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ENDOMETRIOSIS IS ASSOCIATED WITH ALTERATIONS IN THE RELATIVE ABUNDANCE OF PROTEINS AND IL-10 IN THE PERITONEAL FLUID

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1. ABSTRACT

A growing body of evidence suggests that endometriosis modulates the microenvironment of peritoneal cavity. Therefore, in this study, we compared the protein profile of peritoneal fluids from normal fertile women with those from patients with infertility, and patients with mild to severe endometriosis. Two-dimensional gel electrophoresis of peritoneal fluids from normal subjects exhibited a distinct and reproducible pattern of proteins in the size ranges of approximately 35 to 80 kD and pI close to 4.5 to 6.6. Infertility without evidence of endometriosis was not associated with changes in the relative abundance of proteins present in the peritoneal fluid. However, mild endometriosis was associated with a mild reduction in the amount of several peritoneal protein spots with the approximate molecular weights of 35-40 kD and pI close to 5.7-6.0. These changes became markedly apparent in the peritoneal fluid of patients who suffered from the severe form of this disease. Severe endometriosis was also associated with appearance of protein spots in the gels that were not detectable in the peritoneal fluids of normal subjects. Consistent with these data, enzyme-linked immunosorbent assay showed that moderate to severe endometriosis was associated with markedly elevated levels of IL-10 in the peritoneal fluid. Reverse transcription followed by polymerase chain reaction amplification using primers specific to IL-10 confirmed presence of IL-10 mRNA in cells derived from peritoneal fluids. These findings show that endometriosis is associated with disturbed secretion of proteins into the peritoneal cavity and with an elevated level of IL-10 in the peritoneal fluid. The studies also show cells resident in peritoneum as a major source of IL-10.

2. INTRODUCTION

Endometriosis is defined as the presence of endometrial tissue composed of endometrial glands, stroma or both outside the endometrial cavity. This disease can be associated with pelvic pain, dysmenorrhea, abnormal bleeding and infertility (1). Various lines of evidence suggest that a number of abnormalities exist in the endometriotic tissues, the peritoneal fluids and the endometrium of patients with endometriosis (reviewed in 2,3). The endometrial tissues that reside in ectopic foci exhibit a number of differences from the eutopic endometrium such as expression of interleukin-1 receptor antagonist (4), IL-6 receptor (5), HLA-DR (6), proliferation of endometrial cells (7) and secretion of proinflammatory cytokines (8). Furthermore, it has been suggested that the eutopic endometrium in patients with endometriosis

exhibits abnormal characteristics such as aberrant expression of ß3 integrin (9). Additional abnormalities have been reported in the peritoneal fluids of patients with endometriosis. This includes an increased number of peritoneal macrophages (10, 11), decreased natural killer cell activity (12), increased levels of angiogenic (13), and chemotactic (14) activities, the presence of a unique 32-kD protein (15), the increased synthesis and secretion of complement component-3 (16) and increased levels of CA-125 (17). Elevated levels of cytokines such as bioactive or immunoreactive IL-1, IL-2, IL-4, IL-6, IL-8, IFN? ?TNF-? , RANTES, and TGF-ß have been reported in the peritoneal fluids of women with endometriosis (18-26). It has been suggested that these factors may contribute to the infertility that is frequently observed in patients with endometriosis (2, 18). In particular TNF-? has been shown to be both embryotoxic and spermatotoxic (27). In view of the presence of macrophages and cytokines in the peritoneal fluid of women with this disease, it has been suggested that the development of endometriosis may be immune mediated. IL-10 has emerged as a cytokine with immunosuppressive and immune modulatory activities (28-31), although conflicting reports on the levels of this cytokine in the peritoneal fluid of patients with endometriosis have been described (32-34).

In this report, the impact of endometriosis on peritoneal fluid proteins was examined by two-dimensional gel electrophoresis patterns of proteins in peritoneal fluids of normal fertile control patients, as compared with those in the peritoneal fluids from endometriosis patients. The results of these studies demonstrated changes in the relative amounts of several protein spots. We also quantitated the relative abundance of IL-10 protein in these fluids, as well as IL-10 mRNA in the cells present in the peritoneal fluids.

3. MATERIALS AND METHODS

3.1. Materials

Human IL-10 primers and primers to ß-actin were obtained from Stratagene Inc. (La Jolla, CA). The expected sizes of the amplicons were respectively 432 and 661 (35, 36). The sequence of the forward 5' primer for IL-10 which starts at nucleotide 27 is: AAGGATCCATGCACAGCTCAGCAC and the sequence of the reverse 3' primer which ends at nucleotide 453 is: CTGCACCACTGCCTTGCTCTTATT. The sequences of primers for ß-actin were as follows: 5' primer: TGACGGGGTCACCCACACTGTGCCCATCTA and 3' primer: CTAGAAGCATTTGCGGTGGACGATGGAGGG. These primers were synthesized on a DNA synthesizer and purified by HPLC. To verify the identity of the PCR products, the upper and lower IL-10 amplified bands were removed from agarose gels, purified using the Qiaex II kit according to the manufacturer's recommendations (Qiagen, Chatsworth, CA), cloned into pBluescript II and sequenced. The DNA was sequenced with Sequenase ver. 2.0 (Amersham, Life Sciences, Arlington, IL) using the dideoxy chain termination method (37).

All reagents used in the reverse transcription were obtained from Promega (Madison, WI). The Perkin Elmer Cetus GeneAmp Kit was obtained from Perkin Elmer (Norwalk, CT). The molecular weight standards, ϕX174 RF DNA/Hae III fragments and 123 base pair ladder, were obtained from Gibco-BRL Life Technologies (Gaithersburg, Maryland). These molecular weight standard markers allow size determination of double stranded DNA from 123 to 3,075 base pairs.

3.2. Subjects

The patient population in this study was approximately 50% caucasian, 30% African American and 20% Hispanic women; all were of reproductive age and not using any form of hormonal therapy. Laparoscopic examination with sampling was done to confirm the presence of endometriosis and the severity of disease was staged according to the revised American Fertility Society guidelines (38). The subjects included were normal fertile women undergoing bilateral tubal ligation (n=10), women with infertility and without clinical or laparoscopic evidence of endometriosis (n=10), women with mild endometriosis (revised AFS stages I to II, n=12) and women with severe endometriosis (revised AFS stages III to IV, n=12). Approval of this project was obtained from the review boards of the institution.

3.3. Processing of peritoneal fluids

Cul de sac fluid was obtained within ten minutes after induction of general anesthesia and immediately after the introduction of laparoscopic instruments as described (23). All peritoneal fluid specimens were cultured to verify the absence of microbial contamination. Peritoneal fluids were xanthochromatic in color and there was no gross evidence of contamination with blood. To show that these fluids were not contaminated with blood, hemoglobin levels were measured in these fluids and in the sera of patients. The amount of hemoglobin was measured by Cell-Dyne 3000 (Abbott Laboratories, Chicago, IL). The instrument measures hemoglobin in the range of 0-30 gram/dl. The average level of hemoglobin in the serum samples was 12.5 g/dl whereas the level detected in the peritoneal fluids ranged from 0-0.1 gram/dl which is equivalent to those detected in the buffer used for detection of the background levels. Cells were recovered from the fluids by centrifugation (300 x g, 10 min, 4^{o}C). Both cells and supernatant fluids were flash frozen at -80^{o}C. Previous studies showed that cells recovered from the fluids in such a fashion were comprised of >90% CD45 positive leukocytes (39).

3.4. Two dimensional (2D) gel electrophoresis

The peritoneal fluids analyzed by 2D gel electrophoresis consisted of fluids from 6 normal fertile controls, 6 infertile women, 6 women with mild endometriosis and 6 women with severe endometriosis. The fluids were dialyzed (molecular weight cut-off =8000 kD) against three changes of 1 mM Tris-HCl, pH 8.2 to remove electrolytes and other compounds that potentially interfere with the isoelectric focusing. The protein concentrations of the remaining fluids were then estimated by the method of Bradford (40). Two dimensional gel electrophoresis with isoelectric focusing (IEF) in the first dimension (41) was carried out as described (42, 43). Briefly, 300 µg of dialysis equilibrated proteins were suspended in 25 µl of a

buffer of 5 mM K_2CO_3 containing 9.4 urea, 2% (v/v) nonidet P-40 and 0.5% (w/v) dithiothreitol (DTT). The first dimension of the gel electrophoresis was performed for 18 hours at 400V in 130 mm x 1.2 mm, in 4% (W/V) polyacrylamide gels containing 2% carrier ampholytes (1.6% pH 5-7, Serva; 0.4% pH 3.5-10, LKB). First dimensional gels were equilibrated in 3 ml (3 min at room temperature) of equilibration solution (0.06 M Tris-HCl, pH 6.8; 2% SDS; 100 mM DTT and 10% glycerol) (41). Gels were then stored at -20°C until use. First dimensional gels were applied to the second dimension with the aid of agarose solution (0.06 M Tris-HCl, pH 6.8; 2% SDS; 100 mM DTT; 10% glycerol; 1% agarose and 0.002% bromophenol blue) (41). SDS-PAGE (15% running gel and 5% stacking gel) was then carried out. The gels were then stained with silver as described by the manufacturer (Biorad, Hercules, CA). The pI and molecular weights were estimated from pI/molecular weight standards (Biorad) that were run in both dimensions and the molecular weight markers that were run in the second dimension. The relative optical densities of the spots were obtained by laser scanning densitometry using SigmaGel analysis software (Jandel Scientific Software, San Rafael, CA).

3.5. Enzyme linked immunosorbent assay (ELISA) for demonstration of IL-10

Peritoneal fluid IL-10 levels were quantified using commercially available IL-10 kits (Biosource International; Camarillo, CA). Assays were performed, according to the manufacturer's instruction, on the peritoneal fluids diluted 1:4. The amount of IL-10 in each sample was interpolated from a graph based on optical densities derived from a series of standards. The sandwich ELISA detects both natural and recombinant IL-10 in human serum, plasma or cell culture fluids. The assay is highly specific for IL-10; the lower detection limit of the assay is <5 pg/ml.

3.6. Reverse transcription-polymerase chain reaction

Total RNA was obtained using TriReagent (MRC Inc., Cincinnati, OH), and used according to the protocol specified by the manufacturer. Briefly, cells (10^6-10^7) derived from the peritoneal fluids were lysed in 0.8 ml of TriReagent solution in the presence of glycogen carrier. Supernatant containing RNA was combined with 0.2 ml of chloroform, precipitated with isopropanol and washed with 70% ethanol. The RNA pellet was dissolved in RNAse-free water and incubated at 37°C with 40 U DNAse I (Gibco-BRL Life Technologies) for 30 minutes. The reaction was terminated by the addition of EDTA (20 mM) and incubated for 10 min at 65°C. Total RNA was precipitated overnight at -80°C by the addition of three volumes of absolute ethanol-sodium chloride mixture. The quantity of the RNA was determined spectrophotometrically.

Total RNA was reverse transcribed to cDNA as described (Rappopolee *et al.*, 1988; Sambrook *et al.*, 1989). Briefly, total RNA was reverse transcribed in a 20 µl volume containing 2 µg RNA; 0.2 µg oligo(dT), 1.25 mM of each of dATP, dCTP, dGTP, dTTP; 5 U AMV reverse transcriptase; 10 mM MeHgOH, 88 mM ß-mercaptoethanol; 10 U RNAsin; 100 mM Tris-HCl (pH 8.3); 40 mM KCl and 10 mM $MgCl_2$. After 60 minutes of incubation at 42°C, the reaction mixture was heated to 95°C for 3 minutes. Following addition of 5 U of AMV, the reverse transcription was carried out for an additional 60 minutes. After a final incubation at 95°C for 3 minutes, reverse transcription was terminated by placing the reaction mixture at 0°C.

PCR was carried out as described (44, 45). Briefly, 1 µg of reverse transcribed RNA was amplified with 0.5-1 µM of each of the 5' and 3' primers specific for IL-10 in a 50 µl reaction volume containing 1.25 U AmpliTaq DNA polymerase, 1.25 mM $MgCl_2$, 20 µM of each of dATP, dCTP, dGTP, dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and sterile distilled water. Negative control tubes received nonreverse transcribed RNA to verify absence of contaminating DNA. Positive control tubes received all the reagents in the reaction mixture, however, the primers used were specific for ß-actin. The reaction mixture was overlayered with 50 µl of mineral oil and the tubes were heated for 5 minutes at 95°C. After initiation of temperature cycling with a Dual-Block Thermal Cycler (Ericomp, San Diego, CA), samples were amplified for 35 cycles. The denaturation temperature was 95°C for 1 minute, annealing temperature was 55°C for 1 minute and the extension temperature was 72°C for 2.5 minutes. Temperature cycling was concluded with a final extension at 72°C for 10 minutes and the reaction products were maintained at 4°C. Amplified products were resolved in a 2% agarose gel and the bands were visualized by ethidium bromide staining. The ϕX174 Hae III RF DNA fragments and the 123 basepair DNA ladder were used as molecular weight markers.

3.7. Statistical analysis

Data were analyzed using SigmaStat statistical software (Jandel Scientific, San Rafael, CA). Kruskal-Wallis one way analysis of variance (ANOVA) on ranks was used to compare the values among various groups. Levene median test was used to determine homogeneity of variances. When significant differences were noted with ANOVA, the Dunns method was used to compare results of normal group with the patient groups.

4. RESULTS

The peritoneal fluids from four patient groups were subjected to two-dimensional gel electrophoresis. In order to ensure reproducibility, the fluids from six individual patients per group were examined. The peritoneal fluids from normal controls exhibited approximately 73 protein spots that were resolvable by silver staining of the gels (Figure 1A). Comparison of the spots found in these two-dimensional gels with those observed in the peripheral blood showed that several major protein spots were the same as those present in the peripheral blood (Figure 1B). Immunblotting revealed that one of the major protein spots corresponds to albumin with a molecular weight of 70 kD (open arrowhead a, Figure 1 A-D). Except for these proteins, the protein spots within peritoneal fluids were

distinctly different from those found in the peripheral blood. The protein spots observed in women with infertility and without evidence of endometriosis did not differ from those found in the normal healthy controls (data not shown). In contrast, endometriosis was associated with changes in the peritoneal fluid proteins, as detectable by two-dimensional gel electrophoresis. Mild endometriosis led to only minimal loss of proteins in the peritoneal fluid in the size ranges of approximately 35 to 40 kD and pI between 5.7 to 6.0 (closed arrowheads, Figure 1C). On the other hand, in the severe form of the disease, a greater decrease of the same protein spots was seen (closed arrowheads, Figure 1D).

Several protein spots appeared in the peritoneal fluids of patients with severe endometriosis that were not readily detectable in the peritoneal fluids of normal controls or patients with either infertility or mild endometriosis. For example, in peritoneal fluids from women with severe disease, a mean relative optical density of 2295 was observed for the protein spot with a molecular weight above 110 kD and pI close to 5.0 (curved arrow 1, Figure 1D), whereas the mean relative optical densities of the same spot in the normal controls was 422±76(SD). In the fluids from severe endometriosis patients, the mean relative optical densities of the protein spots with a molecular weight close to 20 kD and pI of 5.6 and 5.7 (curved arrows 2 and 3, Figure 1D) were respectively 2059±234 and 1077±646. The mean relative optical densities of the same spots in the normal subjects were respectively 448±301and 117±17. These findings show a two to four fold increase in the amount of these proteins in severe endometriosis.

The two-dimensional gel electrophoresis data indicated that the presence of endometriosis markedly altered the type and amount of proteins present and/or secreted into the peritoneal fluid and that the severe form of this disease was associated with a clear increase in the amount of specific proteins. Peritoneal fluid is known to contain a variety of growth factors and pro-inflammatory cytokines, the levels of which are generally reported to be increased in the peritoneal fluids of patients with endometriosis (18-21, 46-48). Less clear, however, is the role of immunosuppressive cytokines, such as IL-10, in endometriosis. We found that the amounts of IL-10 in the peritoneal fluids from infertile women or women with mild endometriosis did not significantly differ from those observed in the normal control subjects ($p=0.5216$, Figure 2). In marked contrast, the amounts of IL-10 in the peritoneal fluids of women with severe endometriosis was elevated more than 10-fold relative to all other patient groups ($p=0.000126$). In addition, our previous studies demonstrated no significant differences in the amount of several cytokines in relation to the phase of the menstrual cycle in these fluids (5, 23).

The leukocytes present within the peritoneal fluid have been shown to be a major source of the cytokines detected within this fluid (48-51). We therefore looked for the presence of IL-10 mRNA within these cells. In all the samples examined, reverse transcription of RNA isolated from cells derived from peritoneal fluids followed by amplification of the cDNA with amplimers specific to IL-10 showed presence of an amplified product of the expected size (432 base pairs; Figure 3). However, an additional band was also observed in these samples. To show that presence of such a band was not related to the presence of contaminating DNA, the reverse transcription was omitted. Such samples failed to produce an amplified product. Reverse transcription followed by amplification using primers specific to ß-actin as a positive control revealed an amplicon with a size different than the IL-10 amplicon and with a size expected for ß-actin amplicon (Figure 3, lane 2). The amplified product of IL-10 was obtained in all the RT-PCR reactions regardless whether RNA was derived from normal subjects or any of the patient groups.

To verify the identity of the IL-10 RT-PCR products, the upper and lower bands visible on the agarose gel were cloned into pBluescript II vector and sequenced. The upper band contained all the 432 bp of IL-10 sequence expected from amplification of the cDNA with IL-10 specific primers. Although truncated at its 5' end, the sequence of the lower band also exactly matched the IL-10 sequence.

5. DISCUSSION

In this study, we have shown that endometriosis was associated with changes in the amounts and types of proteins that predominate within peritoneal fluid. Two-dimensional gel electrophoresis demonstrated that these alterations consisted of the loss of several major protein spots and the appearance of protein spots that were not detectable in peritoneal fluids from normal subjects. Consistent with the latter findings, we showed by ELISA that severe endometriosis was associated with a significant increase in the amount of IL-10 protein in the peritoneal fluid, which was correlated with IL-10 mRNA expressed in the peritoneal cells present in this fluid. These findings corroborate previous reports which indicate the presence of endometriotic foci within the peritoneum markedly alters the peritoneal micro-environment (10-17).

In general, two basic mechanisms have been postulated as playing a role in the intraperitoneal changes which occur in endometriosis. Endometriotic tissue differs significantly from normal endometrium and, therefore, may itself be a source of these changes via secretion of unique proteins and cytokines. Several groups have demonstrated that polypeptides synthesized and released by endometriotic tissues were different from those released from eutopic endometrium (15, 52, 53). The number of cells exhibiting proliferating cell nuclear antigen was shown to differ in endometriotic tissue as compared with the eutopic endometrium (7). Immunoreactivity for IL-1 receptor antagonist was found to be absent in ectopic endometrium but was detected in eutopic endometrium (4). We demonstrated that ectopic endometrial stromal cells exhibit significantly elevated spontaneous release of IL-6 and were nonresponsive to IL-6 mediated growth inhibition which was associated with reduced IL-6 receptor expression (5). In contrast, eutopic endometrial stromal cells spontaneously released little IL-6, showed high expression of IL-6

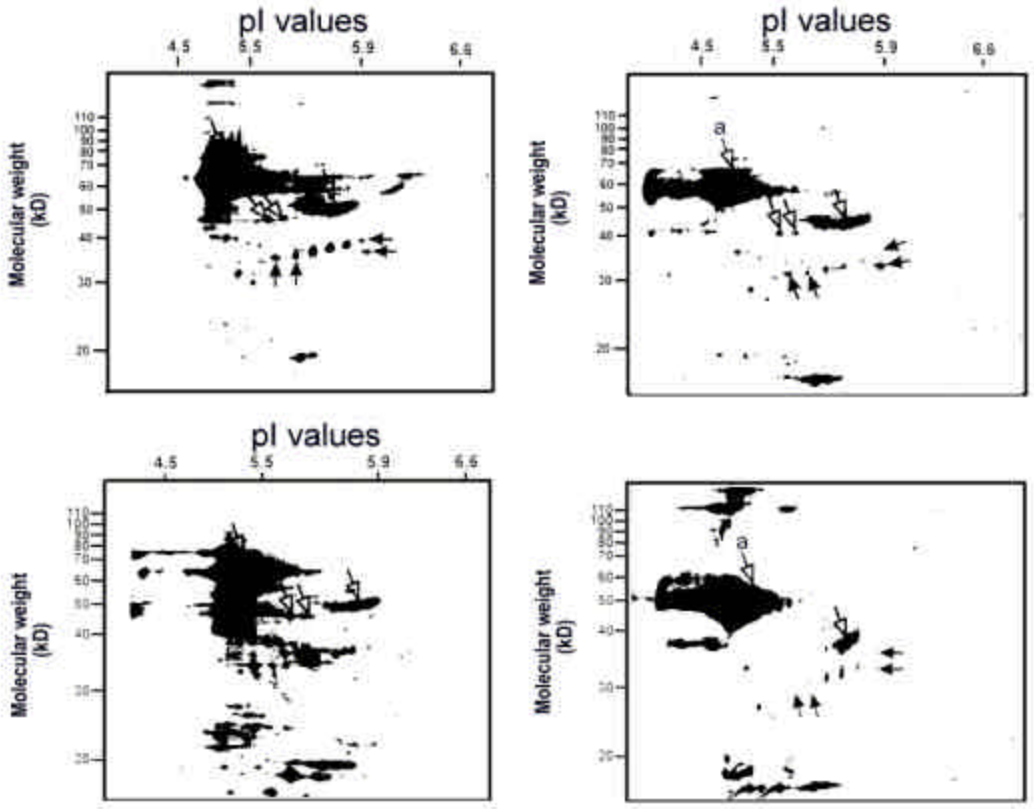

Figure 1. Two-dimensional gel electrophoresis from proteins in peritoneal fluid. The proteins within peritoneal fluids were examined by two-dimensional gel electrophoresis followed by silver staining as indicated. The subject population consisted of normal fertile control women (n=6, Figure 1A), women with infertility and without clinical or laparoscopic evidence of endometriosis (n=6, not shown), women with mild endometriosis (n=6, Figure 1C), and women with severe endometriosis (n=6, Figure 1D). The data presented are representative of all the specimens that were examined. 1A: Normal control peritoneal fluid. 1B: Peripheral blood from control. 1C: Mild endometriosis peritoneal fluid. 1D: Severe endometriosis peritoneal fluid. Note that some protein spots found in peripheral blood (both cells and plasma; blank arrows) as shown in Figure 1B are also observed in the peritoneal fluid (Figure 1A, 1C and 1D). Immunoblotting showed that the major protein band with a molecular weight of 70 kD (shown by open arrowhead a) is albumin. A decrease was noted in the relative abundance of several protein spots (black arrows) in the peritoneal fluid of patients with mild endometriosis (Figure 1C) as compared with those found in the peritoneal fluids of control subjects (Figure 1A). Severe endometriosis was associated with a marked reduction or disappearance of several protein spots in the peritoneal fluid (black arrows; Fig 1D) and a dramatic increase in the amount of several other protein spots (curved arrows; spots numbered as 1-3 in Figure 1D).

receptor and were growth inhibited by this cytokine (54). On the other hand, it is conceivable that the presence of endometriotic implants may alter the number of peritoneal cells, their phenotype or activation status. In this regard, the natural killer activity of lymphocytes derived from peritoneal fluid was shown to be decreased in endometriosis patients (12). Peritoneal fluids of patients with endometriosis were found to contain factors that activate macrophages (48) and pelvic macrophages of women with endometriosis were found to be activated (39, 49) and their maturation to be disturbed (50). Among the many intraperitoneal changes that occur in this disease, significant increases in the amounts of a variety of cytokines have been described. Elevated levels of IL-1, IL-2, IL-4, IL-8, TNF-α, RANTES, and TGF-ß have been reported in the peritoneal fluids from women with endometriosis and generally, the increase in the amount of cytokine correlated with the severity of the disease (18-20, 22, 24, 26). We found significantly elevated levels of IL-6 in the peritoneal fluids from patients with severe endometriosis (5) and that peritoneal leukocytes from women with disease exhibited increased spontaneous IL-6 production (23). Similarly, Rana *et al.* (25) found that elevated levels of IL-8 and TNF-α in the peritoneal fluids of women with endometriosis correlated with enhanced activity of peritoneal macrophages from these patients to synthesize and secrete these cytokines.

Conflicting results have been reported on the amount of IL-10 in the peritoneal fluids of women with endometriosis. Elevated levels of this cytokine have been

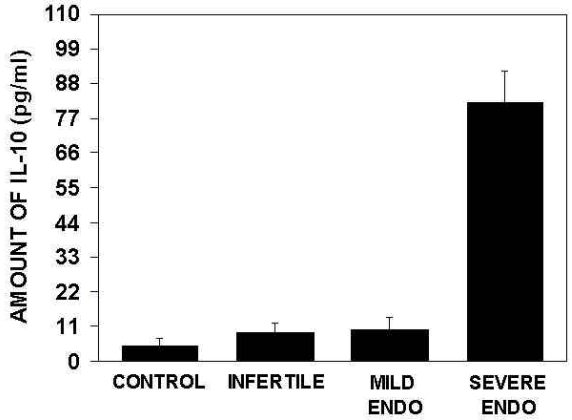

Figure 2. Levels of IL-10 (pg/ml±SD) in the peritoneal fluids, as determined by ELISA. The subject population consisted of normal fertile women (n=10, control), women with infertility and without clinical or laparoscopic evidence of endometriosis (n=10, infertile), women with mild endometriosis (n=12, mild endo), and women with severe endometriosis (n=12, severe endo).

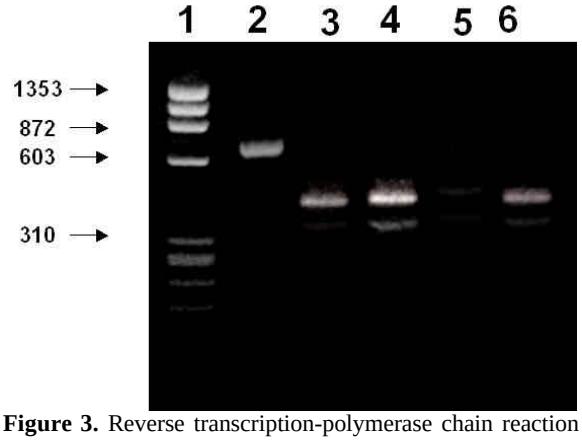

Figure 3. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of ß-actin and IL-10 mRNA in cells derived from peritoneal fluid. The subject population consisted of normal fertile control women (n=6), women with infertility and without clinical or laparoscopic evidence of endometriosis (n=6), women with mild endometriosis (n=6), and women with severe endometriosis (n=6). The data presented are representative of all the specimens that were examined. Lane 1: ϕX174 RF DNA/Hae III fragments. Lane 2: RT-PCR of ß-actin mRNA from cells derived from peritoneal fluid of a patient with mild endometriosis. Lane 3: RT-PCR of IL-10 mRNA from cells derived from peritoneal fluid of a normal control patient. Lane 4: RT-PCR of IL-10 mRNA from cells derived from peritoneal fluid of a patient with infertility. Lane 5: RT-PCR of IL-10 mRNA from cells derived from peritoneal fluid of a patient with mild endometriosis. Lane 6: RT-PCR of IL-10 mRNA from cells derived from peritoneal fluid of a patient with severe endometriosis. The molecular weights of the ß-actin (661 base pairs) and the IL-10 (432 base pairs) amplicons correspond to the size of expected amplified products. The sequences of the upper and lower bands in lanes 3-6 matched the reported sequence of human IL-10.

demonstrated in patients with this disease, as compared to normal fertile women undergoing tubal ligation (33, 55), although no determination of IL-10 as a function of disease stage was assessed in either of these studies. In contrast, McLaren and colleagues (32) found no difference in the levels of IL-10 in the peritoneal fluids of women with and without endometriosis, although a reduction in the amount of IL-13 in these fluids was observed in women with disease. D'Hooghe *et al.* (34) reported no differences in the amounts of several cytokines, including IL-10, in either peritoneal fluid or peripheral blood from women with superficial versus deeply infiltating endometriosis. Rana and coworkers (25) observed no differences in IL-10 protein levels in the peritoneal fluids of patients with severe disease, as compared to normal fertile women, although the peritoneal macrophages from the endometriosis patients exhibited IL-10 mRNA, as well as increased basal and stimulated synthesis of IL-10 protein. It was postulated that this discrepancy could be a function of assay conditions used, and may reflect masking of IL-10 bound to proteins within peritoneal fluid or increased IL-10 metabolism in women with disease. Wu and colleagues (56) also showed that peritoneal macrophages from women with endometriosis exhibited higher lipopolysaccharide induced IL-10 production as compared to normal controls.

Studies suggest that IL-10 may play a role in the regulation of auto-antigen driven responses (57) and, therefore, could be part of the molecular repertoire driven by auto-reactive T cells specific to endometrium which may be present in the peritoneal fluid of patients with endometriosis. In this regard, we have previously demonstrated the presence of a unique CD8/S6F1+ cytotoxic peritoneal T cell subpopulation (39). The increased secretion of IL-10 may also be part of the general response to the signal(s) that leads to an increased level of many cytokines in peritoneal fluids of patients with endometriosis (18-21). On the other hand, since IL-10 actively inhibits the secretion of cytokines such as IL-1, IL-6, and TNF-α (30, 31), the increased levels of IL-10 in the peritoneal fluids of patients with severe endometriosis may be secondary and reflect a reactive response to the elevated levels of cytokines in these fluids. Consistent with this concept, when the activity of IL-10 was neutralized in mice by the administration of neutralizing anti-IL-10 antibodies, significant changes in the levels of cytokines such as IL-6, IFNγ and monokines appeared in the peripheral circulation (58). Furthermore, it has been suggested recently that the progression of endometriosis may be associated with a shift characterized by the induction of cell mediated immunity via elaboration of Th1 cytokines, including IL-2, IL-12 and IFNγ observed in mild disease, towards the production of an immunosuppressive response in severe disease associated with the production of Th2 cytokines including IL-4, IL-6 and IL-10 (2, 24).

In summary, the present report demonstrates that both mild and severe endometriosis was associated with significant changes in the relative abundance of proteins present within the peritoneal fluid. These studies also show that severe disease was associated with a dramatic increase in the amount of IL-10 present in this fluid. Taken

together, these findings provide further evidence that the presence of ectopic endometrium within peritoneal cavity markedly alters the micro-environment of peritoneal fluid.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. Galle PC: Clinical presentation and diagnosis of endometriosis. *Obstet Gynecol Clin N Am* 16, 29-42 (1989)

2. Harada T, T. Iwabe & N. Terakawa: Role of cytokines in endometriosis. *Fertil Steril* 76, 1-10 (2001)

3. Lebovic, DI, M. Muelier & R.N. Taylor: Immunobiology of endometriosis. *Fertil Steril* 75, 1-10 (2001)

4. Sahakian V, J. Anners, A. Haskill & J. Halme: Selective localization of interleukin-1 receptor antagonist in eutopic endometrium and endometriotic implants. *Fertil Steril* 60, 276-279 (1993)

5. Rier SE, P.N. Zarmakoupis, X. Hu & J.L. Becker: Dysregulation of interleukin-6 responses in ectopic endometrial stromal cells: correlation with decreased soluble receptor levels in peritoneal fluid of women with endometriosis. *Clin Endocrinol Metab* 80, 1431-1437 (1995)

6. Ota H & S. Igarashi: Expression of major histocompatibility complex II antigen in endometriotic tissue in patients with endometriosis and adenomyosis. *Fertil Steril* 60, 834-838 (1993)

7. Li S-F, K. Nakayama, H. Masuzawa & S. Fuji: The number of proliferating cell nuclear antigen positive cells in endometriotic lesions differs from that in the endometrium. *Virchows Archiv A Pathol Anat Histopathol* 423, 257-263 (1993)

8. Akoum A, A. Lemay, I. Paradis., N. Rheault & R. Maheux: Secretion of interleukin 6 by human endometriotic cells and regulation by proinflammatory cytokines and sex steroids. *Hum. Reprod* 11, 2269-2275 (1996)

9. Lessey BA, A.J. Castelbaum, S.W. Sawin, C.A. Buck, R. Schinnar, W. Bilker & B.L. Strom: Aberrant integrin expression in the endometrium of women with endometriosis. *J Clin Endocrinol Metab* 79, 643-649 (1994)

10. Syrop CH & J. Halme: Cyclic changes of peritoneal fluid parameters in normal and infertile patients. *Obstet Gynecol* 69, 416-418 (1987)

11. Haney AF, J.J. Muscato & J.B. Weinberg: Peritoneal fluid cell populations in infertility patients. *Fertil Steril* 35, 696- 698 (1981)

12. Oosterlynck DJ, C. Meuleman, M. Waer, M. Vandeputte & P.R. Koninckx: The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. *Fertil Steril* 58, 290-295 (1992)

13. Oosterlynck DJ, C. Meuleman, H. Sobis, M. Vandeputte & P.R. Koninckx: Angiogenic activity of peritoneal fluid from women with endometriosis. *Fertil Steril* 59, 778-782 (1993)

14. Leiva MC, L.A. Hasty, S. Pfeifer, L. Mastroianni, Jr & C.R. Lyttle: Increase chemotactic activity of peritoneal fluid in patients with endometriosis. *Am J Obstet Gynecol* 168, 592-598 (1993)

15. Nothnick WB, T.E. Curry, K.N. Muse, S.N. London & M.W. Vernon: Detection of a unique 32-kd protein in the peritoneal fluid of women with endometriosis. *Fertil Steril* 61, 288-293 (1994)

16. Issacson KB, M. Galman, C. Coutifaris & C.R. Lyttle: Endometrial synthesis and secretion of complement component-3 by patients with and without endometriosis. *Fertil Steril* 53, 836-841 (1990)

17. Barbati A, E.V. Cosmi, R. Spazini., R. Ventura & G. Montanino: Serum and peritoneal fluid CA-125 levels in patients with endometriosis. *Fertil Steril* 61, 438-442 (1992)

18. Taketani Y, K. Tong-Ming & M. Mizuno: Comparison of cytokine levels and embryo toxicity in peritoneal fluid in infertile women with untreated or treated endometriosis. *Am J Obstet Gynecol* 167, 265-270 (1992)

19. Khorram O, R.N. Taylor, I.P. Ryan, T.J. Schall & D.V. Landers: Peritoneal fluid concentrations of the cytokine RANTES correlate with the severity of endometriosis. *Am J Obstet Gynecol* 169, 1545-1549 (1993)

20. Oosterlynck DJ, C. Meuleman, M. Waer & P.R. Konickx: Transforming growth factor-ß activity is increased in peritoneal fluid from women with endometriosis. *Obstet Gynecol* 83, 287-292 (1994)

21. Buyalos RP, V.A. Funari, R. Azziz, J.M. Watson & O. Martinez-Maza: Elevated interleukin-6 levels in peritoneal fluid of patients with pelvic pathology. *Fertil Steril* 58, 302-306 (1992)

22. Eisermann J, M.J. Gast, J. Pineda, R.R. Odem & J.L. Collins: Tumor necrosis factor in peritoneal fluid of women undergoing laparoscopic surgery. *Fertil Steril* 50, 573-579 (1988)

23. Rier SE, A.K. Parsons & J.L. Becker: Altered interleukin-6 production by peritoneal leukocytes from patients with endometriosis. *Fertil Steril* 61, 294-299 (1994)

24. Hsu, C-C, B-C. Yang, M-H. Wu & K-E. Huang: Enhanced interleukin-4 in patients with endometriosis. *Fertil Steril* 6, 1059-1064 (1997)

25. Rana, N., D.P. Braun, R. House., H. Gebel, C. Rotman, & W.P. Dmowski: Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. *Fertil Steril* 65, 925-930 (1996)

26. Iwabe T, T. Harada, T. Tsudo, M. Tanikawa, Y. Onohara & N. Terakawa: Pathogenetic significance of increased levels of interleukin-8 in the peritoneal fluid of patients with endometriosis. *Fertil Steril* 69, 924-930 (1998)

27. Schneider EG, D.R. Armant, T.S. Kupper & M.L. Polan: Absence of a direct effect of recombinant interleukin and cultured peritoneal macrophages on early embryonic development in the mouse. *Biol Reprod* 40, 825-833 (1989)

28. Spits H & M.R. de Waal: Functional characterization of human IL-10. *Int Arch Allergy Immunol* 99, 8-15 (1992)

29. Moore KW, A. O'Garra, M.R. de Waal, P. Vieira & T.R. Mossmann: Interleukin-10. *Annual Rev Immunol* 11, 165-190 (1993)

30. Howard M, A. O'Garra, H. Ishida, M.R. de Waal & J.D. Vries: Biological properties of interleukin 10. *J Clin Immunol* 12, 239-247 (1992)

31. Howard M & A. O'Garra: Biological properties of interleukin 10. *Immunol Today* 13, 198-200 (1992)

32. McLaren J, G. Dealtry, A. Prentice, D.S. Charnok-Jones & S.K. Smith: Decreased levels of the potent regulator of monocyte/macrophage activation, interleukin-13, in the peritoneal fluid of patients with endometriosis. *Hum Reprod* 12, 1307-1310 (1997)

33. Ho HN, M.Y. Wu, K.H. Chao, C.D. Chen, S.U. Chen & Y.S. Yang: Peritoneal interleukin-10 increases with decrease in activated CD4+ lymphocytes in women with endometriosis. *Hum Reprod* 12, 2528-2533 (1997)

34. D'Hooge TM, L. Xiao & J.A. Hill: Cytokine profiles in autologous peritoneal fluid and blood of women with deep and superficial endometriosis. *Arch Gynecol Obstet* 265, 40-44 (2001)

35. Voorhis WCV: Coculture of human peripheral blood mononuclear cells with Trypanosoma cruzi leads to proliferation of lymphocytes and cytokine production. *J Immunol* 148, 239-248 (1992)

36. Nakajima-Iijima S, H. Hamada, P. Reddy & T. Kakunaga: Molecular structure of the human cytoplasmic ß-actin gene: interspecies homology of sequences in the introns. *Proc Natl Acad Sci USA* 82, 6133-6137 (1985)

37. Sanger F, S. Nicklen & A.R. Coulson: DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463-5467 (1977)

38. American Fertility Society: Revised American Fertility Society classification of endometriosis: *Fertil. Steril* 43, 351-352 (1985)

39. Becker JL, R.H. Widen, C.S. Mahan, T.R. Yeko, A.K. Parsons & W.N. Spellacy: Human peritoneal macrophage and T lymphocyte populations in mild and severe endometriosis. *Am J Reprod Immunol* 34, 179-187 (1995)

40. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254 (1976)

41. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250, 4007-4021, (1975)

42. Bravo R, J.V. Small, S.J. Fey, L. Mose & J.E. Celis: Architecture and polypeptide composition of HeLa cell cytoskeleton. Modification of cytoarchitectural proteins during mitosis. *J Mol Biol* 159, 121-143 (1982)

43. Celis JE, B. Gesser, H.H. Rasmussen, P. Madsen, H. Leffers, K. Dejgaard, B. Honroe, E. Olsen, G. Ratz, J.B. Lauridsen, S. Mouritsen, M. Hellerup, A. Andersen, E. Walbum, A. Celis, G. Bauw, M. Puype, J. Van Damme & J. Vanderkerckhove: Comprehensive two-dimensional gel protein databases offer a global approach to the analysis of human cells: the transformed amnion cells (AMA) master database and its link to genome DNA sequence data. *Electrophoresis* 11, 989-1071 (1990)

44. Rappopolee DA, D. Mark, M.J. Banda & Z. Werb: Wound macrophages express TGF-/ and other growth factors in vivo: Analysis by mRNA phenotyping. *Science* 241, 708-712 (1988)

45. Sambrook J, E.F. Fritsch & T. Maniatis: Molecular cloning. A laboratory manual. Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1989)

46. Koyama N, K. Matsurra & H. Okamura: Cytokines in the peritoneal fluid of patients with endometriosis. *Int J Gynecol Obstet* 43, 45-50 (1993)

47. Ryan IP, J.F. Tseng, E.D. Schriock, O. Khorram, D.V Landers & R.N. Taylor: Interleukin-8 concentrations are elevated in peritoneal fluid of women with endometriosis. *Fertil Steril* 63, 929-932 (1995)

48. Ueki M, T. Tsurunaga, T. Ushiroyma & M. Ueda: Macrophage activation factors and cytokines in peritoneal fluid from patients with endometriosis. *Asia-Oceania J Obstet Gynecol* 20, 427-431 (1994)

49. Halme J, S. Becker, M.G. Hammond, M.H.G. Raj & S. Raj: Increase activation of pelvic macrophages in infertile women with mild endometriosis. *Am J Obstet Gynecol* 145, 333-337 (1983)

50. Halme J, S. Becker & S. Haskill: Altered maturation and function of peritoneal macrophages: possible role in pathogenesis of endometriosis. *Am J Obstet Gynec*ol 156, 783-789 (1987)

51. Halme J: Release of tumor necrosis factor-alpha by human peritoneal macrophages in vivo and in vitro. *Am J Obstet Gynecol* 161, 1718-1725 (1989)

52. Sharpe KL, R.L. Zimmer, W.T. Griffin & L.L. Penney: Polypeptides synthesized and released by human endometriosis differ from those of the uterine endometrium in cell and tissue explant culture. *Fertil Steril* 60, 839-851 (1993)

53. Sharpe-Timms KL, M. Piva, E.A. Ricke, K. Sarewicz & Y.L. Zhang: Endometriotic lesions synthesize and secrete a haptoglobin-like protein. *Biol Reprod* 58, 988-994 (1998)

54. Zarmakoupis PN, S.E. Rier, G.B. Maroulis & J.L. Becker: Inhibition of human endometrial stromal cell proliferation by interleukin 6. *Hum Reprod* 10, 2395-2399 (1995)

55. Punnonen J, K. Teisala, H. Ranta, B. Bennett & R. Punnonen: Increased levels of interleukin-6 and interleukin-10 in the peritoneal fluid of patients with endometriosis. *Am J Obstet Gynecol* 174, 1522-1526 (1996)

56. Wu MY, H.N. Ho, S.U. Chen, K.H. Chao, C.D. Chen & Y.S. Yang: Increase in the production of interleukin 6, interleukin 10 and interleukin 12 by lipopolysaccharide-stimulated peritoneal macrophages from women with endometriosis. *Am J Reprod Immunol* 41, 106-111 (1999)

57. Bacchetta R, M. Bigler, J-L. Touraine, R. Parkman, P.A. Tovo, J. Abrams, M.R. de Waal, J.E. de Vries & M.G. Roncarolo: High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* 179, 493-502 (1994)

58. Ishida H, R. Hastings, J. Kearney & M. Howard: Continuous anti-IL-10 antibody administration depletes mice of Ly-1 B cells but not conventional B cells. *J Exp Med* 175, 1213-1220 (1992)

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