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Significantly increased concentration of soluble urokinase-type plasminogen activator receptor in the blood of patients with pelvic inflammatory disease

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ABSTRACT

Background: To determine expression levels of urokinase-type plasminogen activator (uPA), soluble urokinase-type plasminogen activator receptor (suPAR), plasminogen activator inhibitor-1 (PAI-1) in plasma and to identify gene polymorphisms specific to patients with pelvic inflammatory disease (PID) and healthy controls.

Methods: Enzyme-linked immunosorbent assay and polymerase chain reaction–restriction fragment length polymorphism were used to measure plasma levels and polymorphisms in uPA, suPAR, and PAI-1 among seventy healthy controls and 64 PID patients before and after they received routine treatment protocols.

Results: The levels of plasma uPA (ng/ml) and soluble suPAR (pg/ml) were significantly increased in PID patients (uPA: 0.57 ± 0.03; suPAR: 1372.04 ± 68.20) when compared to that in normal controls (uPA: 0.55 ± 0.06, $p = 0.002$; suPAR: 1192.46 ± 51.98, $p = 0.04$); moreover, suPAR decreased significantly after treatment when compared to levels noted in the same patients (1220.06 ± 58.14; $p = 0.003$) after they received treatment. The increased expression of suPAR was significantly correlated with WBC counts ($r = 0.382$, $p = 0.002$, $n = 64$) in blood as well as the plasma levels of CRP ($r = 0.441$, $p < 0.0001$, $n = 64$) and uPA ($r = 0.426$, $p < 0.0001$, $n = 64$) of PID patients prior to receiving treatment.

Conclusions: Increased plasma suPAR could be a biological marker for the diagnosis of PID and may reflect a new focus in targeted therapy for pelvic inflammatory disease.

1. Introduction

Pelvic inflammatory disease (PID) is currently the most common inflammatory disease worldwide [1–3] and is associated with serious long term consequences, such as infertility, chronic pelvic pain, and ectopic pregnancy [4–6]. The plasminogen activation system has been reported to play an important role in mediating fibrin degradation in the inflammatory process [7–9]. Plasminogen activator (PA) is a specific serine protease that binds to a cellular receptor and then converts the inactive protein plasminogen to the active enzyme plasmin [10,11]. Two such plasminogen activators that are noted to be important in the inflammatory process include urokinase (uPA) and tissue type (tPA). Urokinase plasminogen activator and its receptor (uPAR) are thought to regulate pericellular matrix degradation, cell migration and cell adhesion during the inflammatory process [12–14]. Plasminogen activator activity in bovine endometrial tissues is significantly increased among cows with severe endometritis [15]. Urokinase-type plasminogen activator receptor (uPAR) has been reported to play an important role in the defense against pathogens [13,14,16]. It has been demonstrated that uPAR may become cleaved at its cell surface anchor to form a free soluble receptor (suPAR) in blood, and plasma suPAR concentrations have been noted to be increased among patients with rheumatoid arthritis [12] and women with preeclampsia [17]. Plasminogen activator inhibitors (PAIs) contribute to the down regulation of plasminogen activators and are present in body fluids and tissues [9,18]. Up-regulation of uPA and PAI-1 has been found in the peritoneal fluid of women suffering from PID when compared with normal controls [18,19]. The increased concentration of uPA was thought to stimulate fibrinolysis and prevent adhesion formation; however, an increase in PAI-1 likely reflects an underlying response that reduces fibrinolysis during the inflammatory process [18]. However, using laparoscopy to obtain peritoneal fluid is risky and inappropriate in most cases. Therefore, it is highly

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recommended that the significance of non-invasive strategies on the diagnosis and therapy of pelvic inflammatory disease be explored.

Single nucleotide polymorphisms (SNPs) in uPA system genes, such as uPA (rs4065) C/T SNP, uPAR (rs344781) T/C SNP, and PAI-1 (rs1799889) 4G/5G SNP, which are located within the promoter or other regulatory regions, have been suggested to affect gene expression [20–23]. Recently epidemiological studies have found that these gene polymorphisms may increase susceptibility to inflammation-related disease processes, including sepsis [22], atherosclerosis [23], rheumatoid arthritis [12] and acute pancreatitis [24]; however, their potential association with pelvic inflammatory disease has not been investigated. We hypothesized that gene polymorphisms of the *uPA* system could be associated with the development of pelvic inflammatory disease. Therefore, we determined the relationship between plasma protein concentrations, of uPA, suPAR, and PAI-1 and pelvic inflammatory disease. Furthermore, we evaluated the distributions of uPA, uPAR, and PAI-1 gene polymorphisms among PID patients and normal controls and assessed whether protein expression levels of these genes were altered among different genetic polymorphisms to estimate the impact of these polymorphisms on expression levels.

2. Materials and methods

2.1. Subjects and specimen collection

This was a hospital-based case–control study. We worked with gynecologists at Chung Shan Medical University Hospital in Taichung, Taiwan. Between April 2006 and August 2011, 64 women who were diagnosed with PID by gynecologists (Wang PH, Tee YT, and Lin LY) based on the criteria of the national guidelines for pelvic inflammatory disease was recruited as a case group [25,26]. Seventy healthy women who visited the Department of Obstetrics and Gynecology or the Department of Family Medicine for a health examination, e.g., cervical Papanicolaou smear and breast examination, were randomly selected as healthy controls. Healthy controls were matched with respect to demographic and clinical data, i.e., age (matched to within 5 years), race, ethnicity, socioeconomic status, resident area, cigarette smoking status, and alcohol drinking status. Healthy women with risk factors associated with PID were excluded from the control group. The diagnosis of PID conformed to the minimal criteria determined by the Centers for Disease Control and Prevention (CDC) including lower abdominal pain or pelvic pain of no other origin with one of the following criteria: uterine tenderness or adnexal tenderness or cervical motion tenderness. To maximize specificity and reduce the chance of a delayed or missed diagnosis, in addition to the criteria mentioned above, the patients were required to have at least one of the following minor criteria: oral temperature > 38.3 °C, abnormal vaginal or cervical mucopurulent discharge, an abundance of white blood cells (WBCs) on microscopic inspection of vaginal secretions, increased C-reactive protein (CRP), increased erythrocyte sedimentations, or laboratory documentation of *Neisseria gonorrhoeae* or *Chlamydia trachomatis*. Women who were pregnant, breast feeding, taking oral contraceptives or antibiotics to treat other forms of inflammatory diseases, who had systemic diseases or cancers that originated from the pelvic organs (e.g., the cervix or ovaries) or who had undergone a gynecologic operation within 2 months prior to admission were excluded from the study. Whole blood was collected from the seventy healthy controls and 64 PID patients before and after they received treatment based on the routine protocols suggested by the CDC. The recommended parenteral regimens were cefotetan or cefoxitin plus doxycycline or clindamycin plus gentamicin. All PID patients were admitted to the ward unit of the Department of Obstetrics and Gynecology, Chung Shan Medical University Hospital. They were given antibiotics intravenously for at least 3 days and for an additional 24 h after they were afebrile. Thereafter, oral antibiotics were given until day 14 of treatment. Pre-treatment blood samples were obtained before PID patients received treatment protocols, and post-treatment blood samples were obtained one week after treatment was initiated. All blood samples were analyzed for nonspecific inflammatory markers, such as WBC and C-reactive protein (CRP) [27,28]. In addition, plasma samples were analyzed for the expression of uPA, suPAR, and PAI-1. Both the technician who measured levels of uPA, suPAR, and PAI-1 as well as the clinical laboratory staff who measured WBC counts, neutrophil counts, lymphocytes, and CRP were blinded to this study. The blood samples obtained for measurement of uPA, suPAR, and PAI-1 were placed in tubes containing EDTA and were immediately centrifuged and stored at −80 °C. The study was performed with the approval of the Chung Shan Medical University Hospital Institutional Review Board, and written informed consent was obtained from each patient.

2.2. Measurements of uPA, suPAR, and PAI-1 levels by ELISA

The uPA, suPAR, and PAI-1 levels in the plasma samples were analyzed using human uPA, suPAR, and PAI-1 ELISA kits, respectively (R&D Systems, Abingdon, UK). From each plasma sample, 100 μl was directly transferred to the microtest strip wells of the ELISA plate and was then assayed according to the manufacturer's instructions. Absorbance was measured at 495 nm in a microtest plate spectrophotometer, and plasma levels of uPA, suPAR, and PAI-1 were quantified by a calibration curve that used human uPA, suPAR, and PAI-1 as a standard, respectively [29].

2.3. Genomic DNA extraction

Venous blood from each subject was drawn into Vacutainer tubes containing EDTA and stored at 4 °C. Genomic DNA was extracted using QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacturer's instructions. DNA was dissolved in TE buffer [10 mmol/l Tris (pH 7.8), 1 mmol/l EDTA] and then quantitated by measuring OD260. The final preparation was stored at −20 °C and used as a template for polymerase chain reaction (PCR) [30].

2.4. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP)

The primer sequences and the restriction enzyme for analysis of uPA, uPAR, and PAI-1 gene polymorphisms have been described elsewhere [30]. PCR was performed in a 10 μl volume containing 100 ng DNA template, 1.0 μl of 10 × PCR buffer (Invitrogen, Carlsbad, CA), 0.25 U of Taq DNA polymerase (Invitrogen), 0.2 mmol/l dNTPs (Promega, Madison, WI, USA) and 200 nmol/l of each primer (MDBio Inc. Taipei, Taiwan). A 10 μl aliquot of PCR product was subjected to digestion by each restriction enzyme (New England Biolabs, Beverly, MA) at 37 or 55 °C for 4 h. After digestion, the products were separated on a 3% agarose gel stained with ethidium bromide. As a result, for the C/T SNP of uPA, the T allele yielded 187- and 104-bp products, while the C alleles yielded a 291-bp product; for the T/C SNP of uPAR, the C allele yielded 200- and 108-bp products, while T alleles yielded a 308-bp product; for the 4G/5G SNP of PAI-1, the 5G allele yielded 74-, 56-, and 33-bp products, while the 4G alleles yielded 107- and 56-bp products.

2.5. Statistical analysis

The experimental results are presented as the mean ± SE. The estimated parameters (uPA, suPAR, and PAI-1 levels) were skewed quantitative variables even though we attempted to transform them to achieve normality, so non-parametric statistics methods were used. A Mann–Whitney U test was used to compare differences in plasma levels of uPA, suPAR, and PAI-1, as well as WBC counts and C-reactive protein (CRP) levels between healthy women and PID patients prior to receiving the treatment protocols. A Wilcoxon signed-rank test was