvani & Templeton, 2002). Peritoneal fluid contains a variety of free-floating immune cells, including macrophages, natural killer (NK) cells, lymphocytes, eosinophils and mast cells (Gazvani & Templeton, 2002). Macrophages are the most abundant cells among the peritoneal leukocytes. It is well known that macrophages are the main source of different cytokines, growth factors and other biologically active substances in the peritoneal fluid. Recent studies have also suggested that peritoneal fluid of women with endometriosis contain an increased number of activated macrophages and other immune cells and high amount of proinflammatory cytokines and growth factors, which exert a paracrine action on endometrial cells (Minici et al., 2007). So, endometriosis can be considered to be an inflammatory disease (Vigano et al., 2004). Possibly, this peritoneal inflammation might facilitate the implantation and growth of ectopic endometrial tissues. But we don't know exactly yet, whether endometriosis is caused by peritoneal inflammation? Or does endometriosis lead to inflammation caused by inappropriate immune response to endometrial debris? Can the inappropriate peritoneal environment directly influence lesions formation, or may be the intrinsic dysfunctions of endometrial cells play the main role in endometriosis development?

To concretize the role of cellular and humoral immune components of peritoneal fluid in their interaction with endometrial tissue in endometriosis and also to elucidate the local immune mechanisms participating in endometriotic lesions formation we attempted: a) to define the regulatory mechanisms of apoptosis and invasiveness of eutopic and ectopic endometrium in women with endometriosis, b) to elucidate the influence of autologous peritoneal macrophages and humoral factors of peritoneal fluid upon the parameters of eutopic endometrial tissue apoptosis and invasiveness in endometriosis women, c) to establish the changes in the functional state of peritoneal macrophages of women with endometriosis and d) to estimate the character of macrophages activity in response to autologous stimulation of endometrial cells.

## 2. Methods of investigation

### 2.1 Subjects

The study group consisted of 80 women of reproductive age undergoing diagnostic laparoscopies for infertility or pelvic pain. The presence and extent of the disease were determined laparoscopically and staging was performed according to the revised American Fertility Society classification (1985). Mild endometriosis (stage 1-2) was diagnosed in 51 women (64%), severe endometriosis (stage 3-4) was noted in 29 patients (36%). Laparoscopy was performed prior to the initiation of any treatment. Samples of peritoneal fluid and paired eutopic and ectopic endometrial biopsies were obtained from women with endometriosis. Endometrial tissue and peritoneal fluid from 30 control women without endometriosis, who underwent surgical sterilization were also collected. Informed consent was given by each woman participating in our study, according to local Ethical Committee protocol. Samples of peritoneal fluid from the Douglas pouch were aspirated into sterile tubes. Samples of ectopic and matched eutopic endometrium were collected into sterile flasks with isotonic saline solution and were immediately recruited into the study.

### 2.2 Co-cultivation of endometrial tissue and peritoneal macrophages

To evaluate the possible effect of peritoneal environment on the apoptosis and invasive capacity of endometrial tissue, the samples of eutopic endometrium of 10 women without endometriosis and 10 women with endometriosis were cultivated in the presence of autologous peritoneal fluid or autologous peritoneal macrophages. Samples of peritoneal fluid were centrifuged at 2000 g for 10 minutes to remove cellular component. Bloody samples of peritoneal fluid were excluded from our study. Enriched population of peritoneal macrophages was obtained from peritoneal fluid using standard Ficoll-Verografin gradient centrifugation (d-1.078) with subsequent removing of lymphocytes using standard procedure of macrophages adherence to plastic. The percentage of CD14+ macrophages in received fraction was 93-95% as it was established by flow cytometry analysis.

Endometrial tissue was minced by scissors into pieces of 1-2 mm in diameter. Approximately 40-50 mg of endometrial tissue were placed in 24 well plate, in 2 ml of whole RPMI 1640 medium with 2 mM of glutamine, 5% fetal calf serum and antibiotics supplemented with autologous peritoneal fluid or with peritoneal macrophages. Ratio of peritoneal fluid and whole RPMI 1640 in medium for cultivation was 1:1. The final

concentration of macrophages in culture media was $2 \times 10^6$ cell/ml. Endometrial explants were cultured at $37^0$ C and 5% $CO_2$ for 24 hours. Samples of endometrium or peritoneal macrophages, cultured in the same conditions only in RPMI 1640, were used as controls. After termination of cultivation, endometrial tissue was washed up in phosphate-buffered saline (PBS) twice and was taken for subsequent RNA isolation or for enzymatic isolation of stromal endometrial cells. Peritoneal macrophages were collected from wells, filtered through 6 layers of gauze and analyzed using flow cytometry.

## 2.3 Quantitative real-time RT-PCR

The level of mRNAs expression of different factors, regulating apoptosis and invasiveness in endometrial tissue, was investigated using quantitative real time RT-PCR (reverse transcription- polymerase chain reaction). Total RNA was isolated from the whole endometrial tissue using the acid guanidinium thiocyanate-phenol-chloroform method. RNA was converted to complementary DNA (cDNA) using random hexamers and murine leukaemia virus reverse transcriptase (Promega, USA). Reverse transcription was performed at $70^0$C for 3 min and $37^0$C for 90 min. For real time quantitative RT-PCR, gene-specific primers and probes for human β2-microglobulin (housekeeper gene), XIAP, caspase-3, HSP27, MT-1, MMP-2, MMP-9, TIMP-2, TIMP-1 were designed and constructed in the Laboratory of Gene Engineering of National Research Center for Hematology (Moscow, Russia) using the Vector NTI Advance 10 design program (Invitrogen, USA). Commercial kit "Immunoscreen" (Gene Engineering of National Research Center for Hematology, Moscow, Russia) was used to perform real-time quantitative RT-PCR. For the thermocycle reactions and the detection of the fluorescence signals, iCycler iQ Multi-Color Real Time PCR Detection System (BIO-RAD Laboratories, California, USA) was used. To assess the number of cDNA copies in every sample, the standard curves for studied genes were constructed using control cDNA dilution series. As the controls sequences of cloned corresponding genes were used. For each endometrial sample, the amount of copies of housekeeper gene (β2-microglobulin) and specific genes were determined from the appropriate standard curve, generated by iCycler iQ software. The amount of specific gene was subsequently divided by the amount of housekeeper gene to obtain normalized specific gene value and results were presented as the ratio in a sample in the order of $10^3$ per µl for MT-1, XIAP, caspase-3, MMP-2, TIMP-2, $10^4$ per µl for HSP27 and $10^5$ per µl for MMP-9 and TIMP-1.

## 2.4 Flow cytometry

The surface expression of the number of functional receptors and intracellular production of proinflammatory cytokines by peritoneal macrophages was estimated with monoclonal antibodies using flow cytometry method. The following monoclonal antibodies were used in this study: FITC-conjugated anti-human-CD45, CD36, CD204, IL-1beta, IL-8, Vimentin antibodies and PE-conjugated anti-human CD14, HLA-DR, CD49e, CD11b, CD95, CD95L, IL-6, IL-12, TNF alpha antibodies (BD Biosciences, USA). Intracellular staining procedure was carried out according to the manufacturer's instructions using the FIX & PERM cell permeabilization reagents (Invinrogen, Camarillo, CA, USA). The amount of apoptotic endometrial stromal cells after incubation with peritoneal fluid or with macrophages was assessed using commercial kit with Annexin V and propidium iodide (CALTAG

Laboratories, USA). Flow cytometry analyses were performed on FACScan (Becton Dickinson, USA) using CellQuest Pro software. Data from forward versus side scatter was obtained to analyze the CD45+CD14+ macrophage population and Vimentin+ endometrial stromal cell population.

## 2.5 ELISA

The content of IL-8, MCP-1 and calprotektin in the peritoneal fluid of women with and without endometriosis was assessed by ELISA (enzyme-linked immunosorbent assay) using commercial kits (Bender MedSystems, Austria).

## 2.6 Cell invasion assays

The influence of peritoneal fluid and culture media of 24-h cultures of peritoneal macrophages of women with endometriosis upon invasiveness of eutopic endometrial cells of women with endometriosis was estimated in the 2D Matrigel system using BD BioCoat Matrigel Invasion Chamber (BD Biosciences, USA). 50 µl of prepared endometrial stromal cells isolated from eutopic endometrium of women with endometriosis by enzymatic method were placed on top of porous membrane coated with Matrigel. Autologous peritoneal fluid or pooled culture media of 24-h cultures of peritoneal macrophages of women with endometriosis were added to the lower chamber and then incubated overnight. At the end of the incubation period the upper side of the membrane was cleaned with a cotton wool bud. For assessment of the number of invaded cells, the filters were stained with hematoxylin and eosin and mounted on glass microscope slides. Total number of cells that had invaded onto the underside of the filter was counted manually at 200x magnification.

## 2.7 Statistics

Results were presented as the mean ± standard error. All variables were checked for normal distribution with the Kolmogorov-Smirnov test. All the parameters studied showed a normal distribution. Student's t-test was used to compare results from different groups. Statistical significance was defined as $p < 0.05$.

## 3. Impairment of the apoptosis and invasiveness of endometrial tissue in endometriosis

The growing bodies of evidence indicate that the impairment of endometrial cell's apoptosis and the increase of tissue proteolysis supposedly are involved in the pathogenic mechanisms of endometriosis.

## 3.1 Regulation of apoptosis in the endometrium of women with endometriosis

Apoptosis is a fundamental process responsible for maintaining homeostasis in multicellular organisms (Mei et al., 2010). In contrast to necrotic cell death, which is usually a result of trauma, programmed cell death is a physiological process. The mechanisms of apoptosis are highly complex and sophisticated, involving an energy dependent cascade of molecular events. Two major apoptotic routes exist in mammalian cells: the intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. The intrinsic pathway is

characterized by the permeabilization of the outer mitochondrial membrane and the release of several pro-apoptotic factors into the cytosol. These include cytochrome c, Smac/Diablo, AIF (apoptosis-inducing factor), and serine protease HtrA2/Omi (Elmore 2007). These proteins activate the caspase dependent mitochondrial pathway. Cytochrome $c$ binds and activates Apaf-1 (the apoptotic protease activating factor-1) as well as procaspase-9, forming an "apoptosome" (Hill et al., 2004). The clustering of procaspase-9 in this manner leads to caspase-9 activation. Different molecules from IAP family (inhibitors of apoptosis proteins) can inhibit activity of Smac/DIABLO and HtrA2/Omi on this stage of apoptosis (Schimmer et al., 2006). The control and regulation of these apoptotic mitochondrial events occurs through members of HSP (heat shock proteins) family, acting as inhibitors of apoptosis. Proteins from Bcl-2 family also regulate the mitochondrial stage of apoptosis and these factors can be either pro-apoptotic or antiapoptotic. Till date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. These proteins have special significance since they can determine if the cell is committed to apoptosis or else abort the process. The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins (Elmore 2007). The extrinsic pathway is activated by engaging death receptors such as Fas and Tumor Necrosis Factor Receptor (TNFR) with their cognate ligands. This leads to the formation of the membrane-bound death inducing signaling complex (DISC), which recruits the initiator procaspase-8 by the adaptor protein FADD. The intrinsic pathway responds to "intracellular" signals such as DNA damage, oncogene activation, nutrient deprivation, and lineage information (Elmore 2007). The final common end point of both pathways is initiation of execution stage of apoptosis and result in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Elmore 2007). Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD and CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies (Elmore 2007). So, the apoptosis process is very complex and regulated numerous pro- and anti-apoptotic factors.

The role of apoptosis in endometriosis development is studied very intensively at last time. It was demonstrated that apoptosis aids in maintaining cellular homeostasis during the menstrual cycle by eliminating aging cells from the functional layer of the uterine endometrium (Agic et al., 2009). In the normal endometrium apoptosis was detected in the glandular epithelium of late secretory and menstruating endometrium, while very little apoptosis was detected during the proliferative or at the beginning of the secretory phase (Harada et al., 2004). Eutopic endometrium from women with endometriosis has some differences in apoptosis compared with normal endometrium. These differences could contribute to the survival of the regurgitating endometrial cells into the peritoneal cavity and the endometriosis development. It was found, that the percentage of apoptosis in sloughed endometrial cells was greatly reduced among women with endometriosis, implying that the number of surviving cells that enter the peritoneal cavity is greater in women who develop endometriosis (Harada et al., 2004). An increased expression of anti-

apoptotic factor Bcl-2 and decrease expression of pro-apoptotic factor Bax were found in proliferative eutopic endometrium from women with endometriosis (Meresman et al., 2000). These differences could contribute to the survival of regurgitating endometrial cells into the peritoneal cavity and development of endometriosis (Taniguchi 2011). In ectopic endometrium of women with endometriosis the level of Bcl-2 expression was significantly increased in stromal cells (Harada et al., 2004). In endometriotic lesions the alterations in expression of different apoptosis-associated genes, such as PTEN, p52 and Bcl-2 were also demonstrated (Nezha et al., 2008). Authors thought that these changes are analogous in tumor tissue and evidence about possible malignant transformation of endometriotic tissue. At the same time there are some data about absence of significant changes of apoptotsis both in eutopic and in ectopic endometrium in women with endometriosis (Hassa et al., 2009). So, the study of the mechanisms, regulating apoptosis in endometriosis, need to be continued.

We also have checked out the expression of mRNA of some genes, possessed both pro- and anti-apoptotic action, in eutopic and ectopic endometrium of women with endometriosis. It was found that the expression of genes, regulating apoptosis, in endometrial tissue of women with endometriosis significantly differed from that in healthy women (Table 1).

| Parameter, copies number/μl | Endometrium, control (n=7) | Eutopic endometrium, endometriosis (n=15) | Ectopic endometrium, endometriosis (n=15) |
|---|---|---|---|
| mRNA MT-1 | 361.89±124.74 | 46.56±23.76 * | 42.36±17.42* |
| mRNA XIAP | 0.38±0.18 | 22.51±9.62* | 2.90±1.59x |
| mRNA caspase-3 | 0.72±0.33 | 0.38±0.09 | 3.80±1.25*xx |
| mRNA HSP27 | 0.23±0.05 | 0.22±0.08 | 1.75±0.55*x |

Table 1. The level of the expression of mRNAs genes, regulating apoptosis, in the eutopic and ectopic endometrium of women with endometriosis (* – given in comparison to the endometrium of healthy women, *- $p<0.05$; x - given in comparison to the eutopic endometrium of women with endometriosis, x – $p<0.05$, xx- $p<0.01$)

In eutopic endometrium of women with endometriosis the expression of anti-apoptotic factor XIAP (X-linked inhibitor of apoptosis) mRNAs were statistically increased compared to that in the endometrium of women from the control group.

In ectopic endometrial tissue the high levels of caspase-3 and heat shock protein 27 (HSP27) mRNAs expression were seen. Both in eutopic and ectopic endometrium of endometriosis women the low level of metallothionein-1 (MT-1) mRNA expression was found compared to that in the endometrium of women without endometriosis.

Thus, the profile of genes, regulating apoptosis, was different in eutopic and ectopic endometrium in endometriosis except the expression of MT-1 mRNA which was decreased both in eutopic endometrium and in endometriotic lesions. It is known that metalloteoneins (MTs) are a group of ubiquitous low-molecular-weight proteins essential for the protection of cells against heavy metal ion toxicity. These molecules also are directly involved in regulation of cell growth, differentiation and apoptosis in different pathological conditions and in tumour as well (Inoue et al., 2009). Earlier the low level of MT-1 protein was demonstrated in ovarian endometriomas (Wicherek et al., 2006).

Likely, diminishment of the MT-1 synthesis in endometrium of women with endometriosis might lead to the impairment of apoptosis control during this pathology. Another important apoptosis regulator, XIAP, is one of the members of IAP (inhibitory apoptosis proteins) family (Mufti et al., 2007). IAPs proteins are selectively bind and inhibit caspases-3, -7 and -9. XIAP is the only member of this family able to directly inhibit both the initiation and execution phase of the caspase cascade. XIAP is frequently over-expressed in malignant cells and is associated with poor clinical outcome (Schimmer et al., 2006). According to our data XIAP synthesis was significantly increased in eutopic endometrium in endometriosis, but in endometriotic lesions the level of XIAP mRNA expression didn't differ from that in normal endometrium. The high level of XIAP mRNA expression in eutopic endometrium possibly can lead to the decrease of spontaneous apoptosis in endometrium of women with endometriosis and in menstrual endometrium as well. This phenomenon might be responsible for the elevation of viability of endometrial cells in peritoneal cavity and play an important role at the initial stages of endometriotic lesions formation. But the absence of the elevation of XIAP synthesis in ectopic endometrium evidence in favor of the benign character of endometriotic lesions growth. Likely, the growth of already formed endometriotic tissue isn't under the XIAP control. Evidently, mechanisms, regulating endometrial XIAP synthesis, are different in eutopic and ectopic endometrium. The same was noted for the caspase-3 and HSP27. Only in ectopic endometrium we have seen the high level of the caspase-3 and HSP27 synthesis compared to that in endometrium of healthy women. From one side, these results are controversial, because caspase-3 is one of the main proapoptotic factors, a central effector caspase involved in numerous apoptotic pathways (Voss et al., 2007). On the contrary, HSP27 is well known inhibitor of apoptosis because the most heat shock proteins and HSP27 as well have strong cytoprotective effects and behave as molecular chaperone for other cellular proteins (Schmitt et al., 2007). HSP27 can bind to pro-caspase-3 to prevent its cleavage and activation by caspase-9 (Pandey et al., 2000). It does so by directly sequestering cytochrome c when released from the mitochondria into the cytosol (Schmitt et al., 2007). But as it is known HSP27 can interact with different partners implicated in the apoptotic process. For example, under stress conditions HSP27 increases IκBα ubiquitination/degradation, which results in an increase in NFκ-B activity and increased survival (Parcellier et al., 2003). So, the high level of HSP27 synthesis in endometriotic lesions can protect cells form apoptosis in caspase-independent pathway. Possibly the thin balance between mechanisms inducing and inhibiting apoptosis exists in endometriosis tissue. The growth of the endometriotic tissue was accompanied by the high level of synthesis of anti-apoptotic molecules HSP27. These changes are similar to that in the cancer cells and might be responsible for the invasion of endometrium into the peritoneum in the process of lesions formation. But in contrast to tumor cells the high level of caspase-3 mRNA expression in endometriosis lesions might provide the benign character of its growth.

Thus, both literature data and our own results evidence the impairment of apoptosis regulation in endometriosis. It must be special noted, that mechanisms of regulation of apoptosis in eutopic and ectopic endometrium are different. In eutopic endometrium the expression profile of apoptosis-related genes possibly contribute to the increase of viability of endometrial cells. But in endometriotic lesions we found the simultaneously elevated expression of both pro- and anti-apoptotic genes. Likely, this phenomenon might be responsible for benign type of ectopic growth of endometrium in endometriosis.

### 3.2 Changes of the invasiveness of endometrium in endometriosis

It has been shown that early lesions formation is an invasion event that requires breakdown of the extracellular matrix (ECM) proteins (Nap et al., 2004). The ECM consists of collagens, proteoglycans and glycoproteins, including fibronectin and laminin (Curry & Osteen, 2003). The ECM has become recognized as a key regulatory component in cellular physiology, providing an environment for cell migration, division, differentiation, anchorage, and, in some cases, an ultimate fate between cell survival and cell death. Additionally, the ECM is important in metabolic processes, influencing cellular proliferation, differentiation and apoptosis and it serves as a repository for biologically active growth factors (Nap et al., 2004). The highly regulated control of ECM turnover and homeostasis occurs, in part, by the action of a specific class of proteolytic enzymes. Supposedly, enzymes which belong to the system of plasminogen and matrix metalloproteinase family play the important role in ECM remodeling during endometriotic lesions formation (Vinatier et al., 2001).

Plasminogen, a ubiquitous protein secreted by liver, is activated into a protease plasmin by two types of activators. These are tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which bind to a specific cellular receptor (uPA-R). uPA initiates pericellular proteolysis of the ECM. This system is perfectly controlled by powerful inhibitors, some at the level of the plasminogen activators (type 1 and type 2 Plasminogen Activator Inhibitor, PAI-1 and PAI-2, respectively), and others at the level of the plasmin. uPA plays an important role in uterine physiology and in menstruation (Vinatier et al., 2001). Some results evidence about involvement of plasminogen system in mechanisms of endometriosis development. It was found that concentrations of uPA and PAI-1 were strongly increased both in endometriotic lesions and in matched eutopic endometrium of women with endometriosis compared to that in healthy women (Bruse et al., 1998). But there are some works which didn't find any differences in the expression of the proteins from plasminogen system in endometrial tissue in endometriosis (Vinatier et al., 2001). So, the role of these enzymes in endometriosis is not clear yet.

In last years the role of proteases from matrix metalloproteinase (MMPs) family in mechanisms of endometriosis establishment is intensively investigated. It was found that the MMP system is actively involved in the control of different aspects of reproductive function. In the ovary and uterus, MMP system regulates the dynamic structural changes that occur throughout the menstrual cycle (Curry & Osteen, 2003). The MMPs family consists of several structurally related $Zn^{2+}$-dependent secreted endopeptidases. Currently, it is recognized more that 26 endopeptidases that include four broad classes: the collagenases, gelatinases, stromelysins, and membrane type enzymes (MT-MMPs). These proteinases exhibit numerous structural and functional similarities. All of them have conserved domain structures and specific domains related to substrate specificity and recognition of other proteins (Amalinie et al., 2010). Common features of the MMPs family include: 1) the presence of zinc in the active site of the catalytic domain, 2) synthesis of the MMPs as preproenzymes that are secreted in an inactive form, 3) activation of the latent zymogen in the extracellular space, 4) recognition and cleavage of the ECM by the catalytic domain of the enzyme, and 5) inhibition of enzyme action by both serum-borne and tissue-derived metalloproteinase inhibitors or TIMPs in the extracellular environment (Curry & Osteen, 2003). Although similarities exist in the structure of the MMPs, there are also distinct differences in the recognition and specificity for components of the ECM. For

example, collagenases (MMP-1, MMP-8, and MMP-13) degrade native fibrillar collagen of types I, II, III, V, and XI. Gelatin, another important protein of ECM, is susceptible to a wide range of tissue proteinases, including the gelatinases MMP-2 and MMP-9. The stromelysin enzymes (MMP-3, MMP-7, MMP-10, and MMP-11) act on a broad and diverse array of ECM substrates. Both gelatinases and stromelysins are capable of degrading major constituents of basement membranes, including type IV collagen, laminin, and fibronectin. In addition to degrading the ECM, the MMPs exhibit activity toward other MMPs, growth factors, and cytokines such as IGF binding proteins, epidermal growth factor (EGF), TNF-α (Curry & Osteen, 2003).

Involvement of MMPs in endometriosis development was suspected after collagen breakdown products were found in the peritoneal fluid of patients with endometriosis (Spuijbroek et al., 1992) and since that time many studies have demonstrated that the pattern of MMPs expression in endometrium and peritoneal fluid of women with endometriosis significantly differ from that in healthy women (Nap et al, 2004). Increased levels of MMP-1, MMP-2, MMP-7 and MMP-9 were detected in peritoneal fluid of patients with endometriosis (Amalinie et al., 2010). At the same time the amount of the MMP-13 in peritoneal fluid of patients was significantly decreased in comparison to that in healthy women (Laudanski et al., 2005). In eutopic endometrium of women with endometriosis the elevation of the expression of MMP-1, MMP-2, MMP-3, MMP-9 was found (Di Carlo et al., 2009; Shaco-Levy et al., 2008). Diminishment of the expression of MMPs inhibitors TIMP-1 and TIMP-2 in endometrium of endometriosis patients was also noted (Colett et al., 2004; Uzan et al., 2004). The study of the character of MMPs production at the level of ectopic endometrium had shown that MMP-2 and MMP-3 overexpression were related to the infiltrative nature of endometriotic lesion (Uzan et al., 2004). It was also shown that circulating mRNA for MMP-3 was significantly higher in peripheral blood of patients with endometriosis than in control patients, regardless of the degree of endometriosis severity (De Sanctis et al., 2011). In a prospective, blinded, longitudinal study MMP-2 and MMP-9 were more likely to be detected in the urine of patients with endometriosis than in controls (Becker et al., 2010). Thus, practically all received data evidence about the increased production of MMPs both at the systemic and local level in endometriosis. But there are some reports that contradict these results. For example, Colett with coworkers didn't find any difference in the MMP-2 production in endometrial tissue of women with endometriosis in comparison with healthy women (Colett et al., 2004). Very low production of MMP-9 by endometrial cells was seen in endometriosis women (Sillme et al., 2001). So, the role of MMPs in endometriosis pathogenesis is still unclear.

The majority of works are only descriptive and functional involvement of MMPs in the development or progression of endometriosis has not been proven. It is very difficult to study the ectopic endometrium in humans as controlled experiments are limited, because it is not possible to monitor the disease progression without repeated laparoscopies which is difficult on many grounds. In such cases animal models are an extremely important tool in elucidating the pathogenesis of the disease. Animals with experimental endometriosis let us to evaluate the different stages of ectopic lesion formation. Both nonprimate and primate models have been used to study endometriosis for many years. Nonprimates, including rodents, do not undergo spontaneous disease, but it can be induced using either autologous uterine tissue or human endometrium (Story and Kennedy, 2004). Up to now

the prominent changes of MMPs production in animals with experimental endometriosis have been demonstrated. The level of MMP-3 and MMP-9 was significantly higher in endometriotic lesions in rats with experimental endometriosis than in matched eutopic endometrium (Machado et al., 2010; Cox et al., 2001). Suppression of MMP-2 and MMP-9 activity in mice led to the inhibition of endometriosis progression in animals with the decrease of endometriotic lesions weight (Chen et al., 2010). Investigation of the role of MMPs in human endometrial explants in a chicken chorioallantoic membrane model (CAM) of endometriosis allowed to establish that endometrium both of healthy women and patients with endometriosis showed a statistically significant increase of MMP-1 mRNA expression 24, 48, 72, and 96 hours after transfer to the CAM and no statistically significant difference regarding the MMPs mRNA expression was shown for endometrium of healthy women and endometriosis patients (Juhasz-Boss et al., 2010). Earlier we have also studied the synthesis of mRNA of MMP-2 and TIMP-2 in rat ectopic endometrium at different stages of experimental endometriosis development. We found that the dynamic of MMP-2 mRNA expression in ectopic experimental lesions was characterized by its significant increase at $7^{th}$ day after transplantation comparing to that in native uterine endometrium with subsequent declining to normal values at $14^{th}$ and $21^{st}$ days after surgery. The level of TIMP-2 mRNA expression on the contrary was sharply decreased at early and middle stages of endometriotic lesions formation and was elevated at the late stage of experimental endometriosis comparing to that in intact uterine endometrium (Sotnikova et al., 2010). So, during the first 7 days of experimental endometriosis lesions development, the balance of pro- and anti-proteolytic activity in endometrial tissue was shifted towards MMP-2 prevalence. It is known that during this period the maximal level of endometrial tissue invasion and cell proliferation are seen in rat endometriosis model. Our results evidence about the coincidence of high level of MMP-2 expression and high tissue's invasiveness in early experimental endometriosis lesions and allow us to suggest the direct involvement of MMP-2 in invasion of endometrial cells at the initial stage of endometriosis development.

The role of MMP-2 and MMP-9 in endometriosis development has a special interest. It was demonstrated that these two gelatinases actively participate in tumor invasion and progression (Amalinei et al., 2010). Initially, MMP-9 or gelatinase-B, was considered a key MMP in the invasion and metastasis, overexpressed by cancer cells and induced by several cytokines, growth factors and oncogene products (Okada et al., 2001), and its inhibition resulting in loss of metastatic potential. Subsequent studies demonstrated that the activation ratios of pro-MMP-2, not of pro-MMP-9, correlate with lymph node metastasis in breast, lung, thyroid and digestive tract carcinomas (Amalinei et al., 2010). MMP-2 has been shown to play a key role in promotion of invasiveness both of normal and neoplastic cells. Cellular localization in many tumor tissues indicates that MMP-2 mRNA appears to be localized to the stromal fibroblast adjacent to the sites of tumor invasion (Amalinei et al., 2010). It was also shown that MMP-2 and MMP-9 expression are closely associated with the parameters of tumor aggressiveness (Karahan et al., 2007) and tumor growth is reduced by the absence of MMP-2, and the metastatic processes is reduced by the lack of MMP-9 ( Egeblad & Werb, 2002). But MMP-2 and MMP-9 are not tumor specific and are involved in ECM remodeling in a wide range of non-neoplastic processes, including embryonic development, trophoblastic invasion, angiogenesis, T-cell transmigration and wound healing (Amalinei et al., 2010).

Endometriosis often is defined as benign tumor. Taking into account literature data about action of MMP-2 and MMP-9 during tumor progression we checked out synthesis on these MMPs and its inhibitors TIMP-2 and TIMP-1 in eutopic and ectopic endometrium of healthy women and women with endometriosis. We found, that in eutopic endometrium of women with endometriosis the expression of MMP-2, TIMP-2 and TIMP-1 mRNAs were statistically increased compared to that in the endometrium of women from control group. In ectopic endometrial tissue the high levels of MMP-2, MMP-9 and TIMP-1 mRNAs expression were seen (Table 2). So, the distinct characteristic of endometriotic lesion is the high level of MMP-2 and MMP-9 synthesis which might provide invasive character of ectopic endometrium growth. But the eutopic endometrium doesn't posses such invasive activity. Evidently, some factors may influence upon MMPs synthesis in endometrial tissue of women with endometriosis elevating the mRNA expression of MMP-2 and MMP-9.

| Parameter, copies number/µl | Endometrium, control (n=5) | Eutopic endometrium, endometriosis (n=17) | Ectopic endometrium, endometriosis (n=18) |
|---|---|---|---|
| mRNA MMP-2 | 0.89±0.27 | 2.40±0.63* | 5.89±2.29* |
| mRNA TIMP-2 | 4.09±0.74 | 13.53±3.94* | 6.44±1.59 |
| mRNA MMP-9 | 0.27±0.16 | 1.37±0.62 | 13.78±5.04*x |
| mRNA TIMP-1 | 2.72±1.33 | 24.44±9.02* | 87.73± 31.19* |

Table 2. The level of the expression of mRNAs for genes, regulating invasiveness, in the eutopic and ectopic endometrium of women with endometriosis (* – given in comparison to the endometrium of healthy women, *- p<0.05; x – given in comparison to the eutopic endometrium of women with endometriosis, x – p<0.05)

It must be noted that the balance of pro- and anti-proteolytic enzymes synthesis was different in eutopic end ectopic endometrium (Fig.1). In eutopic endometrium we have seen the significant prevalence of the mRNA expression of specific inhibitors of MMPs - TIMP-2 and TIMP-1 upon the activity of MMP-2 and MMP-9, respectively. But in ectopic endometrium the level of mRNA expression of MMP-2 and TIMP-2 was practically the same (Fig.1). This situation allows us to compare the growth of ectopic endometrium with tumor growth. It is well known that in urothelial cancer patients the mean MMP-2/TIMP-2 ratio in patients with recurrence is significantly higher than that in patients without recurrence and the disease-free survival of patients with high MMP-2/TIMP-2 ratio is extremely poor compared with that of patients with lower ratios (Gohji et al., 1996). It was demonstrated also that evaluation of MMP-2: TIMP-2 mRNA balance may constitute an early prognostic indicator of human cancer (Onisto et al., 1995). Evidently, the elevation of the balance of MMP-2 and TIMP-2 in endometriotic tissue results in increase of invasive potential of ectopic endometrium. But mechanisms regulating such changes need to be further elucidated. It can be supposed that peritoneal fluid factors can directly influence upon endometrial invasiveness. But in our case we didn't observe such changes in the balance of MMP-9 and TIMP-1. Moreover, the synthesis of TIMP-1 in ectopic endometrium was very high. From one side these changes can be connected with the specific action of TIMP-1 during tumor progression. It was shown that in certain situations TIMP-1 can act as growth promoting factor and/or antiapoptotic factor to the cancer cells (Mannello & Gazzanelli, 2001). And high level of TIMP-1 synthesis might be attributive to enhance the invasive

growth of ectopic lesion. But from another side, it was shown that excessive levels of TIMP-1 protein such as those secreted by endometriotic lesions into the peritoneal cavity negatively affects the reproduction function of rats with experimental endometriosis (Stilley et al., 2010). It was also shown that the rat embryo treated in vitro with endometriotic peritoneal fluid concentrations of TIMP-1 developed abnormally, and rats treated with a TIMP-1 function-blocking antibody had normal zygote, follicle and embryo quality (Stilley et al., 2010). Summarizing their results authors hypothesize that excessive TIMP-1 was deleterious to ovulation and embryo development because endometriotic lesion-secreted TIMP-1 can translocate to ovary/or oviduct and cause poor preimplantation embryo quality, developmental arrest and the subsequent embryo loss found in endometriosis (Stilley et al., 2010). Thus, the high level of TIMP-1 synthesis in ectopic endometrium might be associated with mechanisms of infertility development in women with endometriosis.
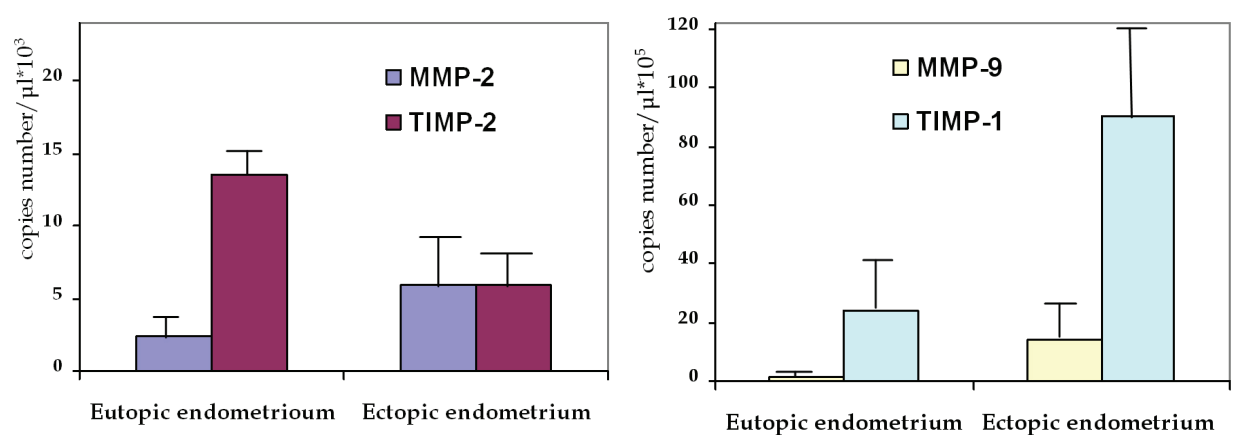


Fig. 1. Comparative characteristic of the synthesis of the enzymes with pro- and anti-proteolytic activity in eutopic and ectopic endometrium of women with endometriosis

Thus, endometriotic lesion establishment and growth are accompanied by the impairment of regulation of apoptosis and invasion in endometrial tissue. These changes at the level of eutopic endometrium possibly are associated with the increase of the viability of endometrial cells and participate in early lesions formation events. At the level of already formed ectopic endometrium we found the significant increase of invasive capacity and elevated synthesis of both pro- and anti-apoptotic factors which might be responsible for the benign type of ectopic lesions growth. Earlier it was suggested that many differences observed between eutopic and ectopic tissue of a patients with endometriosis can be explained as the direct consequence of the different environment of peritoneal fluid (Hara et al., 2004). So, the next step of our investigation was to study the possible peritoneal fluid factors influence upon apoptosis and invasiveness of endometrial cells.

## 4. Influence of peritoneal macrophages and peritoneal fluid upon mechanisms, regulating apoptosis and invasiveness of endometrium in endometriosis

The mechanisms by which regurgitated endometrial cells are cleared from the peritoneal cavity in the majority of women are poorly understood yet. However, it has been suggested that a peritoneal microenvironment might subserve this role (Vigano et al., 2004). There are

two main factors which can contribute to the mechanisms regulating endometrial apoptosis and invasion – soluble biologically active factors of peritoneal fluid, including numerous cytokines, growth factors, metabolites of arachidonic acid, reactive oxygen species, etc. and peritoneal macrophages, the most abundant leukocytes population in peritoneal fluid. To estimate the possible influence both of humoral and cellular components of peritoneal fluid upon the apoptosis and invasiveness of endometrial cells which entered the peritoneal cavity due to the menstrual reflux, we had incubated explants of eutopic endometrial tissue in the presence of autologous peritoneal macrophages or in the presence of autologous peritoneal fluid. After termination of incubation we assessed the level of expression of genes regulating apoptosis and invasiveness in endometrial tissue.

## 4.1 Influence of peritoneal macrophages and peritoneal fluid upon apoptosis of eutopic endometrial tissue in healthy women and in women with endometriosis

Accumulation evidences suggest that apoptosis helps to maintain cellular homeostasis during the menstrual cycle through the elimination of senescent cells from the functional layer of the uterine endometrium during the late secretory and menstrual phases of the cycle (Vigano et al., 2004). It was proposed that in healthy women the majority of menstrual reflux cells undergo programmed cells death and do not survive in the peritoneal fluid (Gebel et al., 1998; Nasu et al., 2009). On the contrary in women with endometriosis the percentage of menstruated endometrial cells undergoing apoptosis is greatly reduced, increasing the number of surviving cells that could continue to exhibit physiological activity (Vigano et al., 2004). In our work we have checked out the influence of peritoneal fluid factors upon apoptosis endometrial cells both in healthy women and in women with endometriosis to elucidate the mechanisms responsible for elimination of menstrual endometrial cells from peritoneal cavity in women with and without endometriosis.

It was found that in women without endometriosis incubation of the endometrial explants with macrophages had led to the significant increase of the caspase-3 and MT-1 mRNAs expression in endometrial tissue (Table 3). Peritoneal fluid in this case didn't influence upon apoptosis-related gene expression in endometrial tissue.

| Parameter, copies number/μl | Endometrium +RPMI 1640 (control) (n=10) | Endometrium+ autologous macrophages (n=10) | Endometrium + autologous peritoneal fluid (n=10) |
|---|---|---|---|
| mRNA MT-1 | 21.51±2.59 | 54.19±13.90* | 68.79±21.68 |
| mRNA XIAP | 0.73±0.15 | 0.73±0.16 | 1.26±0.62 |
| mRNA caspase-3 | 15.69±5.91 | 39.51±9.64* | 131.21±73.54 |
| mRNA HSP27 | 5.28±3.02 | 8.57±0.45 | 13.27±2.45 |

Table 3. The influence of peritoneal macrophages and peritoneal fluid upon the expression of mRNAs of genes, regulating apoptosis, in endometrium of healthy women (* – given in comparison to the control, *- $p<0.05$)

We also estimated the amount of apoptotic endometrial stromal cells in endometrium of healthy women after its incubation with macrophages or with peritoneal fluid in standard test with Annexin V and propidium iodide (Pi) staining. It was established that both

macrophages and peritoneal fluid of healthy women increased the amount of apoptotic Annexin V+ endometrial cells (Table 4). The elevation of the amount of apoptotic cells was noted predominantly in population of endometrial stromal cells, entered in the late/irreversible apoptosis.

| Parameter, % | Endometrium +RPMI 1640 (control) (n=5) | Endometrium+ autologous macrophages (n=5) | Endometrium + autologous Peritoneal fluid (n=4) |
|---|---|---|---|
| Annexin V+ | 63.36±2.86 | 78.14±4.21* | 81.90±1.24*** |
| Annexin V+Pi- | 25.62±2.96 | 29.54±2.24 | 27.55±2.35 |
| Annexin V+Pi+ | 37.74±4.12 | 53.23±4.25* | 54.35±2.25** |

Table 4. The influence of peritoneal macrophages and peritoneal fluid upon apoptosis of eutopic endometrium stromal cells in healthy women (* – given in comparison to the control, *- $p < 0.05$, **- $p < 0.01$, ***- $p < 0.001$)

So, our results let us to conclude that in physiological conditions the endometrial cells are effectively eliminated from the peritoneal cavity due to induction of apoptosis by peritoneal macrophages. Peritoneal fluid, according to our data, is less important for induction of apoptosis in endometrium of healthy women. Only macrophages of women without endometriosis are capable to increase the synthesis one of the most important pro-apoptotic factor - caspase-3 in autologous endometrial tissue. Earlier it was shown that the control of tissue homeostasis by induction of apoptosis in aging and transformed cells is one of the important macrophages function (Gordon & Freedman, 2006). It is also known that apoptotic cells are rapidly engulfed by phagocytes in a process akin to macro pinocytosis. Normally, the uptake of apoptotic cells is accompanies by the induction of anti-inflammatory cytokine synthesis of phagocytes (Erwig & Henson, 2007). Our results have shown that MT-1 synthesis significant elevated in endometrium of healthy women after its incubation with peritoneal macrophages. It is known that metallothioneins demonstrated strong antioxidant properties and able to scavenge a wide range of reactive oxygen species thus serving as anti-inflammatory mediators (Inoue et al., 2009). So, the increase of MT-1 synthesis in endometrial cells, undergoing apoptosis, might be one of the factors, participating in the development of anti-inflammatory environment in the process of endometrial apoptotic cells uptake by phagocytes. Summarizing our data about the influence of peritoneal fluid factors upon apoptosis of endometrial cells in healthy women it can be concluded that the increase of the amount of endometrial stromal cells at the late stages of apoptosis after its incubation with autologous peritoneal macrophages or with peritoneal fluid is physiological process that prevent the survival and growth of menstrual endometrial cells in peritoneal cavity.

In women with endometriosis the response of endometrial cells upon the influence of peritoneal fluid factors significantly differed from that in healthy women. In vitro incubation of endometrial explants of women with endometriosis with the autologous peritoneal macrophages had led to the decrease of MT-1, XIAP, and caspase-3 mRNAs expression (Table 5). In these conditions the peritoneal fluid of endometriosis women significantly increased the level of HSP27 mRNA expression (Table 5) and diminished the amount of early apoptotic endometrial stromal cells (Table 6).

| Parameter, copies number/µl | Endometrium +RPMI 1640 (control) (n=10) | Endometrium+ autologous macrophages (n=10) | Endometrium + autologous peritoneal fluid (n=10) |
|---|---|---|---|
| mRNA MT-1 | 43.95±7.88 | 13.02±4.03** | 26.82±7.12 |
| mRNA XIAP | 0.47±0.19 | 0.04±0.01* | 0.19±0.06 |
| mRNA caspase 3 | 18.52±8.20 | 0.42±0.22* | 2.81±1.09 |
| mRNA HSP27 | 14.52±6.20 | 12.86±5.52 | 70.32±26.19* |

Table 5. The influence of peritoneal macrophages and peritoneal fluid upon the expression of mRNAs of genes, regulating apoptosis, in eutopic endometrium of women with endometriosis (* – given in comparison to the control, *- $p<0.05$)

| Parameter, % | Endometrium +RPMI 1640 (control) (n=8) | Endometrium+ autologous macrophages (n=8) | Endometrium + autologous peritoneal fluid (n=5) |
|---|---|---|---|
| Annexin V+ | 59.96±4.67 | 54.76±3.68 | 45.35±3.38* |
| Annexin V+Pi- | 24.59±1.88 | 20.35±1.54 | 17.38±2.46* |
| Annexin V+Pi+ | 35.00±5.28 | 34.79±3.57 | 28.00±3.11 |

Table 6. The influence of peritoneal macrophages and peritoneal fluid upon apoptosis of eutopic endometrium stromal cells in women with endometriosis (* – given in comparison to the control, *- $p<0.05$).

Thus, the influence of both peritoneal macrophages and peritoneal fluid of women with endometriosis upon apoptosis of autologous eutopic endometrium was opposite to that in healthy women. Peritoneal macrophages decreased the expression of pro-apoptotic genes, and peritoneal fluid increased the synthesis of anti-apoptotic protein HSP27, diminishing the early apoptosis of endometrial stromal cells. We have seen only one exception from the anti-apoptotic action of peritoneal fluid factors in endometriosis. Peritoneal macrophages of women with endometriosis decreased the level of mRNA expression of apoptosis inhibitor factor XIAP. We thought that this effect might be responsible for the phenomenon of low expression of XIAP gene in ectopic endometrium in vivo. But in the general the action of peritoneal factors of women with endometriosis was directed to the inhibition of apoptosis of autologous endometrial cells. Evidently, this action of peritoneal fluid factors might serve as one of the fundamental mechanisms leading to survival of endometrial cells in peritoneal cavity and promotion of ectopic lesions formation and growth. Our results are in a good accordance to the literature data. Earlier it was shown that addition of plasma or peritoneal fluid of women with endometriosis to in vitro cultures of neutrophils from healthy donors reduced the percents of apoptotic cells (Kwak et al., 2002). Decrease of the sensitivity of endometrial cells of women with endometriosis to cytolysis by peritoneal macrophages was also noted (Dmowski et al., 1998). But all these works didn't light the mechanisms of apoptosis-inhibitory action of peritoneal fluid and peritoneal macrophages of women with endometriosis.

## 4.2 The influence of peritoneal macrophages and peritoneal fluid upon the invasiveness of eutopic endometrial tissue in endometriosis women

We also established that peritoneal fluid factors directly influenced upon invasive capacity of endometrial cells of women with endometriosis. In vitro incubation of endometrial explants of women with endometriosis with the autologous peritoneal macrophages had led to the decrease of TIMP-1 and TIMP-2 mRNAs expression and the peritoneal fluid of endometriosis women significantly increased the level of MMP-2 mRNA expression by eutopic endometrial cells (Table 7).

| Parameter, copies number/µl | Endometrium +RPMI 1640 (control) (n=6) | Endometrium+ autologous macrophages (n=6) | Endometrium + autologous peritoneal fluid (n=6) |
|---|---|---|---|
| mRNA MMP-2 | 4.83±2.40 | 2.97±0.89 | 20.44±6.19* |
| mRNA TIMP-2 | 10.36±2.60 | 1.76±0.64* | 8.25±2.57 |
| mRNA MMP-9 | 0.18±0.09 | 0.17±0.13 | 0.98±0.68 |
| mRNA TIMP-1 | 10.12±3.91 | 0.52±0.25* | 15.00±6.98 |

Table 7. The influence of peritoneal macrophages and peritoneal fluid upon the expression of mRNAs of genes, regulating invasiveness, in eutopic endometrium of women with endometriosis (* – given in comparison to the control, *- $p<0.05$).

So, in endometriosis both soluble factors of peritoneal fluid and macrophages evidently increased the invasiveness of eutopic endometrial cells. The literature data about stimulatory action of peritoneal fluid from women with endometriosis upon uPA protein expression in endometrial cell culture from women with and without endometriosis are in a good accordance with our results (Cosin et al., 2010).

Results of our experiments, received after estimation of the invasiveness of endometrial stromal cells of women with endometriosis in matrigel system also have shown the prominent stimulatory effect of soluble factors of peritoneal fluid of women with endometriosis on endometrial cells invasiveness. We found the enhanced invasion of endometrial stromal cells in matrigel in the presence of autologous peritoneal fluid or in the presence of supernatants of 24-hours cultures of peritoneal macrophages of women with endometriosis (Fig.2). In every case we estimated the action of autologous peritoneal fluid, and the action of pooled supernatants of peritoneal macrophages cultures. When endometrial cells migrated through membrane pores with matrigel only in presence of culture media RPMI 1640 we have seen the only few migrated cells (coefficient of spontaneous invasion – 53.52±23.33%). In the cases of endometrial stromal cells invasion in the presence of autologous peritoneal fluid, coefficient of invasion was 71.85±2.15%, and addition of supernatants of peritoneal macrophages cultures in culture media elevated the coefficient of invasion to 76.07±1.43% ($p<0.001$ in both cases).

Thus, summarizing our results about the influence of peritoneal fluid factors on apoptosis and invasiveness of endometrial cells in endometriosis, it might be concluded that immunosurveillance in the peritoneal cavity of women with endometriosis promotes conditions favorable for apoptosis inhibition and invasiveness facilitation of endometrial cells. Incapability of macrophages of women with endometriosis to eliminate the ectopic

cells from the peritoneal cavity and the stimulatory effect of humoral peritoneal factors on the synthesis of proteolytic enzymes by endometrial cells might be considered as the important pathogenetic mechanisms of endometriotic tissue formation and growth.
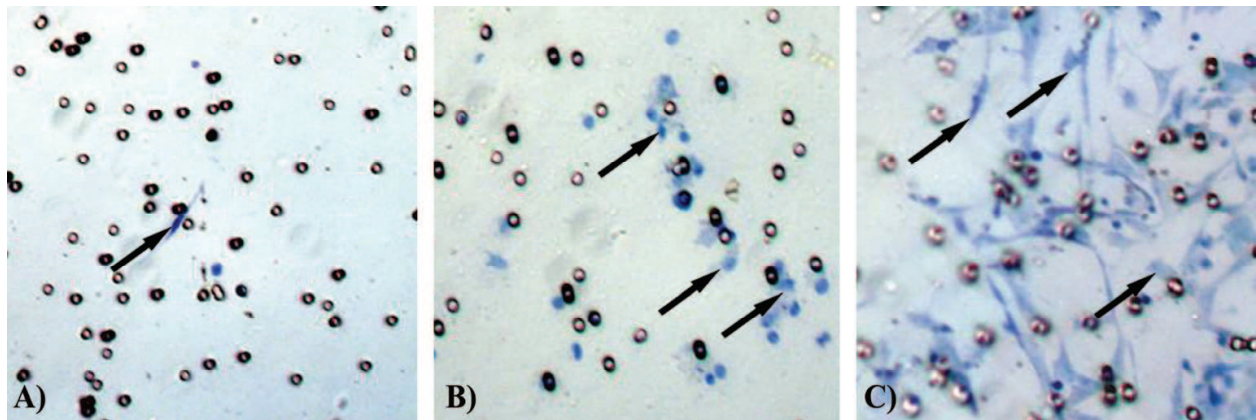


Fig. 2. Invasion of eutopic endometrium stromal cells of women with endometriosis. Holes represent the 8-μm pore in the filter. A- spontaneous invasion of endometrial stromal cells in RPMI 1640 medium; B - invasion of endometrial stromal cells in the presence of the supernatants of 24-h culture of macrophages; C - invasion of endometrial stromal cells in the presence of the autologous peritoneal fluid. Arrows indicate cells. (Original magnification x200)

## 5. Immune response in peritoneal cavity in endometriosis

As we can see from our results and from number of literature data, behavior of endometrial cells, floating in peritoneal fluid, is directly regulated by the peritoneal fluid microenvironment. Deficiency of immune response in peritoneal cavity supposedly might prevent clearance of the retrograde menstrual debris from the peritoneal environment and permits the implantation of misplaced endometrial cells that result in the development of endometriosis (Dmowski & Braun, 2004). To define more precisely the mechanisms attributing to the impairment of immune response in peritoneal fluid, we have studied the functional state of peritoneal macrophages and peritoneal fluid cytokine profile in endometriosis.

### 5.1 Peritoneal macrophages in endometriosis

Macrophages are key cellular constituents of the immune response in peritoneal cavity because macrophages comprise up to 90% of peritoneal fluid cells (Tariverdian et al., 2007). It is well known that macrophages are ubiquitous immune cells that play important role in both innate and acquired immunity (Wu et al., 2004). In addition to protection the body from foreign organisms and antigens, macrophages maintain homeostasis in many tissues and in peritoneal cavity as well through their cytokine production and remodeling capabilities. With regard to female reproduction macrophages contribute to the regulation of the pituitary-gonadal axis and are found throughout female reproductive tissues including the ovary, uterus, oviduct and mammary glands (Wu et al., 2004). Macrophages derive from bone-marrow precursors which when mature enter the bloodstream as monocytes and subsequent migrate into tissues and to various body cavities, where they function primarily

as phagocytes when activated. Within tissues, differentiation of monocytes into macrophages occurs in response to the surrounding microenvironmental context, which directs the acquisition of tissue-specific phenotype. Within most organs macrophages are involved in tissue homeostasis via their ability to execute diverse functional activity, including (i) phagocytosis and degradation of foreign antigens, (ii) matrix dissolution and tissue remodeling,  and (iii) production and secretion of growth factors, cytokines and chemokines (Wu et al., 2004). These effector functions allow macrophages to regulate local immune and inflammatory responses as well as influence normal tissue function.

Macrophages are identified in tissues by their expression of specific proteins markers which are predominantly cell surface receptors. The proteins considered most exclusively restricted to macrophages are CD68 molecules, class II MHC antigens, receptors, that are involved in phagocytosis, including Fc receptors, complement receptors, integrins, mannose receptor, sialoadhesin and scavenger receptors (Wu et al., 2004). Macrophages are considered "professional phagocytes" and can internalize particles much more rapidly and efficiently then other cells due to their expression of specific cell surface receptors, which initiates actin polymerization and internalization of the foreign molecule or organism into a phagosome. Macrophages phagocytose endogenous and exogenous substances, such as cell debris, bacteria and viruses. Macrophages in vivo recognize and internalize apoptotic and necrotic cells (Wu et al., 2004). Macrophages sequestered in the peritoneal cavity remove red blood cells, damaged tissue fragments, apoptotic cells, and probably endometrial cells that gain access to the peritoneal cavity through the fallopian tubes (Dmowski & Braun, 2004).

In endometriotic peritoneal fluid the concentration and number of peritoneal macrophages are significantly increased as compared to healthy controls (Tariverdian et al., 2007). These are large, activated macrophages that produce high levels of smooth-muscle-contracting prostaglandins, such as PGE2 and PGF2α (Gazvani & Templeton, 2002; Dmowski & Braun, 2004). Elevated PGE2 in the peritoneal fluid of endometriosis patients due to macrophages activation have been proposed to subsequently aggravate endometriosis-associated pain by altering uterine and tubal contractility and cause infertility due to a delayed ovum transport (Tariverdian et al., 2007). But despite of activated status macrophages evidently are not capable to effectively control the growth of endometrial tissue in peritoneal cavity of women with endometriosis. Molecular basis for this phenomenon isn't completely elucidated yet.

One of the mechanisms by which abnormally functioning macrophages could contribute to the growth of ectopic endometrial cells is through defective scavenging activity (Sidell et al., 2002). Important function of macrophages in the face of an invading "foreign" material or when encountering cellular debris and apoptotic cells is the scavenger function (Sidell et al., 2002). A family of specific scavenger receptors (SRs) is involved in this activity. This family was aptly named because these receptors have been found to bind and "scavenge" a broad array of modified self and nonself ligands, including apoptotic cells, anionic phospholipids and amyloid and pathogen components (Moore & Freeman, 2006). The SRs are believed to be members of the group of pattern recognition receptors (PRR) that mediate the innate immune host response through recognition of highly conserved pathogen-associated molecular patterns (PAMP). This evolutionarily ancient but highly effective system of host defense enables the immune system to discriminate between "noninfectious self" and "infectious nonself". However, there is a growing body of evidence to suggest that SRs may recognize endogenous neoantigens and apoptotic cells through molecular mimicry of

microbial pathogen ligands (Moore & Freeman, 2006). In the last several years it was found that SRs initiate signaling cascades that regulate macrophages activation, lipid metabolism, and inflammatory programs. In addition, these receptors play role in the induction of apoptosis and apoptotic cell clearance. So, it is possible that SRs are directly involved in the clearance of endometrial debris from the peritoneal cavity. And it is also possible that peritoneal macrophages from women with endometriosis do not express fully functional scavenger receptors. But, now we know very little about the character of scavenger receptors expression by peritoneal macrophages of women with endometriosis. So, the study of the scavenger function of peritoneal macrophages in endometriosis has a special interest.

We have analyzed the expression of some surface functional receptors, such as integrin molecules CD11b and CD49e, HLA-DR molecules, CD95 and ligand for CD95 molecules (CD95L) and scavenger receptors SR-AI (CD204) and SR-B (CD36) as well by peritoneal macrophages of healthy women and women with endometriosis. It was found that peritoneal macrophages of women with endometriosis were characterized by the diminished level of the expression of scavenger receptors A and B type compared to that in healthy women (Table 8). More other, in women with endometriosis the amount of CD11b+, CD49e+, HLA-DR+ and CD95L+ macrophages in peritoneal fluid was also lower than that in healthy women (Table 8). We checked out the correlation between the character of receptors expression and the stage of endometriosis. All women with endometriosis were divided into two subgroups: women with mild and severe endometriosis. In subgroup with mild endometriosis the 1-2 stage of the disease was diagnosed during laparoscopy. Women with severe endometriosis have diagnosed of 3-4 stage of endometriosis during laparoscopic investigation.

| Parameter,% | Control group (n=10) | Endometriosis (n=30) | 1-2 stage of endometriosis (n=20) | 3-4 stage of endometriosis (n=10) |
|---|---|---|---|---|
| CD204 (SR-AI)+ | 68.31±5.37 | 53.20±2.36* | 50.88±3.20** | 56.10±2.15* |
| CD36 (SR-B)+ | 70.86±9.22 | 57.20±2.36* | 56.48±2.96* | 59.53±2.00* |
| CD11b+ | 85.14±1.57 | 71.97±2.30*** | 71.58±2.91*** | 73.10±3.19** |
| CD49e+ | 87.91±2.19 | 80.16±2.34* | 78.03±2.55** | 87.6±4.12 |
| HLA-DR+ | 87.35±2.28 | 78.35±2.43* | 80.59±2.37* | 71.97±6.23* |
| CD95+ | 48.12±3.16 | 51.39±2.54 | 47.46±4.32 | 54.02±3.05 |
| CD95L+ | 60.62±2.42 | 46.37±2.65*** | 45.62±3.42*** | 48.50±3.41** |

Table 8. Membrane expression of functional receptors by peritoneal macrophages of women with endometriosis (* - given in comparison to the control group, * - p<0.05, **- p<-0.01, ***-p<0.001)

Differential analysis of surface macrophages phenotype had shown that the expression of SRs, HLA-DR, CD11b and FasL molecules was significantly decreased both in mild and severe endometriosis compared to analogous parameters in control group (Table 8). The only association between the level of receptor expression and the stage of endometriosis was noted for CD49e molecules. The low expression of CD49e was seen only in women with 1-2 stage of the endometriosis, and in women with 3-4 stage of the disease the amount of CD49+ peritoneal macrophages was completely corresponded to that in healthy women. So,

peritoneal macrophages in endometriosis independently from the stage of the disease are characterized by the low level of expression of functional receptors, such as integrins, scavenger receptors and apoptosis-inducing molecules.

This aberrant expression of the surface receptors might lead to the impairment of peritoneal macrophages function in endometriosis which in turn can contribute to the rescue of endometrial cells from immunosurveillance in peritoneal cavity. It is known that CD11b and CD49e molecules belong to the class of integrins, superfamily of cell adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands and soluble ligands (Takakda et al., 2007). It is known that upon binding extracellular ligands, integrins generate an intracellular signal and, conversely, their functioning can be regulated by signals from within the cell. They serve as transmembrane links between extracellular contacts (other cells or the extracellular matrix) and the actin microfilaments of the cytoskeleton, whose behavior integrins also regulate and modulate. Extracellular ligation of integrins triggers a large variety of signal transduction events that modulate cell behaviors such as adhesion, proliferation survival or apoptosis, shape, polarity, motility and differentiation (Takakda et al., 2007). The low level expression of integrins by peritoneal macrophages might lead to the impairment of the interaction of macrophages with ECM and other cells, including the cells of reflux menstrual endometrium. More other, it is known, that if the peritoneal macrophages are not attached to extracellular matrix components, despite their differentiated status may not be competent scavengers (Sidell et al., 2002). Our results about the simultaneously diminishment of the expression of integrins and scavenger receptors A and B type by peritoneal macrophages of women with endometriosis completely confirmed the hypothesis that the defective scavenger function plays an important role in the immune mechanisms of endometriosis development.

Today the A class of SRs has grown to include 5 members that share common collagen-like domains and homotrimeric structure. SR-A receptors bind oxidized low-density lipoproteins, apoptotic cells, β-amyloid peptide, anionic phospholipids and advanced glycation end-products (Murphy et al., 2005). These receptors have also been implicated in both innate and adaptive immune responses through their recognition of pathogens and pathogen-associated molecules (Moore & Freeman, 2006). The B class of SRs was established with the identification of CD36 as a receptor for oxidized low-density lipoproteins. Unlike the SR-A family, CD36 is a type III (multiple transmembrane domains) receptor that traverses the membrane twice to form a heavily glycosylated extracellular loop with 2 short intracellular tails (Murphy et al., 2005). CD36 has a wider cellular distribution, including monocytes, macrophages, adipocytes, microvascular endothelium, platelets and erythroid precursors. CD36 bind several ligands common to SR-A (β-amyloid, anionic phospholipids, apoptotic cells, advanced glycation end-products), however, it is distinct from SR-A in its ability to bind native lipoproteins and very low-density lipoprotein as well as thrombospondin-I, collagen, fatty acids and pathogen-derived ligands (Moore & Freeman, 2006). As a result of its broad specificity, CD36 has been reported to contribute to a varied list of normal and pathologic processes such as apoptotic cell clearance, fatty acid transport, adhesion, angiogenesis and microbial defense (Murphy et al., 2005). Decrease of SR-AI and SR-B molecules on the surface of peritoneal macrophages of women with endometriosis likely might to contribute to the

impairment of clearance of endometrial cells from peritoneal cavity and facilitate the implantation and growth of ectopic endometrium.

The low expression of FasL molecules by peritoneal macrophages might be attributed to the decrease of apoptosis-inducting function of macrophages in endometriosis. As we have demonstrated above macrophages of women with endometriosis were incapable to induce apoptosis in autologous eutopic endometrium. The observed low expression of FasL molecules on the macrophages surface likely is probably one of the possible mechanisms which impair the apoptosis-inducing function of macrophages in endometriosis. It is known that FasL or Fas ligand is one of the main transmembrane receptors initiating extrinsic pathway of apoptosis (Elmore 2007). The binding of FasL to its receptor Fas on the surface of cell-target results in the binding of the adapter protein FADD, activation of pro-caspase-8 and formation of death-inducing signaling complex (DISC) (Elmore 2007). So, the FasL-positive cells are powerful inducers of apoptosis and impairment of FasL expression on the surface of peritoneal macrophages might be an important factor contributing to inhibition of endometrial apoptosis.

We shown that the expression of HLA-DR molecules by peritoneal macrophages in endometriosis also was decreased comparing to that in healthy women. The same results were received Yamamoto et al. (2008), which observed low HLA expression and particularly reduced HLA-DR in the lipid raft. It is known that HLA-DR molecules are involved in antigen presentation by macrophages (Wu et al., 2004). So, impairment of HA-DR expression might compromise antigen presentation in women with endometriosis, limiting the immune response to peritoneal cavity antigens such as implanted or metaplastic endometrial cells.
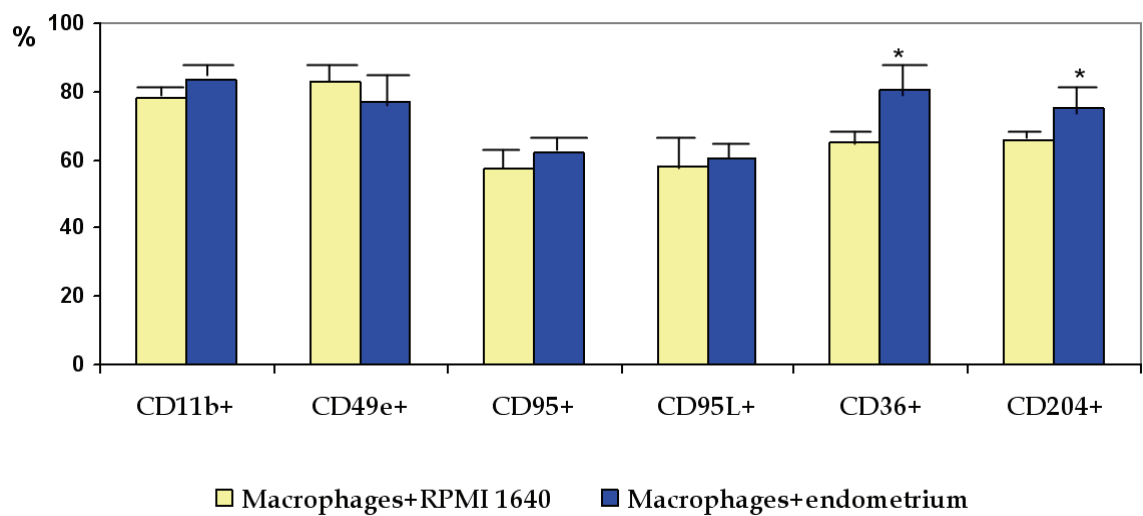


Fig. 3. Changes in the expression of functional molecules and scavenger receptors by peritoneal macrophages of healthy women after its incubation in vitro with autologous eutopic endometrium (* - given in comparison to macrophages incubating only in the presence of RPMI 1640, * - $p < 0.05$).

Limited expression of the surface functional receptors by macrophages in endometriosis can lead to the incapability of macrophages to correctly response upon stimulation by

autologous endometrial cells. We tried to prove this hypothesis and have done series of experiments in vitro for co-cultivation of peritoneal fluid macrophages with explants of autologous endometrial tissue with subsequent estimation of the surface membrane receptors by macrophages. We found that in healthy women the significant elevation of the scavenger receptors A and B type upon macrophages after their incubation with endometrial explants was seen (Fig.3). These results confirmed the suggestion about the direct involvement of SRs in normal response of peritoneal macrophages on the ectopic misplaced endometrium.

On the contrary, in endometriosis women the stimulation of peritoneal macrophages by autologous endometrial cells didn't change the expression of scavenger receptors by macrophages (Fig.4). Moreover, after co-cultivation of macrophages from endometriosis women with eutopic endometrial the significant diminishment of the number of FasL+, CD11b+ and CD49e+ macrophages was seen (Fig.4).
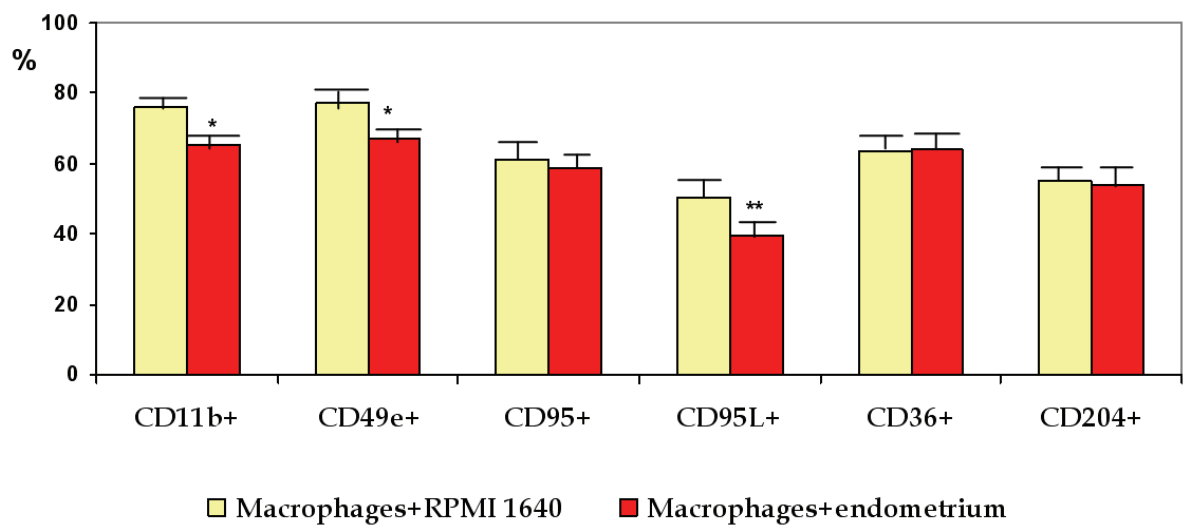


Fig. 4. Changes in the expression of functional molecules and scavenger receptors by peritoneal macrophages of women with endometriosis after its incubation in vitro with autologous eutopic endometrium (* - given in comparison to macrophages incubating only in the presence of RPMI 1640, * - p<0.05).

Supposedly, the initial aberrant expression of functional receptors by peritoneal macrophages results in inadequate response of macrophages to stimulation of self endometrial cells which, in turn escape the immunosurveillance in peritoneal cavity.

## 5.2 Peritoneal fluid cytokine production in endometriosis

The majority of studies up to now have shown significantly increase of the content of cytokines and growth factors in peritoneal fluid of women with endometriosis (Dmowski & Braun, 2004). It is known that cytokines and growth factors are proteins or glycoproteins produced by leukocytes or other cells, and secreted to the extracellular environment. These molecules exert their effects on the same (autocrine) or nearby cells (paracrine activity). They are key mediators of intercellular communication within immune system. Cytokines may have proliferative, cytostatic, chemo atractant or differetiative effects (Berkkanoglu & Arici, 2003). Cytokines possess pleiotropic function

and act in cascade manner. The involvements of cytokines in the pathogenesis of many diseases, connected with inflammation and cells proliferation, such as autoimmune pathology, rheumatoid arthritis, cancer etc, have been proved. The role of cytokines system in pathogenesis of endometriosis was studied very intensively. The increased leukocyte number found in peritoneal fluid of women with endometriosis is likely to be attributable to the enhanced synthesis of various cytokines and growth factors (Dmowski & Braun, 2004). Cytokines also might be produced by endothelial cells, misplaced endometrial cells and ectopic tissue cells. The detailed analysis of the cytokines profile of peritoneal fluid of endometriosis women is above scope of this paper. To receive more information we recommend to direct reader's attention to some detailed reviews (Harada et al., 2001; Gazvani & Templeton, 2002; Kyama et al., 2003; Wu & Ho, 2003; Dmowski & Braun, 2004). Briefly, significantly elevated concentration of numerous proinflammatory cytokines such as IL-1β, TNFα, IL-6, IL-8, IL-12, IL-16, RANTES, EGF, TGF β, CSG, IFG, HGF was found in peritoneal fluid of endometriosis women and practically in all cases investigators conclude that this local sterile inflammation possibly "plays a decisive role in the pathogenesis if the endometriosis". This conclusion is based on the experimental data, showing capability of several proinflammatory cytokines increase the proliferation and growth endometrial cells.  For example, experiments in vitro demonstrated that TNFα and IL-8 collaborate in the proliferation of stromal cells from ectopic endometrium and endometriomata (Harada et al., 2001). It was also shown that TNFα as the factor of peritoneal fluid from women with endometriosis promotes proliferation of eutopic and ectopic endometrial cells (Braun et al., 2002). IL-8 stimulates the adhesion of endometrial cells to fibronectin (Berkkanoglu & Arici, 2003). IL-1β promotes angiogenesis in endometriotic lesions by inducing the angiogenic factors in endometriotic stromal cells but not in normal endometrial stromal cells (Lebovis et al., 2000). But the majority of works are only descriptive and the functional involvement of cytokines in the development or progression of endometriosis has not been proven. Up to now we don't know exactly: is this peritoneal inflammation cause or consequence of the disease. From one side high concentration of growth factors and some cytokines, influencing upon cells proliferation, evidently might promote of endometrium implantation and growth in peritoneal cavity. Bur from another side, as ectopic endometrial lesions are a valuable source of cytokines itself, so peritoneal inflammation may be only the consequence of the presence of endometrial lesions in peritoneal cavity. Solutions of this problem evidently will be very helpful as to the endometriosis pathogenesis comprehension and search of new approaches to the medical treatment of endometriosis as well.

We attempted to trace the connection between cytokine profile of peritoneal fluid of women with endometriosis and possible mechanisms of peritoneal fluid action upon invasiveness of endometrial cells. To this purpose we estimated the concentration of few cytokines with possible action upon cells invasiveness, such as chemokines IL-8 and MCP and protein calprotektin, belonging to the family S100 proteins. We found the significant elevation all above mentioned cytokines in the peritoneal fluid of patients with endometriosis (Table 9). When we analyzed these cytokines content in subgroups of women with mild and severe endometriosis we found, that concentration of these cytokines correlated with the degree of endometriosis: maximal levels of IL-8, MCP-1 and calprotektin were seen in women with severe endometriosis (Table 9).

All of these cytokines are powerful stimulators of cells invasiveness. Earlier it was found that IL-8 directly increases the MMPs activity and invasive potential of endometrial cells in

culture (Mulayim et al., 2004). Calprotektin, comprise the heterodimeric complex from C2a+-binding proteins S100A8 and S100 A9, also participates in cells invasiveness regulation. It was found that enhanced expression of S100A8/A9 gene is the marker of metastatic potential of epithelial tumor cells (Moon et al., 2008). It was also demonstrated that calprotektin is involved in the activation of gene MMP-2 transcription in tumor cell line SNU484 (Yong & Moon, 2007). Chemokine MCP-1 acts as paracrine and autocrine regulator of growth and invasion of prostate tumor (Lu et al., 2009). So, all these cytokines can be stimulators of the eutopic endometrial cells invasiveness in peritoneal fluid. But, the significant elevation of studied cytokines content was noted only in women with severe endometriosis. In cases of mild endometriosis the cytokines concentrations didn't differ from that in healthy women.

| Parameter | Control group (n=10) | Endometriosis (n=35) | 1-2 stage of endometriosis (n=18) | 3-4 stage of endometriosis (n=17) |
|---|---|---|---|---|
| Calprotektin, μg/ml | 0.44±0.17 | 1.28±0.38* | 0.62±0.26 | 1.86±0.65* |
| IL-8, pg/ml | 6.19±3.41 | 19.09±5.00* | 10.83±4.18 | 27.73±9.12* |
| MCP-1, pg/ml | 308.12±29.87 | 528.40±51.45** | 376.56±50.32 | 689.18±74.79***xxx |

Table 9. Cytokine content in peritoneal fluid of women with endometriosis (* - given in comparison to the control group, * - p<0.05, **- p<-0.01, ***- p<0.001; x - given in comparison to the group of women with 1-2 stage of endometriosis, xxx- p<0.001)

It is known, that peritoneal macrophages are the major producers of cytokines in peritoneal fluid (Wu & Ho, 2003). We assessed the production of several proinflammatory cytokines (IL-1β, IL-8, TNFα, IL-6, and IL-12) by peritoneal macrophages in endometriosis. It was found that that character of intracellular production of proinflammatory cytokines by macrophage was correspondent to cytokines content in peritoneal fluid (Table 10). The level of cytokines production directly correlated with the severity of endometriosis and significantly increased only in group of women with 3-4 stages of endometriosis. The only exception we noted for TNFα. We found that that the intracellular production of TNFα by peritoneal macrophages was significantly elevated both in women with mild and severe endometriosis (Table 10).

| Parameter,% | Control group (n=10) | Endometriosis (n=28) | 1-2 stage of endometriosis (n=18) | 3-4 stage of endometriosis (n=10) |
|---|---|---|---|---|
| IL-1β+ | 64.51±3.40 | 74.69±2.90* | 73.47±3.09 | 80.53±3.46*** |
| IL-8+ | 67.10±2.96 | 75.13±2.03* | 68.42±4.14 | 78.17±1.70** |
| TNFα+ | 63.11±3.37 | 74.77±1.95** | 74.72±2.96** | 76.24±2.38* |
| IL-6+ | 52.74±4.18 | 66.63±2.87 | 58.41±3.36 | 66.25±2.70* |
| IL-12+ | 67.35±3.39 | 63.64±2.56 | 63.268±3.32 | 65.53±0.95 |

Table 10. Intracellular production of cytokines by peritoneal macrophages of women with endometriosis (* - given in comparison to the control group, * - p<0.05, **- p<-0.01, ***- p<0.001)

TNFα or tumor necrosis factor α initially was identified for its ability to kill certain cell lines, but now it is known that TNFα have the ability to initiate the cascade of other cytokines and factors associated with inflammatory responses. Earlier it was shown that TNFα increased the adherence of cultured endometrial stromal cells to mesothelial cells (Zang et al., 1993). This finding suggests that the presence of TNFα in peritoneal fluid may facilitate the adherence of ectopic endometrial tissue to the peritoneum and allow implants to develop. There are some experimental data, also evidenced about implication of TNFα in the pathogenesis of endometriosis (Agic et al., 2006). In baboons with laparoscopically confirmed endometriosis, TNFα blockade with p55 soluble TNFα-receptors results in inhibition of the development and growth of endometriotic implants (D'Hooghe et al., 2006). In rats with ectopically transplanted endometrial tissue, the administration of recombinant human TNFα- binding  protein-1 (r-hTBP-1) resulted in defective development of implants compared with controls (D'Antonio et al., 2000). All these data and our own results let us to suggest the possible involvement of TNFα in initial mechanisms of ectopic endometrium implantation and growth. But it must be special noted that the macrophage's production and peritoneal fluid content of the majority of proinflammatory cytokines significantly elevated only at advantage stages of endometriosis.

Thus, we must very careful to speculate about possible involvement of cytokines in the peritoneal fluid in regulation of the behavior of endometrial cells in peritoneal cavity, because the prominent action of peritoneal fluid cytokines generally is seen only in advantage stages of endometriosis. We thought that the impairment of peritoneal macrophages function plays the decisive role in immune mechanisms participating in implantation and growth of ectopic endometrium. Incapability of peritoneal macrophages to act as effective scavengers and adequately to eliminate the misplaced endometrial cells from peritoneal cavity possibly is one of the primary immunological disorders participating in endometriosis pathogenesis. Elevation of proinflammatory cytokines production in peritoneal cavity likely depends on the presence of ectopic lesions and can be stimulated by viable endometrial cells proliferating and growing in peritoneal cavity. Invasion of already implanted ectopic endometrial cells is evidently under the control of proinflammatory cytokines of peritoneal fluid.

## 6. Conclusions

Thus, it is very difficult to distinguish between the participation of endometrial abnormalities and immune impairments in peritoneal fluid in endometriosis pathogenesis. The frontiers between the two mechanisms are not clear-cut and evidently both mechanisms are responsible for endometriotic disease development. From one side, the development and growth of endometriosis lesions are associated with the significant changes in endometrium which are characterized by the high level of the expression of genes, stimulating invasiveness, and imbalanced production of anti- and pro-apoptotic factors in endometrial tissue. There are some differences in eutopic and ectopic endometrium in endometriosis. High level of anti-apoptotic factors XIAP synthesis is the characteristic feature of eutopic endometrium, which can provide high viability of menstrual endometrial cells in peritoneal cavity. But in endometriotic lesions the simultaneously elevated expression of both pro- and

anti-apoptotic genes was seen. Likely, this phenomenon might provide the ectopic but not malignant growth of endometrium in endometriosis. The development and growth of already formed ectopic lesions but not the eutopic endometrium of women with endometriosis is under the control of proteolytic enzymes from metalloproteinases family. We found that some of these differences between eutopic and ectopic endometrium might be due to the impairment of immunosurveillance in peritoneal cavity. Both peritoneal macrophages and humoral factors of peritoneal fluid directly influenced upon apoptosis and invasiveness of endometrial cells. This action might be connected with the changes of functional activity of peritoneal macrophages and local cytokine production. The impairment of the scavenger function of peritoneal macrophages due to the decreased level of membrane expression of integrins, scavenger receptors and apoptosis-inducing molecules FasL is possibly one of the fundamental defects of immune response in peritoneal cavity, which allow endometrial cell to live, proliferate and be implanted. Altered cytokine profile of peritoneal fluid in endometriosis likely can promote the invasiveness of ectopic lesions. Though it can be said that now we don't fully understand the fine immune mechanisms providing the development of endometriosis. More studies into the macrophage functioning and cytokines production as well as clinical experiments on the animal model may improve our understanding of endometriosis pathogenesis and results in the novel therapeutic modalities for endometriosis.

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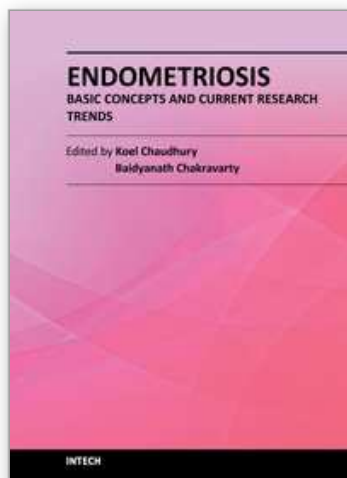
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Edited by Prof. Koel Chaudhury

This book provides an insight into the emerging trends in pathogenesis, diagnosis and management of endometriosis. Key features of the book include overviews of endometriosis; endometrial angiogenesis, stem cells involvement, immunological and hormonal aspects related to the disease pathogenesis; recent research reports on infertility, endometrial receptivity, ovarian cancer and altered gene expression associated with endometriosis; various predictive markers, and imaging modalities including MRI and ultrasound for efficient diagnosis; as well as current non-hormonal and hormonal treatment strategies This book is expected to be a valuable resource for clinicians, scientists and students who would like to have an improved understanding of endometriosis and also appreciate recent research trends associated with this disease.

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