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Pro-inflammatory M1/Th1 type immune network and increased expression of TSG-6 in the eutopic endometrium from women with endometriosis

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CONDENSATION

The prevalence of Th1 related immunity and an increased expression of TSG-6 were demonstrated in the endometrium from endometriotic patients suggesting an endometrial pro-inflammatory immunological imbalance harmful for implantation process.

ABSTRACT

Objective: The study aimed to explore the type 1 and type 2 cytokines expression in the endometrium from women affected by endometriosis compared to controls. The expression of TSG-6, a multifunctional protein involved in several inflammatory disease, was also evaluated.

Study Design

Setting: Experimental clinical study

Patients: 10 patients affected by endometriosis and 11 controls.

Interventions: Patients underwent to an ultrasound transvaginal examination and a diagnostic hysteroscopy in order to exclude any uterine abnormality. All patients underwent endometrial biopsy using a Novak’s curette.

Main outcome measures: The endometrial expression of type 1 (IL-1β, TNF-α, IL-8) and type 2 (IL-10) cytokines, and of TSG-6 was evaluated by immunohistochemistry and by real time PCR. The expression of TSG-6 was confirmed by western blot.

Results: Results of PCR analysis and of immunohistochemistry revealed an increased expression of IL-1β, TNF-α, IL-8 and of TSG-6 in the endometrium of endometriotic patients. IL-10 expression did not show any difference.

Conclusions: An increased expression of pro-inflammatory type 1 cytokines was demonstrated in the endometrium from endometriotic patients, suggesting an endometrial environment harmful for implantation due to the prevalence of Th1 related immunity. An increased expression of TSG-6 was also demonstrated for the first time. Our findings concur to better define the inflammatory imbalance and the abnormal endometrial receptivity, reported in literature, of the eutopic endometrium of women affected by endometriosis.

Keywords: endometriosis, endometrium, implantation, inflammatory cytokines, TSG-6
INTRODUCTION

Implantation is characterized by the interaction of two immunologically and genetically different tissues. During implantation, local and systemic immune factors, cytokines, and growth factors interact with adhesion molecules and other matrix-associated proteins (1). A balance between effectors (i.e., decidual macrophage M1 type [M1] innate immunity and helper T1 [Th1] immunity) and regulators (decidual macrophage M2 type [M2], Th2 cells, regulatory T cells, etc.) is thought to be essential for establishment and maintenance of pregnancy. It is well known that a strong M1/Th1 environment is harmful for pregnancy (2). In fact type 1 cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, are able to inhibit trophoblast invasion, stimulate apoptosis of human trophoblast cells, and enhance decidual macrophage activity, leading to the production of factors harmful to the embryo (3). Recently a new interesting multifunctional protein, tumor necrosis factor-stimulated gene 6 (TSG-6) was found up regulated in inflammation-like processes, in response to the proinflammatory type 1 cytokines IL-1β and TNF- α (4). Due to its role in the extracellular matrix remodeling (ECM) and in the regulation of serine protease activity (5, 6,7,8) it has been suggested that TSG-6 could play essential roles in female fertility. In fact female mice with a targeted disruption of the TSG-6 gene show severe reproductive defects (9).

Endometriosis is a complex gynecological disorder with a prevalence of 10% among women of reproductive age and is associated with chronic pelvic pain, dysmenorrhea and infertility (10). Such symptoms are thought to be the consequence of an inflammatory environment within the pelvis (11). Several studies in literature demonstrated that an altered expression of some cytokines and inflammatory factors also occur in the eutopic endometrium of women with endometriosis (12) suggesting, in such women, an impaired endometrial receptivity (10,13). However, to the best of our knowledge, in none of these studies a wide evaluation of the subset of pro-inflammatory cytokines known as M1 and Th1 type (3) has been performed. Therefore, we aimed to explore the protein and m-RNA expression of the type 1 pro-inflammatory cytokines IL-1β, TNF-α, IL-8 and of the type 2 cytokine IL-10 in the endometrium of infertile women affected by endometriosis as compared to healthy controls. In all samples the expression of TSG-6 was also evaluated.
MATERIALS AND METHODS

Patients

Endometrium was obtained from 10 infertile patients, affected by endometriosis (Endometriosis group) and from 11 fertile healthy control women, with normal menstrual cycles, (Control group). The diagnosis of endometriosis was made according to RCOG guidelines (14). Written informed consent was obtained from all patients enrolled in the study that was approved by the local ethical committee. Exclusion criteria for both groups were as follows: age > 40 yrs, BMI > 30, history of repeated pregnancy loss, corticosteroids treatments or other medical treatments known to interfere with the immune system, known clinical autoimmune disease, thrombophilic conditions requiring anticoagulant therapies. None of the patients enrolled had taken oral contraceptives or other medications known to interfere with the menstrual cycle for at least 6 months before starting the study. In the proliferative phase of the cycle, patients underwent an ultrasound transvaginal examination and a diagnostic hysteroscopy in order to exclude any uterine abnormality. All patients underwent endometrial biopsies using a 3 mm Novak’s curette connected to a 20 mL syringe. Within the endometriosis group five patients underwent endometrial biopsy in the proliferative phase of the cycle, the other five in the secretory phase (Tab 1). Among controls, five patients underwent endometrial biopsy in the proliferative phase the other six in the secretory phase of the cycle (Tab 1). A fraction of the endometrial sample obtained from each patient was prepared for histological examination; a part was stored at -80°C (see below).

RNA extraction and real-time reverse transcriptase polymerase chain reaction (RT-PCR) Endometrial tissues stored at -80°C were homogenised and total RNA isolated using TRizol Reagent (Invitrogen, Carlsbad, CA) according to the TRizol protocol. cDNA was obtained using a random hexamer primer and a SuperScript III Reverse Transcriptase kit as described by the manufacturer (Invitrogen, Frederick, MD, USA). A PCR master mix containing the specific primers was added, along with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The following human primers were used in the RT-PCR analysis:
1. IL-10: forward, 5'- AGGCTACGGCCTGTCATC-3'; reverse, 5'- GGCATTCTTCACCTGCTCCA -3'
2. TNF-α: forward, 5'- CTCCAGGGCGTGCCCTTGTC-3'; reverse, 5'- CTCCAGGGCGTGCCCTTGTC -3'
3. IL-1β: forward, 5'- CTTGAAGCTGATGGCCCTAAA-3'; reverse, 5'- AGTGGTGGTGAGGATTCGT -3'
4. IL-8: forward, 5' - CCA ACA CAG AAA TTA TTG TAA AGC AGC CCT CTT CAA
5. TSG-6: forward, 5'- AGGCCGCCCATCTCGCAAC -3'; reverse, 5'- ACGCCACCACACTCTTTTGC -3'

Ribonucleic acid samples were analyzed spectrophotometrically to determine RNA concentration, yield, and purity. One microgram of RNA was run on a 1% agarose gel to verify ribosomal RNA subunit integrity. The sequence of PCR products and specificity of the reaction were confirmed by semi quantitative PCR, and the size of the products was also confirmed by gel electrophoresis for selected samples. Fluorescence from incorporation of SYBR green into double-stranded PCR products was monitored continuously during cycling at the end of each elongation phase, whereas quantitation of mRNA expression was performed when amplified products were in the log-linear phase. Melting curve analysis demonstrating that each of the primer pairs amplified a single predominant product with a distinct temperature of melting (Tm) was carried out to ensure specificity of the reaction products. Sample mRNA levels were expressed relative to the housekeeping genes by comparing the PCR threshold cycle (CT) between sample complementary DNAs and ß-actin (ßCT). Real-time quantification of mRNA was performed with a SYBR Green I assay, and evaluated using an iCycler detection system (Bio-Rad Laboratories). The threshold cycle (CT) was determined, and the relative genes expression subsequently was calculated as follows: fold change = 2^-ΔΔCT, here ΔΔCT = CT target – CT housekeeping and ΔCT treated – ΔCT control.

Histology and immunohistochemistry

A routine microscopic histopathological study was performed on all sample of endometrium collected using haematoxylin-eosin (H&E) and histological dating was performed according to Noyes criteria (15). For immunohistochemical investigation the following antibodies were used: anti-TNF-β (Santa Cruz, CA, USA), anti-IL-1β (Santa Cruz, CA, USA), anti-IL-8 (Abcam, Cambridge, UK), anti-IL-10 (Peprotec, London, UK), anti-
TSG-6 (rabbit polyclonal anti-TSG-6 [RAH-1], kindly provided by Milner CM, University of Manchester); 4 μm-thick paraffin sections mounted on slides covered with 3, aminopropyltriethoxysilane (Fluka, Buchs, Switzerland) were prepared. Pre-treatment was necessary to facilitate antigen retrieval and to increase membrane permeability to antibody anti-IL-1β, to antibody anti-TNF-α boiling in 0.1 M Citric Acid buffer, to antibodies anti IL-8 and IL-10 for 15 min in Proteolytic Enzyme (Dako, Copenhagen, Denmark), at 20°C. The primary antibody was applied in 1:600 ratio for TNF-α, in 1:4000 ratio for IL-1β, in 1:2000 ratio IL-10, in 1:500 ratio for IL-8, 1:50 for TSG-6 and incubated for 120 min at 20°C. The detection system utilized was the LSAB+ kit (Dako, Copenhagen, Denmark), a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. The sections were counterstained with Mayer’s haematoxylin, dehydrated, cover slipped and observed in a Leica DM4000B optical microscope (Leica, Cambridge, UK).

**Western blot**

Endometrial tissue was homogenized in ice-cold lysing buffer (20 mM HEPES, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% IGEPAL CA-630, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA) containing protease inhibitors. The protein concentration was measured according to Bradford’s method. To analyze the levels of TSG-6 total proteins (30 – 70 μg) were boiled for 5 min in Laemmli buffer and separated by electrophoresis in 10 – 12% denaturing SDS/polyacrylamide gels followed by transfer to Hybond ECL nitrocellulose membranes (GE Healthcare Europe). After saturation of non-specific binding sites with 5% non-fat milk or 1% BSA, both in Tris-buffered saline (TBS) 1° – Tween 20 0.05%, membranes were immunoblotted overnight at 4 °C with the appropriate primary antibody against TGS-6 (1: 4000). The membrane was then stripped by Restore Stripping Buffer (#21059; Pierce, Rockford, Illinois, USA) for 30 min, washed three times and incubated with anti-β-tubulin (1: 2000) (Cell Signal, UK) overnight. Secondary antibodies were conjugated with horseradish peroxidase and immunoblots detected by VersaDoc Image System (Bio-Rad Laboratories, Hercules, California, USA).

**Statistical analysis**
Data were expressed as mean ± standard deviation of the mean (SDM). The distribution of the variables was performed by Kolgomorov-Smirnov test. Differences between two means were analysed using Student-t test. In all instances p=0.05 was taken as the lowest level of significance. The package GraphPad Prism 4 for Windows was used to perform all the statistical analyses.

RESULTS

Clinical features of women enrolled in the study are displayed in Tab 1. No significant difference was found in any of clinical variables analysed except for the number of pregnancies and live births that resulted significantly higher in the control group compared to the endometriosis group (p < 0.001) (Tab 1). The histological examinations confirmed, in all cases, the phase of the menstrual cycle in which endometrial samples were obtained. The expression of cytokines was analysed by immunohistochemistry and by RT-PCR and the results are reported in Fig 1, 2, 3 and 4. The results obtained by PCR analysis revealed that in the endometrium of patients with endometriosis the expression of IL-1β, TNF-α and IL-8 was significantly increased as compared to controls (Fig 1 a, b, c). On the contrast the PCR analysis of IL-10 mRNA expression did not show any difference (Fig 1d). TSG-6 mRNA expression was also explored and resulted significantly increased in the endometrium from endometriotic patients compared to controls (Fig 2a). Representative results of the immunohistochemical evaluation are reported in fig 3, 4. According to PCR analysis, immunohistochemical staining of endometrium specimens revealed a positive reaction localized mainly on the endometrial glands, without differences between menstrual phase, proliferative and secretory, in particular a strong positive reaction for the antibodies anti-IL-1β, and IL-8 (Fig 3) , an intermediate positive reaction for the antibodies anti- TNFα group in endometriotic patients as compared to controls, (Fig 3) and a negative reaction for the antibodies IL-10 in both patients and controls. Immunohistochemistry (Fig 4) and western blot analysis (Fig 2b) confirmed a significant increase in the expression of TSG6, in the endometrium of patients affected by endometriosis when compared to controls. The positive immunohistochemical reaction of TSG6 is wide expressed on all endometrial tissue, but it is more localized on glands. No difference was found in the expression of cytokines as well as of TSG-6 between samples in the proliferative and secretory phase of the cycle in the endometrium of patients affected by endometriosis when compared to controls (Supplementary Figure 1)

ACCEPTED MANUSCRIPT
DISCUSSION

The present study demonstrates, in the eutopic endometrium of patients affected by endometriosis, a significant increase in the expression of pro inflammatory M1/Th1 type cytokines IL-1β, TNF-α, IL-8 (Fig 1,3) and of TSG-6 (Fig 2,4) when compared to controls. These findings are consistent with the hypothesis that in the eutopic endometrium of women affected by endometriosis an altered cytokines network leads to an abnormal endometrial immune environment in which the immunological balance between effectors (M1/Th1 type immunity) and regulators (M2/Th2 type immunity) may be altered, with the prevalence of Th1 related mechanisms. Physiologically, decidual macrophages are characterized by an immunosuppressive phenotype classified as M2, supporting feto-maternal immune tolerance. These macrophage as well as Th2 cells are driven by the presence of IL-4 and IL-10 and concur to protect against rejection of the allogeneic fetus (16, 17). Under the influences of proinflammatory type 1 cytokines IL-1β and IL-8, macrophage differentiates toward an M1 inflammatory phenotype, which promotes TNF-α production and the progression of inflammation (3). Similarly CD4+ T cells, in the presence of proinflammatory cytokines differentiate in Th1 type cells, which concur in the allograft rejection (17). Type 1 cytokines are harmful for pregnancy (2). In the decidua, they promote miscarriage by inhibiting trophoblast invasion. In fact TNF-α stimulates apoptosis of human trophoblast cells (18; 19) and can also influence fetal growth in other ways, as they can activate a prothrombinase, which generates thrombin. Thrombin activation leads to clotting and the production of IL-8, which stimulates granulocytes and endothelial cells to terminate blood supply to the developing placenta (20, 21). Notably our results showed a significant increase of the entire subsets of cytokines, IL-1β, TNF-α and IL-8, known to be associated to a Th1 endometrial environment. On the contrary we did not found any difference in the expression of IL-10, the type 2 immunoregulatory cytokine, able to suppress Th1 cytokines production (22). This allows speculating that the altered endometrial cytokine network may represent an additional mechanism for explaining reduced fertility in patients with endometriosis (10). This hypothesis is supported by several studies in literature, demonstrating an elevated Th1/Th2 cytokine-producing cell ratios in women with multiple implantation failures after in vitro fertilization and embryo transfer (IVF-ET) (22, 23, 24) and in
women with recurrent spontaneous miscarriages (2, 22, 25, 26). A high IL-1β and low IL-10 intra-uterine concentrations were also observed in some unexplained implantation failures after technically optimal IVF-ET (23, 27). The increased expression of type 1 cytokines was not dependent on the menstrual phase (proliferative vs secretory), suggesting that the immunological imbalance of the endometrium in the endometriosis patients is not related to sex steroids fluctuations, but may be due to an intrinsic chronic inflammatory status. Indeed we also demonstrated, for the first time, an increased expression of TSG-6 a multifunctional protein detected in several inflammatory disease with a key role in the extracellular matrix (ECM) remodeling (4). Recent studies demonstrated that TSG-6 is up regulated by the pro-inflammatory cytokines TNF-α and IL-1β (28) suggesting that it could be a component of a negative feedback loop capable of down regulating the inflammatory response (4). Moreover TSG-6 interacts with the serine protease inhibitor (28, 29, 30) and enhances the anti-plasmin activity of plasmin activator (PA), a serine protease involved in the control of ECM turnover (5, 6, 7, 8). We can’t define at the moment the exact role of the increased expression of TSG-6 demonstrated in our study. The four times higher expression of TSG-6 that we found, could be a mechanism of endometrial protection in response to the pro-inflammatory effect of the type1 cytokines, but we can’t exclude that the compensatory increase of TSG-6 could in turn concur to impair the implantation process, due to the TSG-6 anti-plasmin activity. In fact low implantation rates are associated with reduced PA activity (5, 31, 32) and implantation failure is associated with reduced PA expression (33).

In conclusion, to the best of our knowledge, this is the first study focused on evaluating the expression of a wide range of type 1 cytokines functional subset, demonstrating an immunological imbalance toward an M1/Th1 proinflammatory local endometrial environment in women affected by endometriosis. Moreover our study demonstrated, for the first time, an increased expression of a new multifunctional protein, TSG-6, up regulated by the type 1 cytokines and directly involved in the inflammatory processes. Our findings concur to better explain the immunological basis of the abnormal endometrial receptivity of endometriosis patients and add to a growing body of evidences in recent literature, indicating that eutopic endometrium
of women with endometriosis has intrinsic abnormalities in structure, immune components and cytokine production when compared to the endometrium of non-endometriosis women (12, 34, 35, 36).

Further investigations in progress are needed to provide compelling insights in the understanding of endometrial innate and adaptive immunity and to better understand the immunological mechanisms underlying endometriosis infertility.
REFERENCES


Figure captions

**Fig 1:** Expression of IL-1β (a), TNF-α (b), IL-8 (c), IL-10 (d). mRNA levels isolated from the endometrium of endometriosis patients and control women, measured using real-time reverse-transcription PCR. Data are expressed as mean values ± standard deviations of the means. β-actin mRNA expression arbitrarily assumed the value of 1 and was used to normalize the mRNA expression of each sample; p value referred to unpaired t-test analysis.

**Fig 2:** Expression of TSG-6 from the endometrium of endometriosis patients and control women measured using real-time reverse-transcription PCR (2a) and total protein levels of TSG-6 analysed by western blot (2b). PCR data are expressed as mean values ± standard deviations of the means.

β-actin mRNA expression arbitrarily assumed the value of 1 and was used to normalize the mRNA expression of each sample; p value referred to unpaired t-test analysis.

**Fig 3:** Representative figures of immunohistochemical expression of IL-1β, TNF-α and IL-8 the endometrium from endometriosis patients as compared to Controls [CRL]

**Fig 4:** Representative figures of immunohistochemical expression of TSG-6 in the proliferative (P) and secretory (S) endometrium from endometriosis patients as compared to Controls (CRL)
Fig 3
Clinical features and timing of endometrial biopsies (EP: early proliferative, LP: late proliferative, ES early secretory, LS late secretory) of women affected endometriosis and of healthy controls enrolled in the study. (*) Remarks clinical features significantly different between the two groups (p<0.001)

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