Tumour necrosis factor α concentrations in the peritoneal fluid of infertile women with minimal or mild endometriosis are lower in patients with red lesions only than in patients without red lesions

C.Calhaz-Jorge, A.P.Costa, M.Barata, M.C.Santos, A.Melo and M.L.Palma-Carlos

1Human Reproduction Unit, Department of Obstetrics and Gynaecology and 2Lisbon University Haematology and Immunology Centre, Hospital de Santa Maria, Av. Prof. Egas Moniz, 1699 Lisboa Codex, Portugal

3To whom correspondence should be addressed at: Human Reproduction Unit, Department of Obstetrics and Gynaecology, Hospital de Santa Maria, Av. Prof. Egas Moniz, 1699 Lisboa Codex, Portugal. E-mail: calhazjorge@mail.telepac.pt

Tumour necrosis factor α (TNFα) of peritoneal fluid is believed to have important pro-inflammatory and angiogenic activities in the complex mechanisms of development of peritoneal endometriotic lesions. We have evaluated the concentrations of TNFα and macrophages in peritoneal fluid of infertile women with minimal or mild endometriosis and related them to the presence of peritoneal red lesions alone (red lesions only group; n = 11) or their absence (non-red lesions group; n = 36). A group of 39 infertile normo-ovulatory patients with normal pelvic anatomy was used as controls. TNFα concentrations did not differ between controls and either group of patients. Patients with red lesions only had significantly lower concentrations of TNFα in peritoneal fluid (P < 0.05) and had a higher proportion of samples with undetectable concentrations (P < 0.05) than patients without red lesions. The significant difference in TNFα concentrations was present when comparing the groups of patients in the proliferative phase but not in the secretory phase. Macrophage concentrations were not different in the groups. Our findings are compatible with an impairment of macrophage function and therefore lend support to the theory that an inappropriate immunological response of the peritoneal environment to regurgitated endometrium may play a part in the initial phases of endometriotic implants.

Key words: minimal–mild endometriosis/peritoneal fluid/red lesions/tumour necrosis factor α

Introduction

Endometriosis remains an enigma in spite of extensive investigation. At present a composite theory of retrograde menstruation with implantation of endometrial fragments in conjunction with peritoneal factors to stimulate cell growth is the most widely accepted explanation for peritoneal endometriosis (Olive and Schwartz, 1993). Cytokines in the peritoneal fluid are among those factors and are believed to have important roles. Tumour necrosis factor α (TNFα) is a cytokine that is secreted by activated macrophages (Halme, 1989) and has potent inflammatory and angiogenic activities. Increased concentrations of TNF in peritoneal fluid were shown for the first time in patients with endometriosis when compared with women without pelvic pathology (Eiserman et al., 1988). Several other groups reported increased concentrations of TNFα in peritoneal fluid of such patients (Mori et al., 1991; Taketani et al., 1992; Overton et al., 1996; Rana et al., 1996; Harada et al., 1997) although others have found no difference between women with and without endometriosis (Vercellini et al., 1993; Keenan et al., 1995). Possible explanations for the inconsistencies include (i) important differences in the methodologies used for the evaluation of TNFα, with older studies using bioassays and the most recent ones using enzyme-linked immunoassorbent assay (ELISA) or radioimmunoassays, (ii) great variations in the definition of the populations studied, and (iii) the heterogeneity of endometriosis itself. In fact, pelvic endometriosis can occur in three different forms (superficial peritoneal lesions, deep infiltration of fibromuscular tissues, and ovarian cysts) (Nisolle and Donnez, 1997) with superficial lesions showing variable extension and different visual aspects that often co-exist in the same patient.

Peritoneal endometriotic lesions different from the typical, classical ‘powder-burn’ ones were described some years ago (Jansen and Russell, 1986). Several groups confirmed this report and contributed to the general acceptance of the importance of those types of lesions. They were ultimately included in the most recent revision of the American Society for Reproductive Medicine classification of endometriosis (ASRM, 1997). A significant number of studies have shown that red lesions have increased vascularization in the stroma (Nisolle et al., 1993; Matsuzaki et al., 1998), increased epithelial mitotic index (Nisolle et al., 1993; Nisolle et al., 1997), higher incidence of glands with ramifications (Donnez et al., 1992), increased expression of vascular endothelial growth factor (Donnez et al., 1998) and increased capacity to synthesize prostaglandin F (Vernon et al., 1986) when compared with black and white lesions. These findings support the contention that red lesions may be seen as the early stage of endometriotic peritoneal lesions and that they have more metabolic activity than the other types of lesion.

The present investigation was undertaken to evaluate the concentrations of TNFα in peritoneal fluid in relation to the presence of peritoneal red lesions alone or their absence in patients with minimal or mild endometriosis.
Materials and methods

Subjects
A total of 86 women undergoing laparoscopy to evaluate infertility was recruited for the study; 47 patients with minimal or mild endometriosis (stages I and II of ASRM classification) were selected by the existence of either red lesions only (red lesions only group) or black and/or white lesions only (non-red lesions group), whereas the other 39 were free of endometriosis and formed the control group. Patients with both red and non-red lesions were excluded. All the controls had normal pelvic anatomy. Infertility was defined as >12 months of delay in conception. All patients and controls had regular menstrual cycles and were in good health. None had been on hormonal medication in the 2 months prior to the surgical procedure, or had experienced signs of pelvic infection, or had undergone hysterosalpingography; nor had any of them had an abdominal operation in the 3 months before, or been pregnant or breast feeding in the previous 6 months. The study was approved by the local ethics committee and informed consent was obtained from the patients.

Laparoscopies were performed under general anaesthesia and throughout the menstrual cycle except during menstruation. The phase of the cycle was determined on the basis of the last menstrual period, the ovarian findings during laparoscopy and the histology phase pattern of the endometrium (Noyes et al., 1950). In cases in which the menstrual cycle during which laparoscopy was performed had an unusual duration, the date of the menstruation following surgery was also taken into consideration.

Peritoneal endometriotic lesions were diagnosed by their macroscopic appearance according to published criteria (Jansen and Russell, 1986) and categorized as red, black and white lesions as proposed in the latest revision of the ASRM classification (ASRM, 1997). Red lesions were the only type of lesions present in 11 patients and were absent in the other 36 patients (26 with black lesions only, four with white lesions only and six with both black and white lesions).

Peritoneal fluid collection
All visible peritoneal fluid was aspirated from the anterior and posterior cul-de-sac via a second puncture using a 2 mm metal cannula, before any internal manipulation and with the patient still in the horizontal supine position. The peritoneal fluid was transferred into two chilled sterile plastic tubes (one of them with an anti-coagulant, EDTA) and kept on ice until arrival at the laboratory. The non-anticoagulated tube was then centrifuged at 300 g for 15 min and the supernatant removed, divided into aliquots and frozen at −70°C.

Patients with samples contaminated with blood from puncture sites were not included.

Macrophage evaluation
The peritoneal fluid in the anticoagulated tube was diluted in an equal volume of phosphate buffered saline (PBS), underlayered with Hystopaque 1077 (Sigma Diagnostics, St Louis, MO, USA) and centrifuged at 400 g for 30 min at room temperature. Viable macrophages were counted in a Neubauer chamber after staining with trypan blue and their concentration was calculated. Macrophages were identified by their morphological features.

TNFα assays
Quantitative determinations of TNFα were performed using a commercially available enzyme immunoassay kit (Innotest hTNFα; Innogenetics NV, Zwijndrecht, Belgium). The assays were performed blind and according to the manufacturer’s instructions. The kit was said not to have measurable cross-reactivity with other relevant cytokines. Its sensitivity was 4 pg/ml and intra-assay and interassay variation of 6.3% and <10% respectively were found.

Statistical analysis
Continuous variables were analysed using unpaired t-test with Welch correction when the populations showed different variances. As the TNFα concentrations were not normally distributed, median and interquartile values (25% and 75%) were obtained and the Mann–Whitney U test was applied for the differences between groups. Values under the limit of sensitivity of the assay method were considered to be equal to that limit for this kind of statistical analysis. Proportions were analysed by means of Fisher’s exact test for a contingency table. Two-sided P values < 0.05 were considered statistically significant.

Results
The clinical characteristics of the women who participated in this study are summarized in Table I. Age, duration of infertility and proportion of secondary infertility were similar in patients with endometriosis and controls. Also, concentrations of peritoneal macrophages did not differ in the groups (Table I).

TNFα concentration in the peritoneal fluid was under the limit of sensitivity in 43% of patients and 41% of controls (not a statistically significant difference). The measured concentrations were generally low (below 65 pg/ml) except for one high value in the group of patients without red lesions (789.5 pg/ml) which was unexplained and had no influence on the results of statistical analysis.

Table I. Clinical characteristics of patients

<table>
<thead>
<tr>
<th></th>
<th>No endometriosis</th>
<th>Red lesions only</th>
<th>Non-red lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 39)</td>
<td>(n = 11)</td>
<td>(n = 36)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30.0 ± 3.3</td>
<td>29.3 ± 5.0</td>
<td>30.6 ± 4.2</td>
</tr>
<tr>
<td>(22–36)</td>
<td>(18–35)</td>
<td>(19–38)</td>
<td></td>
</tr>
<tr>
<td>Duration of infertility (months)</td>
<td>51.1 ± 25.2</td>
<td>57.6 ± 17.1</td>
<td>50.7 ± 39.4</td>
</tr>
<tr>
<td>(15–120)</td>
<td>(33–96)</td>
<td>(12–236)</td>
<td></td>
</tr>
<tr>
<td>Infertility, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>primary</td>
<td>25 (64.1)</td>
<td>9 (81.2)</td>
<td>28 (77.8)</td>
</tr>
<tr>
<td>secondary</td>
<td>14 (35.9)</td>
<td>2 (18.8)</td>
<td>8 (22.2)</td>
</tr>
<tr>
<td>Macrophages (×10³)/ml</td>
<td>703.3 ± 112.7</td>
<td>673.8 ± 112.1</td>
<td>687.6 ± 130.8</td>
</tr>
<tr>
<td>(470–870)</td>
<td>(530–860)</td>
<td>(430–870)</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD (minimum–maximum)
TNFα concentrations are displayed in Figure 1. No significant difference was found between women without endometriosis and either of the subgroups of women with endometriotic lesions. The concentrations of TNFα were found to be lower in women with red lesions only when compared with patients without red lesions ($P < 0.05$). That difference was statistically significant for women in the proliferative phase ($P < 0.05$ compared with women of the non-red lesions group) but not for those in the secretory phase. The proportion of patients with unmeasurable concentrations of TNFα in their peritoneal fluid was significantly higher ($P < 0.05$) for women with red lesions only than for those without red lesions (Table II). Due to low numbers, the analysis of proportions of cases with unmeasurable concentrations found in women in either the proliferative or secretory phase is meaningless.

No association between concentrations of TNFα and primary or secondary infertility was found in either patients or controls.

**Discussion**

In the present study the concentrations of TNFα in the peritoneal fluid of patients with minimal or mild endometriosis were evaluated and related to the presence or absence of red peritoneal lesions.

TNFα is a macrophage/monocyte-derived polypeptide considered to play important roles in the inflammatory state and angiogenic activity generally accepted as characteristics of the peritoneal fluid of women with endometriosis. Red lesions are probably the first stage of a peritoneal endometriotic lesion and are accepted to have a significantly higher vascular, epithelial and metabolic activity (Vernon et al., 1986; Nisolle et al., 1993, 1997; Matsuzaki et al., 1998) when compared with other lesions. Also, matrix metalloproteinase-1 mRNA was expressed in red lesions but was not detectable in black lesions (Kokorine et al., 1997), suggesting an increased state of tissue remodelling.

Previous reports evaluating TNFα concentrations in peritoneal fluid showed conflicting results with most describing higher concentrations and/or bioactivity (Eiserman et al., 1988; Mori et al., 1991; Taketani et al., 1992; Overton et al., 1996; Rana et al., 1996; Harada et al., 1997) and other groups finding no difference between patients with and without endometriosis (Vercellini et al., 1993; Keenan et al., 1995). Besides differences in methodologies, one important reason for such results may be the difficulty in recruiting homogeneous populations. The variability of the extension of the lesions, the existence of different types of peritoneal lesions (with or without ovarian endometrioid cysts) in the same patient, and the variation in the proportions of each type of lesion create great difficulties in comparing published data. We have tried to avoid as many confusing variables as possible by recruiting patients with minimal or mild endometriosis with either red lesions only or non-red lesions (i.e. black and/or white lesions). Even using such strict criteria, we recognize that some problems in defining the study groups may persist. In fact, the depth of the peritoneal lesions can be unexpected and it has been suggested that deep endometriosis is more influenced by bloodstream factors while superficial implants are more dependent on peritoneal fluid factors (Koninckx et al., 1991).

![Figure 1. Peritoneal fluid concentrations of TNFα in women without endometriosis (controls) or with minimal/mild endometriosis (RL only = women with red lesions only; non RL = women with black and/or white peritoneal lesions only). Horizontal lines within groups represent medians, continuous horizontal line represents minimum detectable concentration. For display, values under the limit of sensitivity of the assay method were considered to be equal to the limit of sensitivity. TNFα concentrations were higher in women without red lesions (median 6.5 pg/ml) than in those with red lesions only (median 4.0 pg/ml), $P < 0.05$, Mann Whitney U test. NS = not significant.](image)

<table>
<thead>
<tr>
<th>Table II. Proportion of patients with minimal or mild endometriosis and unmeasurable concentrations of TNFα in peritoneal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women with red lesions only ($n = 11$)</td>
</tr>
<tr>
<td>TNFα below limit of sensitivity ($n = 20$)</td>
</tr>
<tr>
<td>TNFα above limit of sensitivity ($n = 27$)</td>
</tr>
</tbody>
</table>

$P < 0.05$ by Fisher’s exact test comparing 8/20 (40%) women with red lesions only and TNFα concentration in peritoneal fluid below the limit of sensitivity of the assay method and 3/27 (11.1%) women with red lesions only and TNFα concentration in peritoneal fluid above that limit.
The definition of the control group is also a debatable issue. The presence of microscopic endometriotic lesions in biopsies of macroscopically normal pelvic peritoneum of 6% of infertile patients without visible endometriosis was reported (Nisolle et al., 1990). However, it is not known if peritoneal fluid characteristics of these patients are different from those of other women without endometriosis and the importance of the contamination (if any) of control groups is uncertain. Fertile women have been suggested to be better controls for the study of endometriosis than infertile ones because it is possible that couples with minimal/mild endometriosis as the sole finding in the work-up of infertility are not really different from those with unexplained infertility. We have used infertile controls because it is not usual in our country for healthy women under 40 years of age to choose to be submitted to tubal ligation. By selecting controls with regular menstrual cycles and normal pelvis and without previous pelvic manipulations, we intended to minimize hormonal influences and/or inflammatory sequelae that may alter peritoneal environment.

In our patients, concentrations of TNFα did not differ between the controls and either of the two groups of patients with endometriosis. These results differ from those of another group (Harada et al., 1997), which found higher concentrations of TNFα in the peritoneal fluid of 19 patients with red lesions compared with 19 infertile women without endometriosis. However, our data are not directly comparable because eight of their patients had moderate or severe endometriosis and all participate in the initial phases of development of peritoneal endometriosis.

The concentrations of TNFα in peritoneal fluid were found to be lower in women with red lesions only than in those with black and/or white lesions only. Since TNFα is secreted by peritoneal macrophages (Halme, 1989), we have also evaluated the concentration of these cells. An increase in macrophage concentration in peritoneal fluid has been reported (Haney et al., 1981; Badawy et al., 1984; Hill et al., 1988) in patients with endometriosis but this is not universally accepted. Like other groups (Awadalla et al., 1987; Syrop and Halme, 1987; Zeller et al., 1987), we found no difference between patients and controls. Also, patients with red lesions only and with non-red lesions showed similar peritoneal macrophage concentrations. This result suggests an impaired function of local macrophages in patients with red lesions only and may lend support to the theory that considers the establishment of endometriotic implants to be associated with alterations in cell-mediated immunity (Dmowski et al., 1981). The decreased concentrations of TNFα in peritoneal fluid of women with red lesions only reached statistical significance only in women studied during the proliferative phase. This is in agreement with the assumption that the scavenger mechanisms are most challenged in the days during and after the menstrual period because of refluxed menstrual debris, making a functional impairment more evident.

When performing studies of factors in peritoneal fluid, the fact that the presence and concentration of a factor in the peritoneal fluid may not reflect its functional activity must always be kept in mind. The variety of secretory products in peritoneal fluid and the complex interaction of each one with others and with different receptors makes it difficult to assess the real importance of any isolated factor in the initiation and/or evolution of endometriotic implants. Also, the concepts that peritoneal fluid may stimulate and inhibit growth of superficial endometriosis in different circumstances are not necessarily contradictory. Some support for this assumption comes from the observation that peritoneal inflammation may be inversely and not directly proportional to the extent of pelvic endometriosis as evaluated by the ASRM classification (Haney et al., 1991). The decreased concentrations of TNFα in peritoneal fluid that we have found are not incompatible with the published evidence of an increased tissue activation within red implants possibly related to interleukin-8, vascular endothelial growth factor and/or basic fibroblast growth factor stimulation by TNFα (Yoshida et al., 1997). It also seems conceivable that a lower concentration of TNFα may represent a limiting factor to an otherwise self-promoting system of increased neoangiogenesis (Harada et al., 1999).

In conclusion, our results suggest that women with minimal/mild endometriosis and only red lesions have lower TNFα concentrations in their peritoneal fluid than those without red lesions. This did not relate to the concentrations of peritoneal macrophages. We consider our findings to be compatible with an impairment of macrophage function, thereby giving support to the theory that an inappropriate immunological response of peritoneal environment to regurgitated endometrium may participate in the initial phases of development of peritoneal endometriotic lesions. The finding that differences in concentrations of TNFα in peritoneal fluid showed statistical significance during the proliferative phase reinforces that hypothesis.

References


Received on December 6, 1999; accepted on March 13, 2000