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Association of neutrophil extracellular traps with endometriosis-related chronic inflammation

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ABSTRACT

Objective: To study if neutrophil extracellular traps (NETs) are present in the peritoneal fluid of endometriosis patients. NETs play a crucial role in fighting against microorganisms. However, exaggerated NET production may lead to tissue damage in their vicinity in pathological conditions. Our study evaluates the presence of NETs in endometriosis peritoneal fluid.
Study design: Peritoneal fluid (PF) was collected in a case-control study from 52 women, who underwent either diagnostic or operative laparoscopy. The control group consisted of 17 women with infertility, chronic pelvic pain, simple or functional cysts or irregular bleeding. The endometriosis group, altogether 35 patients, comprised 19 patients with stage I and II and 16 patients with stage III and IV endometriosis. First we tested whether the PF is able to stimulate NET production. Neutrophils from healthy volunteers were treated with the PF of endometriosis patients and controls and NETs were detected with Sytox orange extracellular DNA dye and immunofluorescence microscopy. Then we evaluated if NETs were already present in the collected PF using the specific myeloperoxidase (MPO)-DNA capture ELISA method, based on the MPO associated with the NET scaffold.
Results: The PF of endometriosis patients did not stimulate NET release from healthy granulocytes. However, pre-existent NETs could be detected in 17 endometriosis patients out of 35 (49%). In contrary, in the control group NETs were present in only 3 patients out of 17 (18%), ($p = 0.03$, OR: 4.4). Moreover, the quantification of NETs showed a significantly higher amount of NETs in endometriosis compared to the controls (0.097 vs. 0.02, $p = 0.04$).
Conclusion: This is the first study, which evaluated and described the presence of NETs in the PF of endometriosis patients. Our study shows, that NETs may be involved in the complex pathophysiology of endometriosis.

Introduction

Endometriosis affects approximately 10% of women in the reproductive age [1] and negatively influences quality of life. Retrograde menstruation is still the most prevalent pathogenesis hypothesis; however, the formation of endometriotic lesions depends on the attachment, survival, invasion, and proliferation of the respective cells [2]. The ectopic lesions cause a local inflammation of varying intensity, in which activation of leukocytes, macrophages, and natural killer cells are well documented [3,4]. However, the involvement of neutrophils in the pathomechanism of endometriosis has not been sufficiently explored.

Neutrophil granulocytes are considered the first line of defense against microorganisms [5]. Their classical killing mechanisms are the phagocytosis and intracellular killing [6], where the subsequent apoptosis and clearance by macrophages [7–9] protect the surrounding tissue against the noxious components. A decade ago a novel extracellular killing mechanism was described in which activated neutrophils may expel their entire chromatin, which is scattered with intracellular proteins, serving as catch and kill scaffold against microorganisms [10]. The procedure was designated as NETosis and the expelled structure as neutrophil extracellular traps (NETs). More than 20 different proteins were identified in NETs, originating from the granules, cytoplasm,

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cytoskeleton and peroxisome [11,12]. During NETosis the crucial steps are chromatin decondensation, disintegration of intracellular membranes and release of chromatin threads with the associated proteins [13].

NET formation has an unambiguously beneficial role during infection, since deficiency in NET production, *e.g.* in chronic granulomatosus disease [14,15], or degrading the scaffold by bacterial DNases may lead to severe infections [16–18]. However, the excessive formation of NETs might harm the healthy tissue in their vicinity, as it has been described in acute lung injury [12,19,20], cystic fibrosis [21,22], asthma [23], psoriasis [24], thrombosis [25–27], preeclampsia [28], appendicitis [10], sepsis [29], Crohn's disease [30], systemic lupus erythematosus (SLE) [31–34] and small vessel vasculitis [35].

Based on the nature of endometriosis, which demonstrates similarities with chronic inflammatory and autoimmune disorders [36], we postulated that NETs might play a role in its pathophysiology. Our study is the first that evaluates the presence of NETs in the peritoneal fluid (PF) of endometriosis patients.

Materials and methods

Patients and controls

Fifty-five women were included in a case-control study, who underwent either diagnostic or operative laparoscopy between January 2013 and February 2014 at the Justus-Liebig University, Giessen, Germany. The ethical committee approved the study (95/09). Laparoscopy was performed and the PF was aspirated immediately after entering the abdominal cavity, collected in EDTA syringes, aliquoted and stored at −20 °C.

The control group contained 19 women, on whom either diagnostic laparoscopy due to infertility or chronic pelvic pain or operative laparoscopy, such as tubal sterilization, removal of simple or functional cysts or hysterectomy due to irregular bleeding was performed. One patient was excluded from the control group because of severe hematometra and pelvic inflammation and another one due to pedicle torsion of the adnex and concomitant acute inflammation.

The endometriosis group, altogether 36 patients with histologically confirmed endometriosis, was composed of 19 stage I and II and 17 stage III and IV patients. One patient from the stage III and IV group had to be excluded because of Fitz-Hugh-Curtis Syndrome. The patients were scored according to the revised American Society of Reproductive Medicine (rASRM) criteria and deep infiltrating endometriosis (DIE) was described with the ENZIAN System. Within the endometriosis group 22 patients had DIE.

We did not include patients in the study groups who had concomitant gynecological conditions, such as cystadenoma, dermoid cysts, borderline or malignant tumors, fibroids or polycystic ovarian syndrome, which may influence the results. Medical history, body mass index (BMI) and white blood cell count (WBC) were recorded and the PF was cultured for microorganisms (Table 1).

Neutrophil isolation

Human neutrophils from healthy volunteers were isolated using density gradient separation as described previously [12,37]. Briefly, a double gradient was formed by layering an equal volume of histopaque-1077 over histopaque-1119 (Sigma-Aldrich, Germany). Venous blood was collected in EDTA tubes and layered onto the upper histopaque-1077, by centrifugation at 700 × *g* for 30 min a Granulocytes were concentrated at the 1077/1119 interphase. Cell viability was determined to be 98% by trypan blue dye exclusion. The isolated neutrophils were resuspended in phenol-red free RPMI 1640 medium (Invitrogen, Germany) for further analysis.

Treatment of neutrophils and quantification of NETs

Approximately 10^5 neutrophil cells from healthy donors were seeded per well on a 94-well plate and treated with 100 μl PF from either the control or the endometriosis group for 3 h at 37 °C. As a positive control neutrophils were stimulated with phorbol myristate acetate (PMA) as a typical inducer of NETosis [10]. For negative control, RPMI 1640 Medium (Invitrogen) was added to the neutrophils. Each well was stained with 50 μl of 5 μM Sytox Orange (Invitrogen), which is a cell-membrane impermeable DNA stain, in phosphate buffered saline (PBS) for 10 min at room temperature. Afterwards, the excess of Sytox Orange was removed by centrifugation of the plate at 250 × *g* for 10 min, and 50 μl from the supernatants was discarded. The fluorescence intensity was measured at excitation and emission wavelengths of 545 nm and 590 nm, respectively (FLx 800 fluorescence microplate reader; BIO-TEK Instruments).

Immunofluorescence microscopy of neutrophils treated with PF

Isolated neutrophils from healthy donors were seeded on coverslips and treated either with the PF of endometriosis patients or kept untreated for 3 h at 37 °C. Afterwards, the cells were fixed with 2% paraformaldehyde and blocked with 3% bovine serum albumin in PBS. For NET detection, the neutrophils were incubated with primary mouse anti-DNA Histone H1 (Millipore, Germany), followed by detection with the secondary antibody coupled to Alexa Fluor 555 donkey anti-mouse IgG (Invitrogen, Germany), and 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield mounting medium with DAPI; Vector Laboratories, Burlingame, CA, USA) was used for nuclear DNA detection. Images were taken with fluorescence microscope using MetaMorph imaging software version 7.0 (Leica Microsystems, Wetzlar, Germany).

Myeloperoxidase (MPO)-DNA ELISA

To detect the preexistent NETs in the PF, we used the MPO-DNA capture ELISA method according to Caudrillier and co-workers based on MPO association with NETs [19]. Prior to assessment PF samples were pretreated with 500 U/ml micrococcal nuclease (MNase) from Staphylococcus aureus (Sigma-Aldrich) in order to partially digest NETs and to obtain smaller DNA-MPO particles for the assay. For the capture antibody, 5 μg/ml anti-MPO mAb (Millipore) was coated onto a 96-well plate (dilution 1:500 in 50 μl) overnight at 4 °C. After washing the Plate 3 times (300 μl each), the plate was coated with 1% bovine serum albumin (BSA) in PBS for 1 h. Thereafter, 20 μl of the MNase-treated PF samples was added to the wells with 80 μl incubation buffer containing a peroxidase-labeled anti-DNA mAb (Cell Death ELISA^{PLUS}, Roche; dilution 1:25). The plate was incubated for 2 h, shaking at 300 rpm at room temperature. After 3 washes (300 μl each), 100 μl of peroxidase substrate was added and absorbance at 405-nm wavelength was measured after 20 min of incubation at room temperature in the dark.

Statistical analysis

The descriptive parameters, *e.g.* age, BMI, smoking habits and WBC were tested for normality and compared using the Smirnov–Kolmogorov test. In the Sytox Orange analysis the results were expressed as relative absorbance increases compared to the vehicle (sample-vehicle/vehicle) and the not normally distributed data were compared with the Mann–Whitney-*U* test. In the MPO-DNA ELISA the percentage of NET positive patients was determined