ORIGINAL ARTICLE

CONTENTS

The analysis of cervicovaginal microbiota and immune mediators in patients with adenomyosis

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ABSTRACT

Aim: To determine and characterise changes associated with microbial, inflammatory and immune factors in patients with adenomyosis.

Materials and Methods: 115 female patients were enrolled in the study, including 85 patients with adenomyosis and 30 healthy individuals. Vaginal cleanliness was assessed by collecting vaginal samples and plating on solid bacterial agar. Urogenital infections were determined by the PCR analysis of the cervicovaginal samples. The immunological characterisation was performed using enzyme immunoassay to analyse cervicovaginal lavage for β-defensin-2 and a defined set of cytokines. Antibody titers were quantified in patients' serum using nephelometry.

Results: Patients with adenomyosis displayed disrupted cervicovaginal microflora that was characterised by elevated levels of pro-inflammatory cytokines and antimicrobial peptide β-defensin-2. Serum analysis of patients with adenomyosis revealed higher concentration of IgG, IgM and IgA antibodies compared to healthy control groups.

Conclusions: Analysis of inflammatory and immunological parameters in patients with adenomyosis revealed distinct features of the pathophysiology of adenomyosis. Specifically, patients with adenomyosis had disrupted cervicovaginal microflora with higher prevalence of anaerobic species. Furthermore, patients with adenomyosis had elevated levels of pro-inflammatory cytokines and interleukins in cervicovaginal lavage, and higher levels of IgG, IgM and IgA antibody serum titres. The analysis presented in the paper represents an important step towards determining precise diagnostic parameters and effective therapies to treat adenomyosis.

KEYWORDS: antimicrobial peptides, adenomyosis, cervicovaginal microbiome

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INTRODUCTION

Adenomyosis is a chronic disease of the female reproductive system that affects up to 60% of women of reproductive age worldwide [1]. Adenomyosis is characterised by benign invasion of the endometrium into the myometrium [2]. Disruption of myometrium leads to pathology that affects female reproductive health and includes multiple clinical manifestations including excessive uterine bleeding, dysmenorrhea, chronic pelvic pain, and reduced fertility [3, 4]. Despite well-defined clinical characteristics of adenomyosis, molecular and cellular mechanisms that underlie the disease origin and progression remain poorly understood. Determining the molecular basis of the development of adenomyosis will help design better diagnostic tools and treatment regimens for women.

A number of factors have been proposed to contribute to the development of adenomyosis including angiogenic abnormalities [5], hormonal imbalance [6], and genetic factors [7]. More recently, a number of studies have revealed the importance of the immunological and pathogenic components in a related endometrial condition endometriosis [8, 9]. A local immune dysregulation as well as changes in the microbiome in the genital tract have been shown to play a role in the disease development [10]. Infectious agents including bacteria, viruses and fungi can serve as a trigger to initiate a dysbiosis in the genital tract leading to chronic inflammation and immune dysregulation [11, 12]. The inability of the immune system to resolve inflammation and clear the infection agents results in tissue damage and pathogenesis. Understanding the immunological networks of endometrial-myometrial pathology will be invaluable in defining the biological markers of the disease.

There are multiple lines of defence against the harmful factors that lead to the changes in the female reproductive flora and contribute to the disease progression. The first line of defence includes cytokines and antimicrobial peptides (AMPs) released by immune

cells in response to microbial agent, inflammation and physical damage [13]. There are approximately 39 antimicrobial peptides in cervicovaginal fluid [14] with β-defensins family, cathelicidin and SLPIs being the most dominant AMPs [15]. Multiple reports have demonstrated the importance of AMPs in the protection of vaginal microbiome [16, 17]. However, the role of AMPs in adenomyosis remains poorly understood. Release of AMPs, such as β-defensins, serves as a chemoattractant for immune cells. Following AMPs release, monocytes and dendritic cells migrate to the site of infection and can phagocytose and present pathogen associated molecular patters for T cell recognition. Upon activation, T cells trigger the next line of defence known as the adaptive immunity that involves activation of B cells and production of different antibodies including IgG, IgM and IgA that act at mucosal barriers as well and tissues and blood [18].

In this work, we defined the components of innate and adaptive immunity in the cervicovaginal microenvironment in patients with adenomyosis. We show that both microflora and immune composition is altered when adenomyosis is present, implicating these changes in disease progression.

AIM

To determine and characterize changes associated with microbial, inflammatory and immune factors in patients with adenomyosis.

MATERIALS AND METHODS

PATIENTS INVOLVED IN THE STUDY

115 female patients aged 18-45 years old, who underwent treatment at the clinical bases of the Department of Obstetrics and Gynecology No. 1 of the KhNMU, took part in the study. The patients were assigned into groups as follows. The first clinical group (study group) included 85 (73.9% of the total number of patients enrolled in the study) patients with adenomyosis. The second clinical group (control group) consisted of 30 (26.1% of the total number of patients enrolled in the study) healthy fertile women. All patients underwent a routine general clinical examination during the course of the study, in accordance with the current clinical recommendations of the Ministry of Health of Ukraine. The examination was carried out after obtaining the patient's informed consent for the diagnosis and processing of personal data.

Adenomyosis was diagnosed in patients in the study group based on the diagnostic tools and histological methods of examination of tissues obtained during hysteroscopy. Patient exclusion criteria included extragenital pathology, oncology including leiomyomas, inflammation of the genitals, HPV infection. Patients who were exposed to urogenital viral or bacterial infection in the last 6 months and who underwent antibiotic therapy were excluded from the study.

ETHICS STATEMENT

The study was conducted in compliance with the basic bioethical norms and requirements of the Helsinki Declaration of the General Assembly of the World Medical Association on Human Rights and Biomedicine (1977), in accordance with the provisions of the WHO, the International Council of Medical Scientific Societies, the International Code of Medical Ethics (1983) and the Order of the Ministry of Health of Ukraine No. 690 dated September 23, 2009. The study protocol was approved by the Bioethics Commission of the KhNMU.

[World Medical Association Declaration of Helsinki "Ethical Principles of Medical Research Involving Human Subjects". 2008. URL : https://zakon.rada.gov.ua/laws/show/990_005]

DETECTION OF UROGENITAL INFECTIONS

Vaginal cleanliness was determined by isolating the *Lactobacilli* strains from vaginal samples using standard procedure of culturing bacteria on solid agar and by performing subsequent PCR sequencing.

To determine the presence of anaerobic bacteria, cervicovaginal lavage (CVL) was analyzed for the presence of the indicated microbial agents (Table 1). Bacterial agents were detected by PCR using standard procedure.

IMMUNOLOGICAL CHARACTERISATION

CVL samples were collected and the levels of cytokines were determined by solid-phase enzyme immunoassay method using Stat Enzyme Immunoassay Analyzer Fax ® 303 Plus (Awareness Technology, Inc. USA) according to manufacturer instructions.

ANALYSIS OF ANTIBODIES IN BLOOD

Blood samples were collected during the study and processed immediately post-collection. Serum was separated from whole blood samples by centrifugation at 2,000g for 15 min at 4°C. Separated serum samples were analysed for total immunoglobulins IgG, IgM and IgA using nephelometry.

Table 1. Cervicovaginal cleanliness in the examined patients

Degree of vaginal cleanliness	Study group (n=85)		Control group (n=30)	
	n	%	n	%
I	8	9.4	24	80
II-III	71	83.5	6	20
IV	6	7.1	-	-

% is percentage out of the total number of examined patients per group
n-number of patients
Source: compiled by the authors of this study

Table 2. Concentration of antimicrobial peptide β-defensin-2 and cytokines in the cervicovaginal lavage of the examined patients.

	Study group	Control group
	n=85	n=30
	Antimicrobial peptide (AMP) ng /ml	
β-defensin-2	30.5±3.5*	46.7±3.8
	Cytokines pg /ml	
IL-4	20.0±1.8*	15.3±1.5
IL-8	4.94±0.6*	3.8±0.4
IL-10	8.37±0.2*	7.1±0.9
IL-18	14.0±1.5*	10.9±1.3
TNFα	43.2±4.5*	33.4±3.6
VEGF	228.5±25.1*	173.5±16.9
IFNg	9.54±1.1*	7.9±0.2

Mann-Whitney U test was performed. Mean±SEM is shown where SEM is standard error of the mean. *P<0.05 was considered statistically significant between the control and study groups. n is number of patients per group
Source: compiled by the authors of this study

STATISTICAL ANALYSIS

Statistical analysis was performed using software package "Statistica 10.0 for Windows". For each variation series, we calculated average arithmetic mean (M), average quadratic deviation (σ), average mistake average arithmetic (m). To identify significant differences, Mann-Whitney U test was used. Differences were considered significant at a level of $P < 0.05$.

RESULTS

ANALYSIS OF BACTERIAL COMPOSITIONS IN THE CERVICOVAGINAL SECRETIONS IN PATIENTS WITH ADENOMYOSIS

There were 115 female patients involved in the study and the patients were divided into two groups. Group 1 constituted patients with adenomyosis diagnosis (study group, 85 patients) and group 2 included healthy patients (control group, 30 patients). The patients were 31.2 ± 2.7 years old, with no statistical difference in age between the study or control groups. 30% of the patients in the study group contained cases of gynecological complications and family history of gynaecological conditions compared to 10% in the control group. Average duration of adenomyosis in patients in study group was between 3 to 5 years. The clinical signs of pathology in the study group included pelvic pain (71% of patients), dysmenorrhea (43% patients) and infertility (84% patients).

First, we aimed to determine the degree of vaginal cleanliness among all the patients enrolled in the study. We used previously reported lactobacilli grading method [19] to define four degrees of purity of vaginal flora: Grade I defined as dominant lactobacilli with no other bacteria present, Grade II defined as having lactobacilli and other bacteria present, Grade II defined as few or no lactobacilli with other bacteria present and Grade IV defined as no lactobacilli and no other bacteria present. Vaginal discharge was collected from patients and bacterial flora was examined using PCR (Table 1). Grade I vaginal purity was detected in 24 (80.0%) women of the control group and in 8 (9.4%) patients in the study group. Grade II-III vaginal purity was identified in 6 (20.0%) control group patients and in 71 patients (83.5%) of the study group. Grade IV vaginal purity was present in 6 (7.1%) of cases in patients of the study group and was not detected in any of the patients in

Table 3. Concentration of antibodies in the serum of the examined patients.

	Study group	Control group
	n=85	n=30
IgM (g/l)	1.85 ±0.26*	1.02±0.09
IgG (g/l)	16.6 ±2.5*	12.3 ±1.6
IgA (g/l)	2.33 ±0.34*	1.10 ±0.08

Mann-Whitney U test was performed, Mean±SEM is shown where SEM is standard error of the mean. *P<0.05 was considered statistically significant between the control and study groups. n is number of patients per group
Source: compiled by the authors of this study

the control group. Together, these data indicated that vaginal purity is negatively affected in patients with adenomyosis.

Decrease in vaginal purity suggested that microbial changes may be present in the vaginal environment. Previous work suggested that there is an association between anaerobic microbes and inflammation of endometrium [20, 21] which prompted us to ask whether anaerobic bacteria may play a role in adenomyosis. To address this question, we examined the anaerobic microbial composition of the cervicovaginal flora in the study and control groups. Samples of cervicovaginal lavage were collected and microbial composition was determined by PCR for an indicated set of microbial agents (Fig. 1). Anaerobic microbial agents constituted seven microorganisms that have previously been reported in vaginal microflora [22, 23]. Comparison between the control and study groups revealed that patients with adenomyosis were characterized by the presence of a number of anaerobic microorganisms with the *Streptococcus* spp. *Staphylococcus* spp. and *Eubacterium* spp. being second and third most abundant anaerobe present respectively. A single case of *Candida* spp., and another case of *Mycoplasma hominis* were identified in the study group, suggesting that the presence of these microorganisms is rare yet detectable. Unlike the study group, the vaginal microflora of healthy patients had no presence of any of the anaerobic microorganisms, consistent with the control group exhibiting Grade I degree with vaginal purity (Table 1). Together, these results suggested a possible link between the adenomyosis and the presence of anaerobic species in likely disrupted cervicovaginal microbiota.

Disruption in microflora in the vaginal environment in patients with adenomyosis lead us to examine the local immunological composition. AMPs produced by commensal microbiota are known to be the key mediators of defence against microbial pathogens in female reproductive tract [12]. AMPs are known to fluctuate in response to hormonal changes as well as in certain disease of female reproductive tract [13]. There different types of AMPs in the endometrial fluid [24] and β-defensins are of the most abundant AMPs present. As a representative analysis, we, chose to look at the levels of β-defensin-2 in CVL fluid in the study and control groups. The levels of b-defensins were downregulated in patients with adenomyosis compared to the control group (Table 2). This result supported the link between adenomyosis and dysregulated microflora.

CERVICOVAGINAL INFLAMMATORY CHANGES IN PATIENTS WITH ADENOMYOSIS

We hypothesised that disrupted microbial composition within the vaginal environment may also be accompanied by immunological changes.

We therefore examined the levels of the key immunological regulators including interleukins (IL-4, IL-8, IL-10, IL-18), pro-inflammatory mediators (TNFα, IFNγ) and endothelial growth factor (VEGF) in the CVL in the study and control groups. In CVL, the concentration of IL-8, IL-10 and IL-18 was significantly higher in patients with adenomyosis compared to the control group (Table 2). Since IL-18 stimulates the formation of Th1-type cytokines (IFNγ, TNFα), its increase at the local level may be aimed at limiting inflammatory processes in the reproductive tract. In addition, by secreting Th1-type mediators, IL-18 may contribute to the development of a T-cell immune response. In line with these observations, higher levels of TNFα were detected in patients with adenomyosis compared to the study group (Table 2). IL-4 and IL-10, that are associated with anti-inflammatory responses, were elevated in patients with adenomyosis. The concentration of VEGF was significantly higher in the study group compared to healthy volunteers indicating a possible disruption in the normal angiogenesis that may result in vascular growth and proliferation of ectopic endometrium.

Next, we examined the levels of three subclasses of antibodies IgG, IgA and IgM in the serum of patients of adenomyosis or healthy women. All three subclasses of antibodies were elevated in patients with adenomyosis

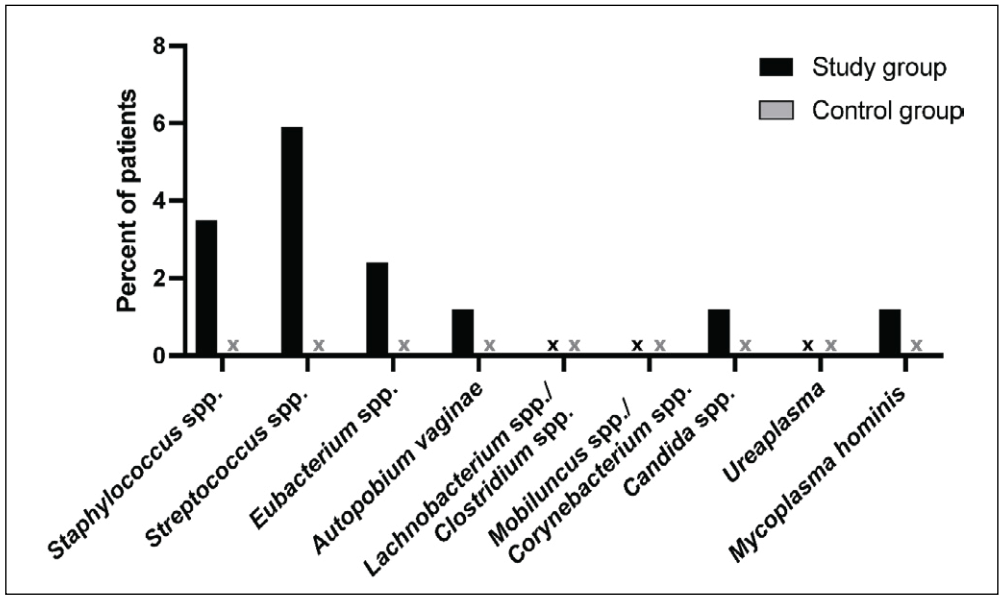

Fig. 1. The presence of anaerobic microbial species in cervicovaginal lavage of patients with and without adenomyosis
The number of patients is presented as a percentage per either study (black) or control (grey) groups. Black bars depict patients in the study group (with adenomyosis, total n=84). No anaerobic bacteria were identified in the control group of patients (n=30) "X" indicates that no patients were identified with a given microbial species.
Authors' own work

compared to the control group, with IgG levels being the most dominant class (Table 3). Collectively these results indicated that the inflammatory microenvironment, defined by elevated levels of pro-inflammatory mediators and increased inflammation-associated immunoglobulins, may be a characteristic of adenomyosis lesions.

DISCUSSION

The results of the study show that adenomyosis may be accompanied by changes in the cervicovaginal microbiome, a drop in b-defensin-2 and changes in the local immunological profile.

Epithelial cells of female reproductive tract are colonized by commensal bacteria that secrete AMPs among others, thereby providing protection against harmful pathogens. Our results showed that patients with adenomyosis displayed disruption in cervicovaginal microbiome that was manifested by the presence of anaerobic microorganisms (Fig. 1, Table 1). Consistent with this observations, other reports indicated that endometriosis-related lesions have distinct microbial compositions [11] and different stages of endometriosis are characterised by specific microbial patters [25]. In our study, we did not look at individual stages of adenomyosis. Furthermore, we only looked at the levels of β-defensin-2, one of many AMPs secreted by commensal bacteria in female genital tract. In future, we would like to determine whether certain stages of adenomyosis present themselves with defined patterns of cervicovaginal microbial composition and expand our analysis to other AMPs in adenomyosis.

The striking difference in pro-inflammatory cytokine profile between the patients with adenomyosis suggests that immune cell composition at the endometrial site differs between the adenomyosis patients and the healthy women. It would be interesting to define immune subsets in the cervicovaginal microenvironment in patients with adenomyosis to pinpoint the dominant immune cell populations that trigger the release of the proinflammatory cytokines identified in the present study. Interestingly, the levels of two anti-inflammatory cytokines IL-4 and IL-10 were similarly elevated in patients with adenomyosis (Table 2), indicating that even these elevated levels of anti-inflammatory cytokines are insufficient to suppress inflammation at the lesion site. Future work will focus on expanding the analysis of cytokine profiles to wider clinical groups to determine whether the cytokines can be used as prognosis markers for the development of adenomyosis [26].

Identifying the association between the elevated antibody levels and the presence of adenomyosis (Table 3) raises an interesting possibility that adenomyosis may have the autoimmune component its pathophysiology, similar to other endometriosis lesions [27]. The elevated levels of the antibodies in the serum of adenomyosis patients suggests that adaptive immunity mediated by T and B cells is activated in these patients.

Follow up studies are necessary to determine whether these antibodies are acting at the site of adenomyotic lesion, thereby exacerbating the disease further. Another outstanding question remains - what are the antigens that are recognised by these antibodies in patients with adenomyosis? Provided the elevated levels of antibodies in patients with adenomyosis recognize self-antigens at the adenomyosis lesion, it would be interesting to investigate whether the mechanism of immunosuppression might be effective at certain stages of adenomyosis development.

Adenomyosis remains to be a topical global problem affecting health of millions of women worldwide and the current treatment does not address prevention or pathophysiological aspects of the disease. The study presented here will contribute to a better understanding of the molecular mechanisms that govern adenomyosis at the cellular level and help advance the development of much needed diagnostic and prevention strategies.

CONCLUSIONS

The pathophysiology of adenomyosis remains understudied largely due to the complexity of the diagnosis and limited understanding of the etiology of the condition. The study presented here revealed several important aspects of the inflammation and immune dysregulation that accompany adenomyosis lesions. The results showed that patients with adenomyosis exhibit disrupted microbial composition within the cervicovaginal environment characterised by high prevalence of anaerobic species. Cervicovaginal lavage collected from patients with adenomyosis was characterized by elevated levels of interleukins (IL-4, IL-8, IL-10, IL-18), pro-inflammatory mediators (TNFα, IFNγ) and endothelial growth factor (VEGF). Furthermore, the serum concentrations of IgG, IgM and IgA antibodies were elevated in patients with adenomyosis compared to healthy individuals. Our study provides an important foundation to further dissect the immunological composition of adenomyosis and ultimately help design better diagnostic and therapeutic tools to treat this debilitating condition.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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